UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI MEDICINA VETERINARIA

PhD Course in Veterinary and Animal Science
Department of Health, Animal Science and Food Safety
Class XXXIII

Development of new oocyte in vitro culture strategies to enhance the outcome of assisted reproductive technologies

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2019/2020

Acqua di monte
acqua di fonte
acqua piovana
acqua sovrana
acqua che odo
acqua che lodo
acqua che squilli
acqua che brilli
acqua che canti e piangi
acqua che ridi e muggi.
Tu sei la vita
e sempre fuggi
(Gabriele d’Annunzio)

Abstract

Fertility preservation has received unprecedented attention nowadays. In addition to cryopreservation and re-implantation of embryos, oocytes, and ovarian tissue pieces, in vitro culture system for follicles/oocytes has been considered an alternative strategy for fertility preservation. Reproduction strategies based on the recovery of oocytes' population from antral follicles are unsatisfactory, and the success of this approach has not exceeded 35% of embryos produced in vitro for over 30 years. The possibility of accessing the reserve of smaller follicles (primordial, secondary, and up to the preantral stage) would amplify the number of gametes available for increasing reproductive potential. Furthermore, this would open enormous prospects for the rescue of fertility in various conditions in the human clinic and genetic rescue in animal breeding and biodiversity preservation programs. However, this would require developing protocols capable of growing immature oocytes to the stage in which they can be matured and fertilized in vitro.

Culture systems to achieve in vitro growth (IVG) of immature oocytes to maturity and subsequent fertilization in vitro (IVF) have been the subject of research for almost 40 years. Several systems that support the growth of later stages of follicle development from rodents have been developed, with some reporting the production of live young, but they are still at an experimental stage, and further research is required before the protocols could be clinically applied.

One of the significant limitations is identifying growth factors, hormones, and nutrients necessary for each specific follicle development stage. This evidence has led to hypothesize the development of culture systems consisting of a step-by-step approach, although no reliable protocols have been developed so far. The oocyte

culture at the early stages of development represents an alternative to maximize the potential source of gamete used for fertility preservation. Several attempts have been made to recreate these conditions in vitro, but no reliable protocols have been developed to date. The lack of knowledge in the mechanisms involved in the early development of the oocyte and this passage from growing to fully grown stage be one of the most critical steps during oocyte development, these still represent the significant limiting factor for this technology.

The studies conducted during the doctorate program led to defining a physiological culture system that successfully differentiated growing bovine oocytes. This study used parameters predictive of oocyte differentiation to evaluate the current technique's efficiency and efficacy.

Based on previous observations from our laboratory, we initially hypothesized that zinc plays a role during the latest stages of oocyte growth and differentiation, particularly in controlling transcription during the final stage of oocyte growth. This first study demonstrated that zinc supplementation improves the meiotic competence of growing oocytes, affects the global transcription activity and the global DNA methylation. This information was used in the next part to better define a culture system for growing oocytes.

The subsequent study provided a 5-days protocol named L-IVCO (long in vitro culture of oocytes) to promote growing oocyte differentiation until the acquisition of meiotic and embryonic developmental competencies in a significantly higher proportion of the published protocols. This study demonstrated that a physiological medium could support a gradual transition of the oocyte from immature to mature stage, thus generating suitably quality blastocysts after fertilization.

In conclusion, our studies provide an improved protocol that can increase the source of fertilizable gametes in preservation programs and gives a prospective approach in human clinics, animal breeding programs, and salvage intervention of threatened species. Moreover, our studies defined a model to perform in-depth studies of the cellular and molecular processes that regulate the acquisition of meiotic and developmental competence during oocyte differentiation.

Acknowledgments

First, I wish to thank my mentor for these last three years, Prof. Alberto Luciano. I appreciate the support both emotional and educational during my time here. Thank you for demanding from me much more than I thought I would be able to do. I express here my gratitude for sharing your wisdom, your time, and your experience. I will never forget your motivation and enthusiasm. Thank you for welcoming me and make this opportunity real.

An exceptional thank you to Prof. Valentina Lodde for your mentorship and friendship. Thank you for welcoming me in your home, I will never forget about it. Thank you for receiving me like your family and for your patience in teaching me as much as you could.

Enormous thanks to Prof. Federica Franciosi for your friendship, your contributions, detailed comments and insight have been of great value to me. Your absolute passion for the research is inspiring. Thank you for all the breaks during the day and “aperitivo” after work, which I was not allowed to pay for.

I wish to thank Prof. José Buratini for your guidance, friendship, and to make the connection between Brazil and Italy, making my Ph.D. possible. Thank you for being a friend, especially outside of Brazil, and for all trips around Milan, helping to disconnect from the work.

Thank you to Dr. Martina Colombo to be more than a friend, to be a family for me. Thank you for your support and to be there for me all the time. You were there every time from the vacations on the beach to the quarantine and to remember me about the deadlines. My life in these last years would not have been the same without

you and your family! “Grazie per tutte le lezioni di italiano e per avere sempre avuto la pazienza di spiegarmi tutto ciò che ti chiedevo”

I wish to thank Dr. Ana Caroline Silva Soares. Without you pushing, guiding, and remembering me about everything, especially deadlines. Thank you for your friendship and advice. Besides, I am tempted to individually thank all of my friends who have joined me on this journey, but this list might be too long, therefore I will simply say thank you very much to you all.

Finally, I want to thank all my family for financial and emotional support. Even living far from home, they were able to be with me all the time, even coming visit me when I was no able to go. I would not be here if they weren’t supporting all my crazy ideas and making everything possible!

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List of abbreviations

ACTB	Beta-actin
AI	Artifcial insemination
ANGPT2	Angiopoietin 2
AREG	Amphiregulin
ART	Artificial reproductive technologies
BMP15	Bone morphogenetic protein 15
bp	Base pair
BSA	Bovine serum albumin
BTC	Betacellulin
cAMP	Cyclic adenosine monophasphate
CC	Cumulus cells
cGMP	Cyclic guanosime monophosphate
CL	Corpus luteum
COC	Cumulus oocyte complex
Cx	Connexins
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DO	Denuded oocytes
E2	Estradiol
EAF	Early antral follicle
EGF	Epidermal growth factors
EGR1	Early growth response 1
EREG	Epiregulin
ERK	Extracellular signal related kinase
EU	5-ethynyl uridine
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF9	Growth differentiation factor 9

GJC	Gap junctions communication
GV	Germinal vesicle
GVBD	Germinal vesicle breaking down
HA	Hyaluronan
HAS2	Hyaluronan synthase 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT1	Hypoxanthine phosphoribosyl transferase 1
IP3	Inositol 1,4,5-triphosphate
IVC	In vitro culture
IVCO	In vitro culture of oocytes
IVEP	In vitro embryo production
IVF	In vitro fertilization
IVG	In vitro growth
IVM	In vitro maturation
IVP	In vitro embryo production
LH	Luteinizing hormone
L-IVCO	Long in vitro culture of oocytes
MAF	Medium antral follicles
MI	Meiosis I
MII	Meiosis II
MOET	Multiple ovulation and embryo transfer
MPF	Maturation promoting factor
mRNA	messenger ribonucleic acid
MT	Metallothionein
MTF-1	Metal response element-binding transcription factor-1
NSN	Non surrounded nucleolus
OPU	Ovum pick up
OSF	Oocyte secreted factor
P4	Progesterone
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDE3	Phosphodiesterase 3
PGC	Primordial germ cells
PVA	Polivinilalcool
PVP	Polyvinylpyrrolidone
RGS2	Regulator of G-protein signaling 2
SLC30a	Solute carrier family 30A
SLC39a	Solute carrier family 39A
SPRY2	Sprouty RTK Signaling Antagonist 2
TPEN	*N*,*N*,*N′*,*N′*-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine
TZP	Trans-zonal projections
ZIP	Zrt- and Irt-like proteins
ZnT	Zinc transporters

Foreword

This thesis is structured as described below.

Chapter 1 consists of an overview of fertility preservation issues that concern humans and farm and wild animal species to save or protect gametes (eggs and sperm), embryos, or reproductive tissues so that stored gametes can be used to have progeny in the future. Implementing reliable oocyte culture protocols could allow human female cancer patients to preserve eggs before gonadotoxic treatments and store germinal material from endangered animal species or animals with relevant genetic traits that will significantly benefit from developing alternative preservation strategies. To address this issue, we propose a culture system for growing bovine oocytes that would enhance the potential source of oocytes that can be submitted to IVP procedure and reach meiotic competence in vitro, offering useful and safe options for fertility preservation but also aiding the understanding of the mechanisms involved in the processes of oocyte growth and differentiation.

The following chapters represent different parts of this research project, and each section has the structure of an article (published or under submission), each with its bibliography at the end.

Chapter 2 is a published article describing the effects of zinc supplementation during in vitro growth of bovine oocytes and assessing the role of zinc in the control of transcription during the latest phase of oocyte growth and differentiation.

Chapter 3 describes advanced physiological approaches for in vitro oocyte development to exploit the ovarian reserve's reproductive potential. It is almost ready for submission. It describes the effects observed in the growing oocyte culture in a

physiological system capable of guiding growing oocytes through final differentiation until the achievement of becoming a blastocyst after in vitro maturation and fertilization.

Chapter 4 comprises two parts. Both represent published articles detailing the in vitro oocyte culture approach. The first section is a visual protocol that originated from the culture system described in Chapter 3. The second section presents in vitro culture methods proposed utilizing physiological conditions to support the specific needs of oocytes at distinct stages of differentiation to expand the source of female gametes for IVP by maximizing the attainment of fertilizable oocytes. The original published articles of chapters 2 and 4 are in the appendix.

A general discussion concludes the thesis with indications of prospects for continuing to improve female reproductive efficiency.

CHAPTER 1

LITERATURE REVIEW

1. Fertility preservation: state of the art

Fertility preservation might be defined as ‘‘the application of surgical, medical, or laboratory procedures to preserve the potential for genetic parenthood in adults or children at risk of sterility before the end of the natural reproductive lifespan’’ [1], thus it is the process of saving or protecting gametes (eggs and sperm), embryos or reproductive tissues so that stored gametes can generate progeny in the future.

The first breakthrough in fertility preservation was 70 years ago with the development of cryobiology which triggered the semen bank industry, especially in cattle and human reproductive health care [2]. With the evolution of this technology others started to emerge, and scientists started to look after female gamete preservation. In humans, it was initially available only to adults with partners, although the cost was a limiting factor [2]. Nowadays, several fertility preservation strategies are being studied, especially for humans [3]. Such as germplasm cryobiology [4, 5]; xenotransplantation [6], in vitro culture of oocytes at different stages of differentiation [4, 7, 8] and ovarian tissue cryopreservation [9, 10]. Thus, artificial reproductive technologies (ART) are still considered critical tools for saving and maintaining fertility in the species.

For example, in human cancer patients, in which the aggressivity of the treatment causes reproductive disorders, the safeguard and preservation of the genetic material is an option. One of the possibilities is ovarian tissue cryopreservation prior to undergoing gonadotoxic treatments but re-implantation of this tissue is the only alternative. This alternative brings a high risk of re-implantation of malignant cells. Another alternative would be the in vitro growth of the oocytes enclosed inside the follicles of the cryopreserved ovarian tissue and its use to generate embryos in vitro by

means of in vitro reproductive technologies [10, 11]. Moreover, culturing immature oocytes in vitro would avoid the administration of hormonal protocols for the patient, prevent multiple surgical procedures and, most importantly, there would be options for fertility preservation/restoration for women who currently have none [12-14].

Fertility preservation is a concern not just in humans but also in farm and wild animal species, as a need for the preservation of genetic material from endangered species or individuals with relevant genetic traits will also greatly benefit from the development of alternative preservation strategies [15]. Options for fertility preservation are emerging as novel techniques from experimental stages to test in patients and clinical trials [2]. Due to ethical issues and the difficulty to have material when the experiments are performed in human tissues, the advance in understanding the bovine physiology is relevant since it has been demonstrated to be comparable anatomically and physiologically to humans, making the cow a suitable model to study mammalian reproduction [3, 16]. Thus, to further optimize fertility preservation strategies, the use of animal in vitro models will certainly benefit progress in both human and animal fertility preservation research [17].

Though, to be able to correctly use these technologies, the development of genome resource biobanking is imperative. It refers to the collection, processing, storage, and use of germplasms (oocytes, embryos, ovarian tissues) that can be used for understanding and sustaining biodiversity [15]. Oocyte and embryo cryopreservation are established procedures, but the ovarian cryopreservation still remains experimental [2, 10]. It is a great advantage in human medicine giving the opportunities for patient autonomy and fertility preservation after cancer treatment [2, 10, 13]. The need for fertility preservation will continue to grow while oncology treatment depends on gonadotoxic treatments, during the current demographic shift

toward postponed motherhood, and until gametes can be generated de novo [1]. Also, it is an advantage for mammals because if it is used properly in association with ART, it has the potential to decelerate the loss of genetic diversity in captive populations by reintroducing the original genetic traits (without removing genetically valuable individuals from the wild) and decrease the interval between generations [15].

New techniques develop faster now than in the past, and it is especially welcome for a subject where timeliness is imperative for personalized treatment. Cells can be preserved indefinitely in the deep cold, but the reproductive window is open for a limited time [2]. Fertility preservation has traveled far from its origin in cryopreservation and holds even greater expectations for the future [2].

2. Preserving germinal resources in female

When fertility needs to be preserved in females, the choice of the specific ART to be used depends on several factors such as age, type of cancers, and available time between diagnosis and the onset of treatment [3]. The most established method for fertility cryopreservation is embryo cryopreservation but banking oocytes are preferable for many reasons and this technology was boosted after vitrification that increased the survival rates of these cells after warming [1, 18]. Moreover, oocyte and embryo cryopreservation require an ovarian stimulation which is generally inappropriate to girls and young adolescents. In these cases, the ovarian tissue cryopreservation is an additional option [18]. This technique is particularly valuable since no hormonal stimulation is required and the ovarian tissue has a high number of primordial follicles that can be used for xenotransplantation [6] or in vitro culture [4, 13].

ARTs have come a long way in the last 30 years in animals and humans and are well established in the clinical practice [19]. The artificial insemination (AI) together

with in vitro embryo production (IVP) are the most commonly ARTs used. The IVP consists of a three-step process involving in vitro oocyte maturation (IVM), in vitro oocyte fertilization (IVF), and subsequent in vitro culture (IVC) of the zygote to the blastocyst stage [20]. The large-scale production of embryos in vitro has been possible in domestic mammals, particularly cattle, for several decades but in terms of efficiency, it remains stable for over 30 years [20]. It seems that a plateau is reached with only small margins for improvement because of the intrinsic biological and/or technical limitation of the source of gametes and of in vitro conditions [19].

Nowadays, the culture of oocytes at early stages of development is available for research purposes, but it could maximize the potential source of cryopreserved gametes and have many clinical applications relevant to fertility preservation [3]. The stock of pre-antral ovarian follicles represents a potential source, which could be ‘unlocked' for reproduction, preservation or research purposes. Pre-antral follicles provide a safe method for the restoration of fertility and they also constitute a genetic pool out of which endangered animal populations can be restored when in vitro follicle culture becomes a mainstream technique [2]. Consequently, these oocytes need a dynamic and variable culture system to support all stages of oocyte development, starting from the activation of the dormant primordial follicle to a stage where oocytes can undergo meiotic maturation [9]. The lack of knowledge in understanding the oocyte's early development mechanisms is an important limiting factor. Moreover, the lack of standard protocols for preserving this genetic material makes this technology still experimental [3].

The development of a culture system for growing oocytes would be an important breakthrough in reproductive medicine and would provide an important model for human fertility preservation. Nowadays, little is known about the reproductive

physiology of individual species of wild mammals, and the potential use of reproductive biotechnologies for safeguarding endangered wildlife species has had partial success. Thus, such culture system can enhance the potential source of oocytes that can be submitted to IVP technologies and reach meiotic competence in vitro, offering useful and safe options for fertility preservation but also help us to understand the mechanisms involved in the oocyte growth and differentiation.

3. Folliculogenesis and oogenesis

3.1. Folliculogenesis

Oogenesis and folliculogenesis are two distinct but strictly connected processes thus, they need to be coordinated to generate a good quality gamete [21, 22]. The formation of the recruited follicle, which will bring to the growth, maturation and ovulation of a fertilizable oocytes, requires several cell types such as those of the theca, granulosa, and cumulus cell layers that surround the oocyte [23]. Thus folliculogenesis can be divided into two distinct phases: (1) the first phase, also known as pre antral phase, is characterized by the change of granulosa cells from flattening to cuboidal shape, their proliferation and beginning of follicle growth and (2) the second phase, the antral phase, is characterized by the increase in the number of granulosa cells, a rapid increase in the oocyte size and the formation of a fluid-filled cavity between granulosa cells, known as antrum (Fig 1. [24, 25]).

In mammals, the whole germ cell compartment of the ovary derives from a group of embryonic stem cells called primordial germ cells (PGC), the development starts during the fetal life and it occurs asynchronously over the lifetime [24, 26]. The

PGCs that are still diploid cells, are located outside the embryo at the base of the allantois and surrounded by the extraembryonic mesoderm, they migrate along the hindgut reaching the genital ridge [27, 28]. The PGCs continue to mitotically proliferate during the migration and after arrival at the genital region; once these cells reach the genital region, they are called oogonia or spermatogonia depending on the embryo genotype. In mammals, like bovine, human, and ovine, the assembly of the primordial follicle starts during fetal development [29, 30] and in bovines, their arrival to the gonads occurs around 35 days of gestation. After this period the PGCs take different fates according to the sex of the embryos, in females the oogonia switch from mitotic to meiotic division. After DNA replication, they enter prophase of the first meiotic division and are now called oocytes [27, 31].

The formation of the primordial follicle occurs between 91 and 144 days of pregnancy in the bovine species. In the primordial follicle, the prophase I arrested oocyte is surrounded by a single layer of approximately six flatten cells and surrounded by a basal membrane [23]. After the day 170 of pregnancy onward, only primordial follicles are present and the oogonial mitosis is discontinued. In cattle, these follicles have their diameter equal or less than 40 μm [23, 25] and the cells are meiotically arrested until they are stimulated to grow, which can take from months to years according to the species [24, 25, 32, 33].

After puberty, some arrested primordial follicles are recruited and leave the pool of arrested follicles to enter in a non-reversible growing phase. The mechanisms involved in the recruitment of the follicles remain to be solved [24, 25]. These primordial follicles constitute the ovarian reserve, from which follicles are recruited for development [34]. The key stages of oocyte development can be summarized as (1) activation of primordial follicle growth; (2) development of laminar pre-antral follicle

stage; (3) antral cavity formation and differentiation of granulosa cells; (4) proliferation of granulosa cells and expansion of fluid-filled antral cavity; and (5) release of the cumulus-oocyte complex (COC) after rupture of the follicle in response to the LH surge [12, 35]. It is important to anticipate here that during the last phase of folliculogenesis the enclosed oocyte resumes meiosis and progress and complete the first meiotic division with the emission of the first polar body and stops again at the metaphase of the second meiotic division. The transition from the prophase I to metaphase II, which occurs within the preovulatory follicle, is called oocyte maturation and the oocyte enclosed in the cumulus mass that is ovulated blocked at the MII stage until fertilization occurs in the fallopian tube.

More precisely, once primordial follicles are recruited to restart their growth, a modification from flattening to cuboidal granulosa cells can be observed and the follicle is called a primary follicle. This stage is characterized by oocytes' growth and an increase in the granulosa cells number [24, 25]. When the follicles reach at least two layers of granulosa cells it reaches the secondary follicle stage it is consistently bigger than the primordial stage (around 100 µm in diameter) and it is characterized by an increase in the number of granulosa cells, a rapid increase in the oocytes' diameter, which is still blocked in diplotene stage, begin of the thecal cells layer formation, mRNA (messenger ribonucleic acid) synthesis in the oocyte, gonadotrophins responsiveness and, in bovines, initial deposition of the zona pellucida [23, 25, 36, 37]. Following, the granulosa cells organization occurs in several layers and an antral cavity with follicular fluid among the cells is formed. This is the end of the pre-antral phase of the folliculogenesis and the beginning of the antral phase and the follicles are now denominated as tertiary follicles [36].

The tertiary follicle is characterized by the production of hyaluronan (HA) and versican by granulosa cells, creating an osmotic gradient fluid into the follicle to create the antrum [38], the formation of two different layers in the theca cells, interna and externa theca; the interna theca becomes vascularized for clearing waste and transporting nutrients while the theca externa is a more loosely associated group of cells but contains smooth muscles cells [24, 36]. At this stage, the granulosa cells intimately related to the oocytes receive signals from the oocyte which establish and maintain a group of cell that can be classified as cumulus cells (CC) and the inner layer (the closest to the oocyte is denominated corona radiata), the granulosa cells that are non-closely related to the oocytes are classified as mural granulosa cells and the cumulus together with the female gamete from the cumulus-oocyte complex (COC) [39, 40].

During the follicle development, the enclosed oocytes reactivate transcription and grow in size in parallel with the follicle until the oocyte reaches the full size and transcriptions stop, in cows, it occurs when the oocyte is around 120-130 µm and the follicle diameter is around 3 mm in diameter [36, 37, 41]. The mammalian oocyte typically reaches its full diameter before the follicle has reached full size [40], in fact, the oocyte reached the growth plateau but the follicle continues to grow and it can reach up to 15 – 20 mm in diameter before ovulation [37].

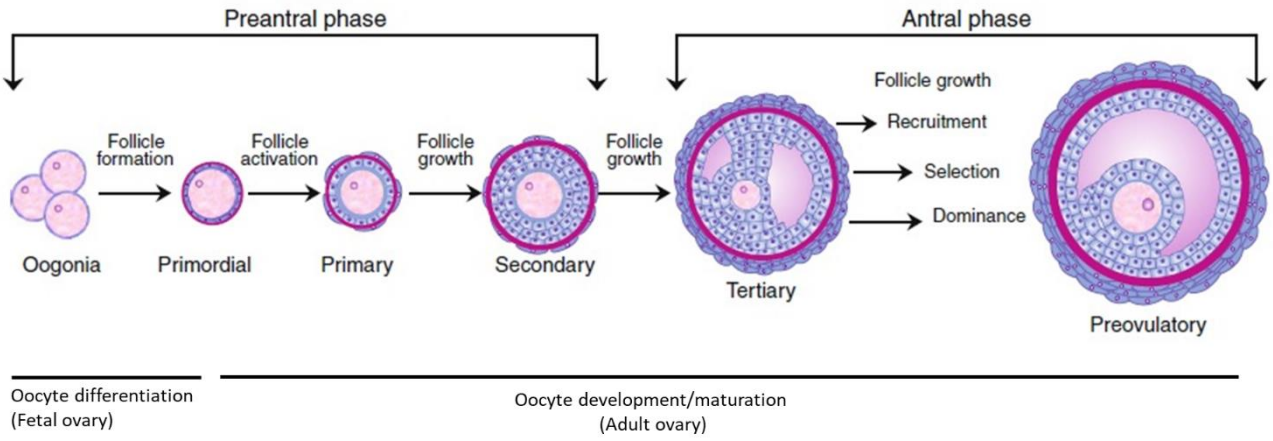

Figure 1. Schematic sequence of folliculogenesis. Pre-granulosa cells enclose each oogonium to form the primordial follicle which has a single layer of flattened granulosa cells, it happens during fetal life. When puberty occurs a pool of primordial follicles initiate their development with follicle and oocyte growth. The primary follicle has a single layer of cuboidal granulosa cells. The secondary follicle has two or more layers of cuboidal granulosa cells and a small number of theca cells. All the preantral follicles have a primary oocyte. When the follicle reaches the tertiary stage, it enters in the antral phase. The tertiary follicle has several granulosa cell layers, theca cells, and primary oocyte and is characterized by an antral cavity that contains follicular fluid. Preovulatory or also called as Graafian follicle is the last stage of follicle development (Adapted from [24, 42]).

In cow, follicular development occurs in waves, the follicular recruitment can occur in two or three waves and only the final wave is an ovulatory one [43]. First, a pool of follicles is recruited and a rise in FSH (follicle-stimulating hormone) content allows their further growth [44]. When the cohort of follicles reaches around 8 mm in diameter starts the dominance period of the follicle where one follicle has a rapid increase in size, becoming a dominant follicle [37, 44]. The selected follicle starts to produce estradiol (E_2) and inhibin to suppress the other follicle's growth, becoming the

only preovulatory follicle. During this period many changes occur such as the size of Golgi complexes are reduced, nuclear membranes become undulated, elongation of the corona radiata and nucleolus vascularization [37, 45]. All these changes occur before the LH (luteinizing hormone) surge [39].

The dominant follicle continues to grow until the LH surge, at this point the oocyte within this follicle undergoes to the final maturation characterized by expansion of CC, meiosis resumption with the germinal vesicle breaking down (GVBD) and progression through the final stage of meiosis I (MI) until it is arrested at metaphase of meiosis II (MII), culminating in the release of the oocyte from the follicle [34, 37, 46, 47].

After ovulation, the corpus luteum (CL) will form and will secrete progesterone (P4) to maintain the pregnancy. If the pregnancy does not occur the uterus starts to produce prostaglandin F2-alpha, which causes the CL regression and the start of the next estrous cycle [48]. It is important to underline that although a large number of primordial follicles are recruited, only a few ovulate while the others regress through a process called follicular atresia to favor the development of the dominant follicle. This step may look like an inefficient process but it is important to select the follicle with the most competent oocyte [49].

Follicular atresia is the term used to identify the loss of follicles that occurs by apoptotic removal of granulosa cells, oocytes, and ultimately the follicle itself [50, 51]. Apoptotic granulosa cells start to appear and gradually increase their number in early atretic follicles, when the majority of granulosa cells undergo apoptosis occurs a disruption in the granulosa layers, then those follicles are eliminated [52]. Morphologically, atresia is induced in the granulosa cells layer, but not in CC or oocytes during the early stages of atresia [52, 53]. In fact, it was demonstrated in vitro, where

the apoptosis occurs in granulosa cells but not in CC, moreover, this study also demonstrates that the dissociation between the cells triggers apoptosis [54].

When the enclosed oocytes are used in in vitro embryo production protocols, commonly adopted criteria is to select antral follicles using morphological parameters [55-57] based on the previously demonstrated correlation between macroscopic parameters and histological analysis of follicular atresia [57]. Non-atretic follicles have a uniformly bright appearance, extensive and very fine vascularization, regular granulosa cells layers and no free-floating particles in the follicular fluid; as they become atretic follicles gradually lose their translucency, become slightly grey and free-floating particles in the follicular fluid can be observed. Lastly, follicles at heavy atretic stages have a dark appearance and enclose very dark CC. This selection is important due to the intimate relationship between the oocyte and granulosa cells and how it can affect the further development of the COC

Although follicular atresia can occur at any stage of follicular development, it appears that the majority of the follicles undergo atresia during late preantral to early antral stage [28, 50, 58, 59]. In fact, follicles in their early antral phase present the highest atresia rate, reaching 40.5% of the follicles atresia, while other stages present lower atresia rates. In almost all mammals, apoptosis seems to be confined to granulosa cells of follicles undergoing transition to the antral stage or to large subordinate antral follicle not selected for ovulation [50, 60]. In fact, most follicles found in an ovary at any time in the estrous cycle are atretic [57] and this appears normal since most follicles do not ovulate and enter in regression phase becoming atretic [61].

In the practice of in vitro embryo production technologies, the selection of non-atretic follicles becomes particularly arduous in early antral follicles, due to the size and the scarce follicular fluid. For this reason, a morphological examination is operated on

the isolated COCs to discard those showing atretic signs [8, 14, 62-64]. This is achieved again by morphological observations such as the presence of more than five compact layers of CC, grossly spherical shape, intact oolemma and homogeneous and finely granulated ooplasm [65-67].

3.2. Oocyte growth, differentiation and competence acquisition

Oogenesis is defined as the complete process of oocyte development and differentiation from the migration of the first germ cell to the genital ridge until the formation of a haploid, mature, and developmentally competent female gamete [68]. The entire process of oocyte development that starts during fetal life and complete at the time of ovulation attain progressive acquisition of meiotic and embryonic developmental competence results from integrated nuclear and cytoplasmic events [69].

After migration to the genital ridge, the PGCs proliferate through synchronous mitotic divisions, leading to the formation of a nest of cells called “germ cell nest” and giving rise to oogonia. In the germ cell nest the cells are connected by intercellular bridges [68, 70, 71] and one cell of the nest will become the primordial oocyte while the others are nurse cells and supply nutrients to the oocyte, thus these cells are loosely surrounded by somatic cells and this grouping of germ cells and somatic cells is called the ovigerous cords [72, 73].

Oocytes separate from the nest by a process called cyst breakdown and they become enclosed in the primordial follicles. Each primordial follicle is composed of one oocyte and some granulosa cells [74]. At this point, the oogonia switch from mitotic to meiotic divisions and are now is called oocytes. Then, they undergo different stages of

the meiosis with a series of events occurring such as the pairing of homologous chromosomes, synapsis and recombination or “crossing-over”. Ultimately they enter in a prolonged resting phase called dictyate stage, which, in the bovine species happens between 75 to 80 days of pregnancy [31, 33, 68, 75].

The prolonged resting phase can take from months to years according to the species [25, 33]. In the ovary, primordial follicles are cyclically recruited to develop. Once the follicles are recruited the oocyte starts its growth phase in which the oocyte’s size increase from less than 30 µm in diameter to more than 120 µm in the tertiary follicle, with changes in the nuclear and cytoplasmic compartments, accumulating molecules that will drive the subsequent steps of oogenesis, fertilization and embryonic development, up to the embryonic genome activation [39].

During the recruitment of primordial follicles, the oocyte is characterized by a single layer of a mixture of 5–14 cuboidal and flatten cells arranged around the oocyte. In general, most ultrastructural features of the ooplasm and its organelles are similar to those described for the resting primordial follicles. Although, it is observed a slight increase in the number of microvilli and the nucleus is eccentrically located in most oocytes, the majority of the mitochondria are round, but elongated and dividing mitochondria are becoming more common [23].

In secondary follicles, zona pellucida starts to form as well as extension of the granulosa cell processes to the oolemma, forming gap junction connexions between these cells and the oocyte, elongated mitochondria are observed, and the number of cytoplasmic vesicles increases. In the tertiary follicle, the oocyte is characterized by a multilayer of granulosa cells surrounding it with small cavities between the granulosa cells in early stages or a single antral cavity, the mitochondria are distributed throughout the ooplasm and the number of Golgi complexes increased [23, 39].

The cytoplasmic and nuclear events occurring in the oocyte must be coordinated to assure an adequate oocyte developmental competence acquisition. In fact, it was observed that these events are temporally correlated [32, 76] and nuclear modifications occur gradually according to the follicle stage, in the primary follicle the nucleolus displays less dense fibrillar centers suggesting that transcription is resuming in the oocyte [36]. In the secondary follicle, cortical granules are present at this stage, and the nucleolus is very active [36]. In the tertiary follicle, as the oocyte approaches its full size, the transcriptional activity starts to cease until it becomes silenced. The nucleolus also begins to form tighter electron-dense foci, which also reflects the global transcriptional silencing [76]. The modifications that occur during this period are crucial for the subsequent early embryonic development [32]. These oocytes still have their chromatin enclosed in the nucleus, which is known as germinal vesicle (GV), they are arrested in prophase I and are known as immature oocytes. These oocytes as commonly referred as GV stage oocytes or immature oocytes, until meiotic resumption occurs, after this, they develop until the MII stage where occurs a second arrest of the meiotic division and these oocytes are known as MII stage or mature oocytes.

According to Lodde et al., 2007 [41], bovine immature oocytes retrieved from early tertiary follicles present decondensed chromosomes and the chromatin is still transcriptionally active. As follicles grow, the enclosed oocytes also undergo nuclear modifications so that the oocytes collected from follicles of a diameter ranging between 0.5 and 8 mm can be classified into 4 stages according to the degree of chromatin compaction and its organization within the nucleus, evaluated by means of fluorescence microscopy: GV0, where diffuse filamentous of chromatin in the whole area of the nucleus can be observed; GV1 is similar to GV0 stage but some foci of chromatin condensation can be observed; GV2 stage, distinct clumps of chromatin

condensation distributed around the nucleoplasm can be observed; and GV3 the chromatin is condensed into a single clump within the nuclear envelope. In this study, the authors also demonstrated that the degree of chromatin compaction and the oocyte diameter are related to the growth of the follicle and the ability of the oocyte to acquire developmental competence, that is the oocytes with a higher degree of chromatin condensation are more suitable to further development (Fig 2) [41].

As anticipated, oocyte transcription is active during the growth period. In bovine oocytes, transcription is activated in the primary follicle and is maintained up to when the oocyte reaches a diameter of around 110 μm in tertiary follicles [39]. In fact, during growth, the oocyte synthesizes a large amount of RNAs. In particular, mRNA accumulates to form a reserve of maternal messengers, that will be used when the oocyte becomes transcriptionally silenced, which occurs when the oocytes terminate its growth phase and well ahead meiotic resumption after the LH surge. It has been shown that as the oocytes progress through the latest stages of its growth, the chromatin starts to gradually condense and concomitantly become gradually transcriptional silent [32, 76]. In fact, it was observed that GV0 oocytes, with uncondensed chromatin, have high levels of transcription; in turn, in the GV3 oocytes, the transcription is ceased. The biggest drop in transcription is temporarily linked with the chromatin condensation in early stages, especially in the transition between GV0 to GV1 oocytes but once bovine oocytes have reached full size, greater than 120 mm in diameter, and the follicle diameter is about 3 mm, the level of transcriptional activity is minimum if not completely arrested [32, 36, 76-78].

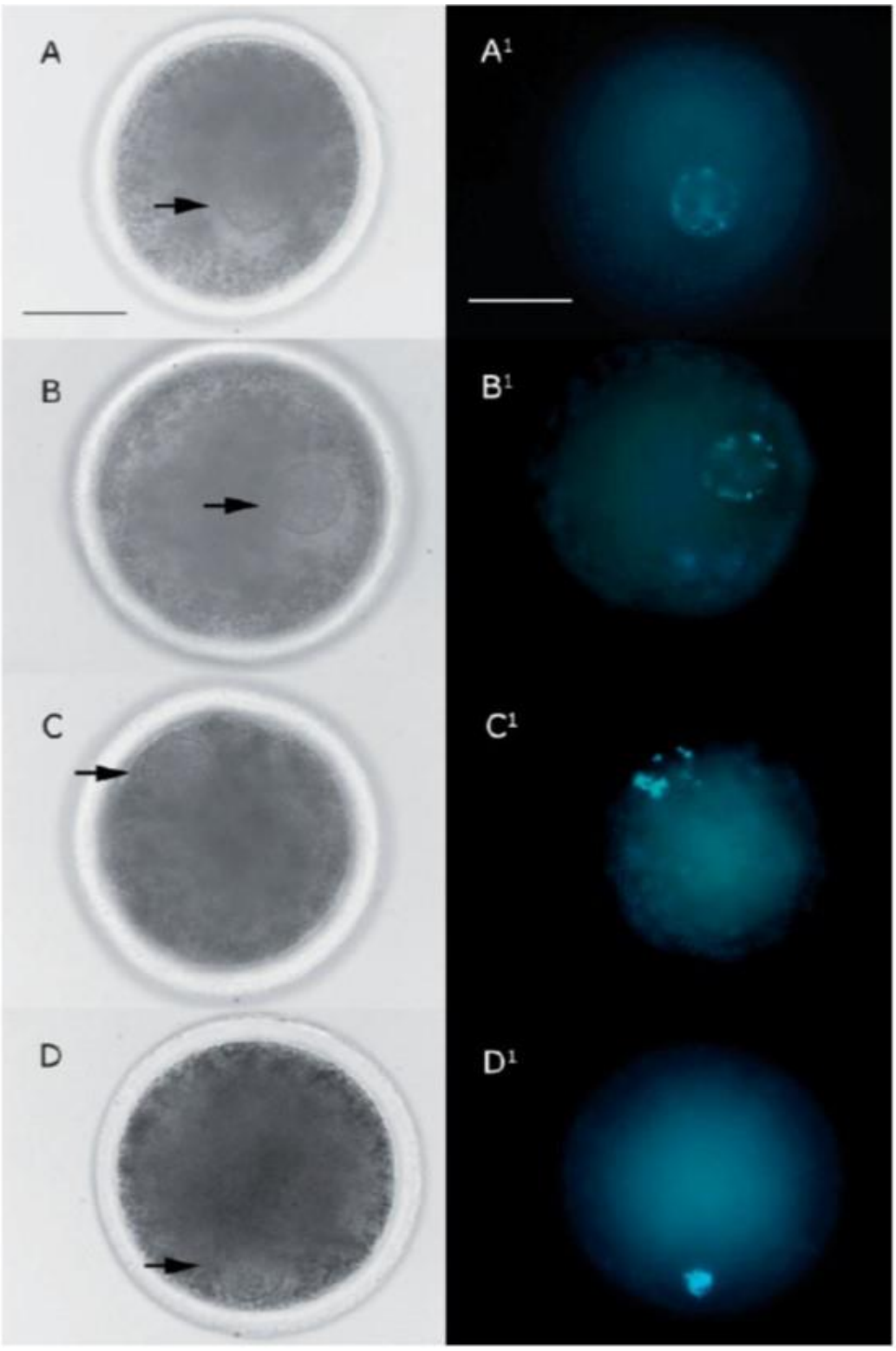

Figure 2. Representative image of oocytes with different configurations of the chromatin enclosed in the GV. Bright-field and fluorescent images after Hoechst 33342 indicating bovine oocytes with GV0 (A, A^1); GV1(B, B^1); GV2(C, C^1); GV3 (D, D^1) configuration [41].

This period of silent transcription lasts until early stages of embryonic development with the major genome activation [79, 80]. The length of the transcriptional silence period varies according to the specie, in bovines, transcription resumes in the embryos at the 8 to 16-cell stage [79-82].

After the LH surge, the oocyte that was arrested in prophase I resume the meiotic division which comprehends the progression period between prophase I to the metaphase II of the meiotic division when the meiotic progression stops again and the oocyte is released in the fallopian tubes through ovulation. During maturation, the oocyte undergoes further ultrastructural modifications and attains full developmental competence, becoming ready to be fertilized [39, 83]. The transition from the Prophase I to the metaphase II stage happens within the ovarian preovulatory follicle [84]

After the LH surge, nuclear modifications can be observed: the oocyte nuclear envelope breaks down (this stage is also known as GVBD stage) and microtubules spindle assemble around the paired homologous chromosomes, then the spindle segregates half of the homologous chromosome in a small cell called first polar body, it is an asymmetric division. The remaining chromosomes are captured by the second spindle formation, when occurs the second arrest until it is fertilized [80]. After fertilization, the oocyte finishes the meiosis II by segregating half of the remaining chromatids into the second polar body. The chromatids inside of the oocyte start to decondense forming the haploid female pro-nucleus and fuse with the haploid male pro-nucleus, forming a diploid cell called zygote [80].

Concomitantly to nuclear maturation, cytoplasmic maturation involves cytoplasmic changes required to prepare the cell for fertilization and embryo development. The oocyte’s cytoplasm typically contains several organelles commonly found in other cells, such as mitochondria, Golgi apparatus and the endoplasmatic

reticulum. It also contains some specific organelles such as cortical granules and acid granules. During maturation these organelles undergo oocyte-specific transformation such as size reduction of Golgi apparatus and alignment of the cortical granules and mitochondria attain a central position and the nuclear envelope disappears. Cytoplasmic maturation remains poorly understood but it is fundamental for the completion of meiosis [85].

While the oocyte matures, a mucified matrix is formed between the somatic cells, creating a space between them, this step is known as cumulus expansion and it is important to make possible the rupture of the follicle and egg release into the oviduct [80]. Moreover, matrix formation is associated with oocyte quality, adhesive and invasive capacity of the oocyte, also it is necessary for fertilization [86, 87]. Suboptimal formation of the matrix can affect the developmental competence of the oocytes [86-88].

3.3. The germinal-somatic compartments crosstalk

The CC are closely related to the oocytes and this proximity ensures an exchange of nutrients and other metabolites, maturation-enabling factors, creating a microenvironment that contributes to differentiation and developmental competence acquisition [89, 90]. The communication between the oocyte and its companion cells occurs in three ways: through gap-junction communications (GJC), trans-zonal projections (TZP) and oocyte secreted factors (OSF).

The GJC are involved in folliculogenesis, oogenesis, as well as oocyte meiotic arrest [91]. In fact, the formation of the GJC starts during the primordial stage of follicle development and persists until later stages of follicular growth [91]. The GJC are

composed by connexons forming a central pore that allows the passage of small molecules such as ions, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), inositol 1,4,5-triphosphate (IP3), nucleotides, amino acids, pyruvate, glucose and small peptides (Fig 3) [92, 93]. Each connexon is composed of six transmembrane proteins called connexins (Cx); the main connexins present in the follicle are Cx43 (GJA1), Cx37 (GJA4) and Cx 32 (GJB1) [92, 94].

During oocyte growth, GJC play a major role in the regulation of chromatin remodeling and transcription during oocyte growth and differentiation. The importance of this kind of communication was firstly reported in the mouse model through mouse knock-out for Cx 37 experiments where oocytes failed to achieve full meiotic competence [91, 95]. Moreover, if mice oocytes at very early stages of their growth are cultured in the absence of CC the transcriptional silence fails to occur [95-97], while in bovines the transcription suddenly drops [41]. It can be due to the growth phase from which the oocytes were isolated. The studies in cows retrieve the oocytes from early antral follicles in pubertal and normal cycling cows [8, 41] while studies in mice were performed in oocytes from pre antral follicles in primed mice [97]. Because of this difference, the time of GJC disruption can affect chromatin structure and function, also oocytes from pre antral follicles can be unable to condensation of chromatin because they are far from being fully grown.

The role of GJC in the modulation of chromatin configuration and transcription was confirmed also in bovine [8, 41], where at the time of collection, oocytes isolated from early antral follicles with a GV0 configuration (and thus transcriptionally active) showed fully open state of GJC, while they progress growth and consequently, in the chromatin condensation they start to show partially opened until completely closed GJC [8, 41].

In vitro, the functionality of GJC can be maintained to ensure gradual chromatin remodeling, contribute to transcriptional regulation during oocyte growth and achievement of meiotic and developmental competence [8]. In the cow model, GJC functionality was experimentally maintained during in vitro culture of oocytes collected from early antral follicles (that are typically GV0) using low concentrations of FSH, similar to the physiological one. The results of this study indicated that when growing oocytes were cultured with a low concentration of FSH the percentage of COC with open GJC is significantly higher when compared to COC cultured with higher concentrations of FSH. Interestingly when high concentrations of FSH were used the GJC functional coupling was not maintained and meiotic resumption was forced to occur bypassing all the necessary chromatin remodeling steps and thus compromising their competence. In addition, it does not support the maintenance of GJC coupling as well while chromatin remains in its uncondensed state [8].

Other components that influence the maintenance of GJC when oocytes are cultured in vitro are the inhibitors of phosphodiesterase (PDE), the enzyme that hydrolyses cAMP [98, 99]. One of these compounds is cilostamide, which specifically inhibits the type 3 PDE (PDE3), that is specifically expressed in the oocytes. In previous studies, bovine oocytes were treated with the uncoupler 1-heptanol to induce GJC disruption and a sudden condensation of chromatin and a decrease in transcription was observed. When cilostamide was added in the hepthanol supplement medium, the COCs were able to maintain the communications and gradual chromatin condensation was observed, demonstrating that cAMP is at least in part involved in the mechanisms by which CC and GCJ coupling mediates chromatin remodeling [8].

GJC are also involved in the control of meiotic arrest and meiotic resumption. It has been shown that GJC closure correlates with meiotic resumption. The process

of meiotic resumption relies on a complex chain of events that includes gonadotropins, steroid intermediates, like meiosis activating sterol [100] and other signaling peptides like kit ligand and the EGF-like peptides, which in turn influence cellular levels of second messengers from the granulosa and theca cells, like cAMP, cGMP, IP_3, DAG, and calcium which are involved in the resumption of meiosis [101, 102].

In vivo, an interruption of cell-to-cell communication is observed after the LH surge, leading to meiosis resumption in virtue of lower levels of cAMP in the oocyte; in vitro, the communications are disrupted when the COC is isolated from the follicle [84, 92] and premature rupture of the communications can affect the oocyte's competence levels [103, 104]. It has been shown that the LH surge leads to the phosphorylation of type 5 PDE (PDE5) in granulosa cells, increasing the cGMP hydrolyzing activity. Also, the LH surge leads to dephosphorylation and inactivation of NPR2 guanylyl cyclase, reducing cGMP production, consequently reducing the cGMP content in the oocyte. This drop in the cGMP level in the oocytes results in a reduction in the levels of cAMP and reinitiating meiosis [105-107].

In vitro, resumption of meiosis is stimulated by high concentrations of FSH which stimulates the production of epidermal growth factors (EGF), such as amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) [101, 102]. EGF interact with their receptor and it starts the phosphorylation of the ERK1/2 (extracellular signal-related kinase) [108, 109]. This leads to the phosphorylation of the CX43 [110], closing the GJC and consequently the oocyte is deprived of inhibitory mechanisms of cAMP and cGMP, leading to meiosis resumption [102, 110, 111].

Finally, the communication between oocyte and its companion cells through secretion of paracrine factors, known as OSF (Fig 3). The oocyte secretes these factors that will act as a regulator of granulosa cells and CC functions [112, 113]. It

was proven that the oocyte controls the proliferation of granulosa cells in preantral and antral follicles through the secretion of factors [114]. As reviewed by Eppig, the oocytes play an essential role during all stages of ovarian development, from initial follicular formation until the disassembly of ovulated COC [115].

There are several OSF, but the most studied ones are the bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9). Their role in communication acts as a signal to stimulate granulosa and CC proliferation [21, 112]. They also function to prevent luteinization of the granulosa cells, inhibit apoptosis, and regulate metabolism. These factors transmit their signals through SMAD2/3 and EGFR-ERK1/2 signaling pathways to alter gene expression and cell function [102]. The communication between CC and oocyte is bidirectional and complementary, for example, oocyte release some OSF, mostly GDF9 and BMP15 that interact with somatic cells, promoting granulosa cells differentiation and proliferation; in turn, the somatic cells produce their paracrine signs such as kit-ligand, activin, inhibin, to coordinate the oocyte growth. This exchange of molecules is continuous and changes dynamically over follicle development [112, 116, 117].

Mutations on the GDF9 gene by homologous recombination in mice do not prevent the recruitment of primordial follicles to the primary stage, although further development beyond the primary stage is not observed in GDF9-null mice [115, 118]. Moreover, the absence of GDF9 alters the oogenesis and folliculogenesis, causing sterility [118].

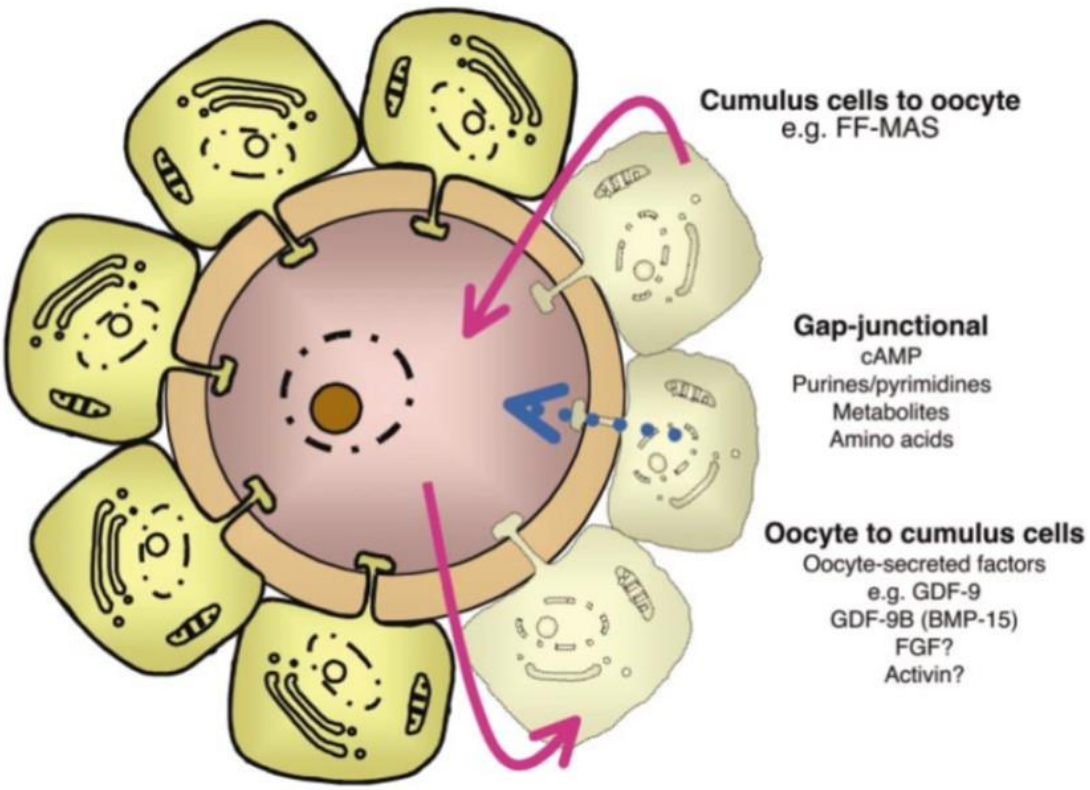

Figure 3. Oocyte granulosa cells communication. Paracrine signaling through oocyte secreted factors (curved arrows) and gap-junction communications exchange (straight arrow) [119].

The importance of communication between oocytes and their surrounding cells is clear. They are involved in several processes during follicle and oocyte development, playing an important role especially in the oocyte's control of granulosa and CC function and differentiation and, inversely, follicular control of the oocyte maturation [102].

3.4. Importance of CC gene expression as a marker of oocyte differentiation

Since CCs and oocytes are so tightly linked, both morphologically and functionally, it is believed that they can reflect the oocyte's quality and, as such, can be used as a source of material to assess non-invasively the oocyte competence and possibly as a main criteria for COC selection [89, 120]. Since CCs contribute to the success of oocyte and embryonic development acting in a meticulous and time-specific manner through the expression of appropriate molecules, the identification of those expressed genes, together with an analysis of their patterns and molecular functions, are powerful tools to gain information about the processes involved in oocyte competence acquisition [89]. For this reason, a better characterization of the molecular determinants of oocyte heterogeneity would be beneficial in understanding basic oocyte biology as well as in improving IVP efficiency [121-123]. This led to the verification of the gene expression patterns in CC and establish a non-invasive approach for the oocyte's fate [89]. In fact, an increasing number of studies were done focusing in the CC characteristics and their predictive potential about the developmental capacity of the oocyte [81, 121, 124-127].

Embryonic developmental competence acquisition is regulated by somatic cells surrounding the oocyte and for this reason, the CC gene expression is an important process triggered by signaling pathways according to the oocyte's stage [127]. The analysis of genome-wide studies has provided interesting molecular function in CC [128-130]; for example, previous studies could associate the presence of RGS2 gene encodes for GTPase-activating protein, it is probably involved in the regulation of granulosa cells response to gonadotrophins and its expression in CC is correlated to oocyte developmental capacity and clinical pregnancy [128, 129]. Besides, recent studies from our laboratory identified a group of genes that are differentially expressed during the transition of growing to fully-grown oocytes which

represent the transition from early antral follicles to middle antral follicles and it can help us to design a culture system more adequate for these oocytes, according to their metabolic requirements [121].

The transcriptomic profiles of bovine oocytes and surrounding CC at different stages of differentiation were assessed by microarray analysis [121, 123, 131] and are publicly available on a transcriptomic online platform (http://emb-bioinfo.fsaa.ulaval.ca/Home/index.html) that has been implemented by collecting and storing transcriptomic data from bovine oocytes, CC and granulosa cells under different physiological conditions and using the same transcriptomic platform and bioinformatic tools from several research groups [122]. After being analyzed and validated, all the collected transcriptomic data sets were subjected to meta-analysis and made available through a web interface called EmbryoGENE profiler, which thus provides dynamic expression profiles of any gene of interest in the selected sample (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/index.html) [122]. In this way, genes that will contribute to more accurate oocyte selection and therefore improve the outcome of the reproductive technologies in livestock can be found.

3.5. The role of zinc during oocyte growth

Zinc transport across biological membranes is carried out by specific systems that work together to control zinc homeostasis. There are two families of membrane transporters involved in the process of cellular zinc buffering: 1) the SLC30A gene family, which encode for 10 zinc transporter (ZnT) proteins that are responsible for the decrease of intracellular zinc; 2) the SLC38A gene family, which encode for 15 ZRT/IRT like proteins (ZIP) that are responsible for replenishing cytosolic zinc from

extracellular compartments. Moreover, a third system involves the function of isoforms of metallothionein (MT), responsible for redox activity and zinc's translocation in a controlled manner into the cellular compartment [132, 133].

To date, less is known about the role of zinc during the early stages of oocyte development, but an increasing amount of studies are starting to reveal an essential role for zinc in the control of oogenesis and early embryogenesis in model organisms [134, 135]. For example, in mouse, the total zinc content increases during oocyte maturation [134, 136] and the chelation of zinc during mouse IVM (using the zinc chelator TPEN) severely impairs meiotic progression [137, 138]. Accordingly, in mice, dietary zinc deficiency during the periovulatory period (i.e when the oocyte undergoes maturation in vivo) causes several defects in ovarian function, such as failure to complete meiosis, a lack of cumulus expansion, and ovulation. Importantly, it has also been shown that dietary zinc deficiency during preconception, which is when the oocyte complete its growth and undergoes final differentiation and remodel its chromatin, does not block meiotic maturation but instead disrupts oocyte epigenetic programming including a decrease in DNA and histone methylation and causes embryonic defects [139]. Besides, zinc has a role in protecting DNA from damage, oxidation and in defending cellular components [140].

These data indicate that zinc plays important role also before meiotic resumption. A previous study mined the transcriptomic profile of CC isolated from oocytes in different chromatin configurations and it revealed that the most upregulated gene was the zinc transporter (SLC39a8, which encodes for ZIP8 protein) [121]. Besides the zinc importance, the culture systems with a defined medium are usually performed in the absence of zinc. However, serum inclusion provides at least minimal zinc concentration for cell growth and survival [140].

Some studies in mouse growing oocytes with uncondensed chromatin have shown that zinc modulation regulates the localization of the metal response element-binding transcription factor-1 (MTF- 1) [141], which is a well-characterized transcription factor that functions as a cellular zinc sensor by coordinating the expression of genes involved in zinc homeostasis [142]. Thus, the zinc supplementation during oocyte growth could benefit the culture system and aid the oocyte during the growth period, when the cells are actively transcribing molecules that will regulate and sustain subsequent stages of oocyte and early embryonic development.

4. Challenge in ART and strategies

4.1. New approaches for in vitro embryo production

During the reproductive life span, only a minimal fraction of the oocytes that are present in an ovary mature, are released in the fallopian tubes upon ovulation and are available for being fertilized and develop into a viable embryo [20]. Most of the IVP protocols use COC that have basically completed the growth phase within the antral ovarian follicle, and hence are referred to as fully-grown oocytes, this COCs can be obtained from follicles with diameter between 2 and 8 mm (middle antral follicles, MAF) [20]. The COC can be obtained in vivo, through OPU (ovum pick up) or from ovaries from slaughtered cows. Then this COC is submitted to several steps, the first one is the in vitro maturation (IVM) which has the aim to bring the oocytes from prophase I (or GV stage) to the metaphase II; then they are co-cultured with the sperm (male germ cell) in a step called in vitro fertilization (IVF). Finally, the longest step is the in vitro culture of embryo (IVC) which lasts 7 days and allows cellular divisions until it reaches

the blastocyst stage (Fig 4). All these steps together are called IVP but this system is not capable to fully support the oocyte and early embryonic development as demonstrated by the fact that it struggles to overcome 35% of efficiency [143].

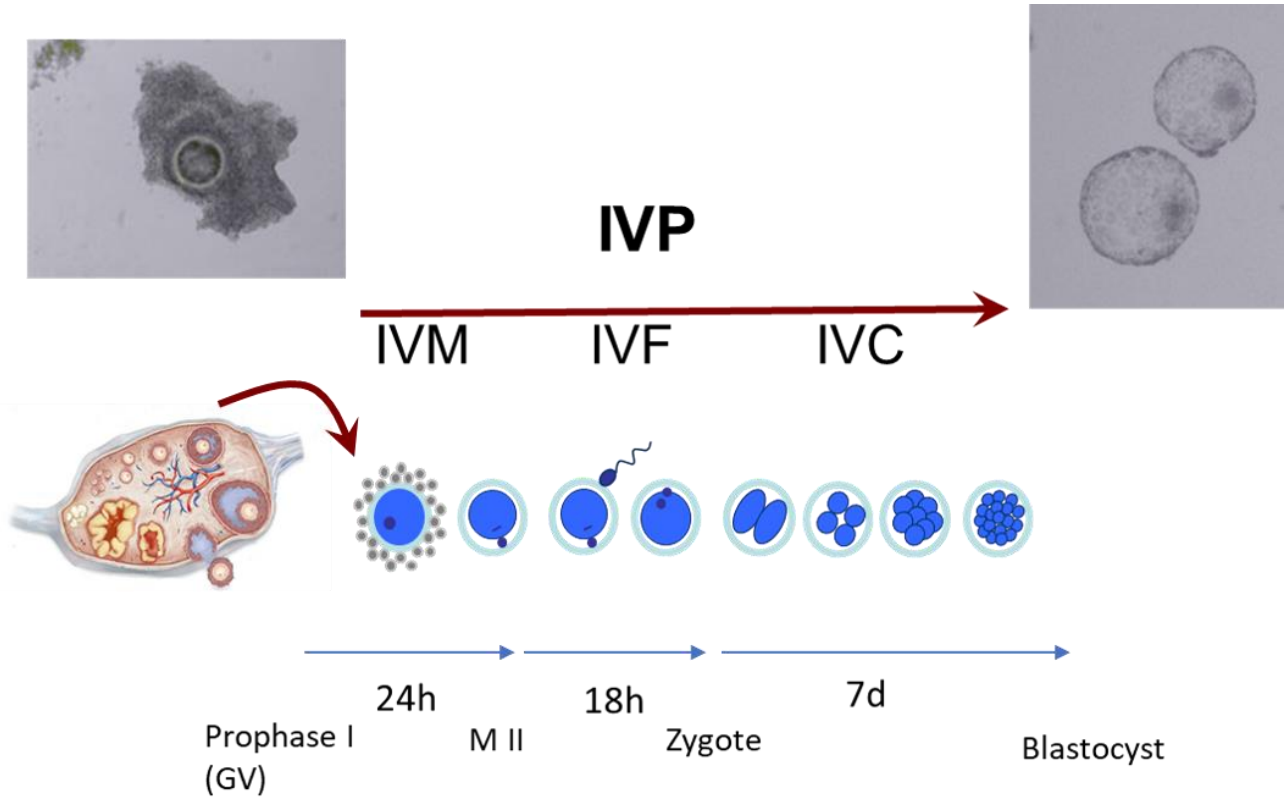

Figure 4. Representative scheme of in vitro embryo production (IVP). The standard IVP process starts with the COC collection from the follicle with a diameter between 2 and 8 mm. Then, the COCs are submitted to several steps such as: in vitro maturation (IVM), which brings the oocyte from prophase I to metaphase II of the meiotic division; after the oocytes are co-cultured with the sperm (male germ cell) in a step called in vitro fertilization (IVF). Finally, they are submitted to in vitro embryo culture (IVC) that allows the mitotic divisions until it becomes a blastocyst.

The IVP efficiency is calculated as the proportion of immature oocytes from MAF that reaches the blastocyst stage of embryonic development [121] and it remains stable for over 40 years, in part due to the limited number of fully grown oocytes that

populate an ovary at a given time which can be retrieved and subjected to standard IVP techniques [19, 143, 144].

To improve the efficiency of the system two options are pointed out: improvement of the IVP steps by itself or higher exploitation of the ovarian reserve. In a standard IVP procedure, the oocytes from antral follicles are used and they are able to resume meiosis once isolated from the ovary, also they have different stages of chromatin condensation within the germinal vesicle (GV1, GV2 and GV3) [41], but small margins for improvement are possible because of the intrinsic biological and/or technical limitation of the source of gametes and of in vitro conditions [19]. The other option is a high exploitation of the ovarian reserve, which represents a potentially large source of oocytes compared with those in antral follicles [33, 58, 145-147] such as oocytes coming from early antral follicles (EAF), which are follicles with diameter between 0.5 and 2 mm (Fig 5). These oocytes are in their growing phase, with uncompacted chromatin dispersed within the germinal vesicle (GV0), they still have active GJ mediated communication between the oocyte and the CC and they are still transcriptionally active, thus they are not able to resume meiosis once isolated from the follicle [8, 41, 65, 148] and consequently, they have low competence to reach the blastocyst stage compared with oocytes from MAF [127, 149, 150].

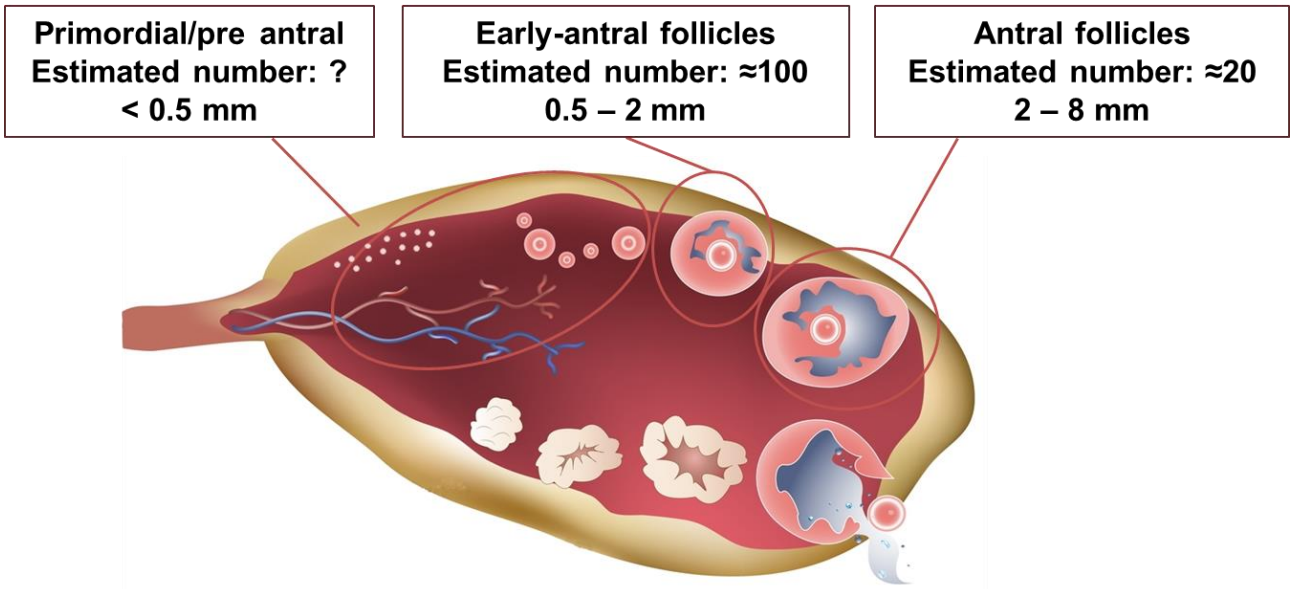

Figure 5. Schematic figure about the ovarian reserve. Figure showing the source of oocytes present in an ovary at a given time in cycling ovary, the number of antral follicles is around 20 oocytes/per ovary and a potentially large source of oocytes compared with those in antral follicles can be observed, these oocytes are enclosed in early antral follicle and represents a source around 5 times higher than the antral follicles (extrapolation of the ovarian reserve based on [33, 58, 145, 146]).

The transition from the growing to the fully-grown stage is one of the critical steps during oocyte development. In cattle, several studies attempted to recapitulate these events in vitro [8, 151-158]. However, to date no reliable protocols have been developed and only limited success has been reported. According to previous studies [41], these growing oocytes constitute a homogeneous population. Conversely, the population of fully-grown oocytes obtained from middle antral follicles is more heterogeneous, a condition that is mirrored by the various degrees of chromatin compaction (GV1, GV2 and GV3) that can be observed [41]. Among these, previous data have shown that GV2 and GV3 oocytes are overall characterized by better quality and higher embryonic developmental competence [41, 76, 121, 159, 160].

Due to the low developmental competence of growing oocytes, they cannot be directly submitted to standard IVM protocols since they are meiotically incompetent. However, they require an additional period of culture that would allow them to complete the growth phase and adequately differentiate. This led to defining tailored culture systems for growing oocytes to promote their developmental competence acquisition.

5. Aim of the study

The techniques based on IVP starting from oocytes isolated from antral follicles have low efficiency because the oocytes are not ready to be submitted to IVP system, but they require an additional period of culture that would allow them to complete the growth phase and properly differentiate. The transition of growing to a fully-grown stage is one of the critical steps, so this study focused on recreating an in vitro culture system that is physiologically similar to the environment the COC come from and supporting the oocyte differentiation and to higher exploitation of the ovarian reserve, pointing to a potentially large source of oocytes coming from early antral follicles [58, 145].

This study's primary purpose is to steer the fundamental mechanisms that regulate the folliculogenesis process in the early antral to antral transition by reconstituting a physiological environment capable of supporting the in vitro differentiation of growing oocytes to becoming capable of maturing, fertilizing, and developing into a blastocyst.

Specifically, the project aimed to:

1. To assess whether zinc plays a role during the latest phase of oocyte growth and differentiation and particularly to the hypothesis that zinc participates in controlling transcription at this stage (Chapter 2).
2. To define a physiological protocol that can sustain the oocyte's coordinated differentiation with the surrounding cumulus cells able to promote oocyte growth, chromatin transition, ability to expand after IVM, meiotic competence acquisition, cumulus expansion ability, and embryonic developmental capability after fertilization (Chapter 3).
3. To take advantage of this technology to exploit the ovarian reserve's reproductive potential and support oocytes' specific needs at distinct differentiation stages and maximize fertilizable oocytes (Chapter 4).

Such a culture system can enhance the potential source of oocytes usable in IVP protocols. Moreover, it supports the oocyte until it reaches meiotic competence in vitro, offering useful and safe fertility preservation options. Lastly, it may represent a formidable tool to understand the mechanisms involved in oocyte growth and differentiation.

CHAPTER 2

THE EFFECT OF ZINC SUPPLEMENTATION ON MEIOTIC COMPETENCE AND TRANSCRIPTION ACTIVITY OF GROWING OOCYTES

Published in:

Reproduction 2020 May; 159(6):679-691

https://doi.org/10.1530/REP-19-0398

Title:

Zinc supports transcription and improves meiotic competence of growing bovine oocytes

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Short Title:

Zinc in bovine growing oocytes

Key Words A minimum of four keywords describing the manuscript

Oogenesis, meiosis, oocyte competence, cumulus cells, transcription, chromatin, zinc transporters, ZIP, Znt metallothionein, SLC39A, SLC30A, DNA methylation

Word count of the full article: 6059

Abstract

In the last years many studies focused on the understanding of the possible role of zinc in the control of mammalian oogenesis, mainly on oocyte maturation and fertilization. However, little is known about the role of zinc at earlier stages, when the growing oocyte is actively transcribing molecules that will regulate and sustain subsequent stages of oocyte and embryonic development. In this study we used the bovine model to gain insights into the possible involvement of zinc in oocyte development. We first mined the EmbryoGENE transcriptomic dataset, which revealed that several zinc transporters and metallothionein are impacted by physiological conditions throughout the final phase of oocyte growth and differentiation. We then observed that zinc supplementation during in vitro culture of growing oocytes is beneficial to the acquisition of meiotic competence when subsequently subjected to standard in vitro maturation. Furthermore, we tested the hypothesis that zinc supplementation might support transcription in growing oocytes. This hypothesis was indirectly confirmed by the experimental evidence that the content of labile zinc in the oocyte decreases when a major drop in transcription occurs in vivo. Accordingly, we observed that zinc sequestration with a zinc chelator rapidly reduced global transcription in growing oocytes, which was reversed by zinc supplementation in the culture medium. Finally, zinc supplementation impacted the chromatin state by reducing the level of global DNA methylation, which is consistent with the increased transcription. In conclusion our study suggests that altering zinc availability by culture medium supplementation supports global transcription, ultimately enhancing meiotic competence.

Introduction

From the time zinc was discovered to be the most abundant transition metal in mouse oocytes (Kim, Vogt et al. 2010), many studies have focused on the understanding of its possible role in the control of mammalian oogenesis. Thus far, most of the studies considered the function of zinc during oocyte maturation and fertilization. These studies seem to indicate that zinc regulates distinct molecular mechanisms during the two stages, in line with the notion that zinc participates in a vast number of biological processes (Maret 2017).

In the immature fully grown oocyte, zinc is implicated in the maintenance of meiotic arrest (Kong, Bernhardt et al. 2012, Tian and Diaz 2012). Conversely, during maturation, there is a rise in total zinc content, which is key for successful oocyte maturation (Kim, Vogt et al. 2010, Bernhardt, Kim et al. 2011, Tian and Diaz 2012, Kong, Duncan et al. 2014, Jeon, Yoon et al. 2015). In the MII stage oocyte the quota of zinc loosely bound to biomolecules in a readily exchangeable form (Outten and O'Halloran 2001, Dean, Qin et al. 2012), referred to as “labile zinc” or “free zinc”, is accumulated in cortical vesicle-like structures that are released in the extracellular compartment upon fertilization through repetitive exocytic events termed “zinc sparks”, which are in turn implicated in the process of meiotic resumption and block of polyspermy (Kim, Bernhardt et al. 2011, Bernhardt, Kong et al. 2012, Zhao, Kwon et al. 2014, Que, Bleher et al. 2015, Duncan, Que et al. 2016, Que, Duncan et al. 2017, Que, Duncan et al. 2019). The molecular mechanisms by which zinc regulates all these processes are just starting to be elucidated. However, in reason of the global transcriptional silencing in the fully-grown oocytes, they must be transcription-independent.

To date, less is known about the role of zinc at earlier stages of oocyte development, i.e. during oocyte growth, when these cells are actively transcribing molecules that will regulate and sustain subsequent stages of oocyte and early embryonic development. The first indication that zinc is fundamental during oocyte growth comes from in vivo studies in the mouse. Specifically, it has been shown that a zinc-restricted diet 3–5 days before ovulation disrupts oocyte maturation as well as embryonic, fetal and placental development at various levels (Tian and Diaz 2012, Tian and Diaz 2013, Tian, Anthony et al. 2014). Moreover, studies in mouse growing oocytes with uncondensed chromatin in the Non Surrounded Nucleolus (NSN) configuration, have shown that zinc modulation regulates the localization of the metal response element-binding transcription factor-1 (MTF-1) (Kong, Duncan et al. 2014), which is a well characterized transcription factor that functions as a cellular zinc sensor by coordinating the expression of genes involved in zinc homeostasis (Andrews 2001).

To further advance the knowledge on the role of zinc during oogenesis, we propose the bovine as experimental model since the changes that occurs within the nucleus during the latest phases of oocyte growth and differentiation before meiotic resumption have been well characterized (reviewed in (Luciano and Lodde 2013, Luciano, Franciosi et al. 2014, Bogolyubov 2018). In cattle, as in other mammals, changes in large-scale chromatin configuration in the Germinal Vesicle (GV) are used as a morphological marker of oocyte differentiation (Zuccotti, Garagna et al. 2005, De La Fuente 2006, Luciano and Lodde 2013, Luciano, Franciosi et al. 2014, Bogolyubov 2018). Specifically, four stages of GV oocytes, from GV0 to GV3, have been characterized by increasing level of chromatin compaction (Lodde, Modina et al. 2007, Lodde, Modina et al. 2008, Lodde, Modina et al. 2009). GV0 oocytes, which typically show uncondensed chromatin, represent the high majority (around 80%) of oocytes

collected from early antral follicles (0.5–2 mm), are still growing, meiotically non-competent, transcriptionally active and display low levels of global DNA methylation. In contrast, oocytes with increasing levels of compaction (GV1, GV2 and GV3) are considered “fully grown” and are typically collected from medium antral follicles, 2-8 mm in diameter. GV1, GV2, and GV3 oocytes are meiotically competent, while they differ in the ability to form blastocyst after in vitro fertilization, with GV1 oocytes being less competent than GV2 and GV3. Notably, a major drop in transcription occurs during the transition from GV0 to GV1 configuration, similar to the mouse non-surrounded nucleolus/surrounded nucleolus (NSN/SN) transition, also described in humans and other mammalian species (reviewed in (Luciano and Lodde 2013). GV0 to GV1 transition also entails increased levels of global DNA methylation (Lodde, Modina et al. 2009).

The bovine model offers the additional following advantages for the purpose of the proposed study. Firstly, a system for the In Vitro Culture of Oocyte, termed IVCO, is available in cattle (Luciano, Franciosi et al. 2011). Thanks to the use of physiological doses of FSH and PDE3 inhibitors, this system is able to promote the crosstalk between oocytes and cumulus cells and in turn the oocyte growth and the increase of their meiotic competence of when they are further subjected to standard in vitro maturation (IVM) condition (Luciano, Franciosi et al. 2011). Secondly, the transcriptomic profiles of bovine oocytes and surrounding cumulus cells at different stages of differentiation are available. Specifically they were assessed by microarray analysis (Labrecque, Lodde et al. 2015, Dieci, Lodde et al. 2016, Labrecque, Fournier et al. 2016) within the EmbryoGENE program (http://emb-bioinfo.fsaa.ulaval.ca/Home/index.html), that has been implemented by collecting and storing transcriptomic data of bovine oocytes, cumulus and granulosa cells under

different physiological conditions using the same transcriptomic platform and bioinformatic tools. After being analyzed and validated individually in their respective publication, all the collected transcriptomic data sets were subjected to meta-analysis as described in (Khan, Fournier et al. 2016) and made publicly available through an interactive web interface called EmbryoGENE profiler, which thus provides dynamic expression profiles of any gene of interest in the selected sample (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/index.html).

Given the above observation, we have used the bovine growing oocyte as experimental model to assess the extent to which zinc plays a role during the latest phase of oocyte growth and differentiation and particularly to the hypothesis that zinc participates in the control of transcription at this stage.

Materials and methods

All chemicals and reagents were purchased from Sigma-Aldrich S.r.l. (Milan, Italy), unless otherwise stated.

EmbryoGENE dataset mining

The expression profile of genes encoding for proteins involved in intracellular zinc homeostasis (Kimura and Kambe 2016) was assessed by mining the publicly available EmbryoGENE Profiler (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/index.html), which contains profiles of microarray expression data from a variety of tissues and conditions, collected by the researchers within the EmbryoGENE Network (http://emb-bioinfo.fsaa.ulaval.ca (Khan, Fournier et al. 2016)). Specifically, the EmbryoGENE

Profiler was mined to assess the expression profiles of: 1) members of the Solute Carrier Family 39 A (SLC39A), which encode for Zrt- and Irt-like Proteins (ZIP); 2) members of the Solute Carrier Family 30 A (SLC30A), which encode for Zn Transporters (ZnT) and 3) members of the protein coding gene Metallothioneins (MT). Three sets of previously published microarray data that cover the period of oocyte growth and acquisition of meiotic and developmental competence, were considered: 1) GEO series accession number GSE48283:oocytes collected from follicle of different size (<3, 3-5, >5-8, and >8 mm)(Labrecque, Fournier et al. 2016); 2) GEO series accession number GSE48376: oocytes with different degree of chromatin compaction (GV0, GV1, GV2 and GV3) (Labrecque, Lodde et al. 2015) and 3) GEO series accession number GSE79886; cumulus cells isolated from oocytes with different degrees of chromatin compaction (GV0, GV1, GV2 and GV3) (Dieci, Lodde et al. 2016). Importantly, each data set was validated by quantitative PCR as reported in each respective publication.

Oocytes collection and culture

Cumulus-oocytes complexes (COC) were collected and cultured as previously described (Lodde, Modina et al. 2007, Luciano, Franciosi et al. 2011). Briefly, bovine (Holstein Friesians) ovaries were harvested at a local abattoir (IT 2270M CE; Inalca S.p.A., Ospedaletto Lodigiano, LO, Italy) from pubertal females subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. The ovaries were transported to the laboratory within 2 hours in sterile saline solution maintained at 26°C. All the subsequent procedures, unless otherwise specified, were performed between

35°C and 38°C. COCs were retrieved from 2 to 8 mm middle antral follicles with a 19-gauge needle connected to an aspiration pump (COOK-IVF, Brisbane QLD, Australia) with a vacuum pressure of -28 mm/Hg. After aspiration, small pieces of ovarian cortex were sliced and examined under a dissecting microscope. COCs were isolated from early antral follicles (0.5 – 2 mm diameter) by rupturing the follicle wall with a 21-gauge needle. COCs were washed in Medium 199 supplemented with HEPES 20 mM, 1,790 units/L Heparin and 0.4% of bovine serum albumin (M199-D) and examined under a stereomicroscope. COCs from early antral follicles were collected within 2 hours. Only compact COCs with five or more complete layers of cumulus cells and a finely granulated homogenous ooplasm were used. The population of oocytes collected from this follicular class typically contain around 80% of GV0 stage oocytes and their mean diameter is 110 µm (Lodde, Modina et al. 2007, Luciano, Franciosi et al. 2011).

The in vitro culture of growing oocyte (IVCO) was performed as previously described with minor modification (Luciano, Franciosi et al. 2011). Oocytes were always cultured in the presence of cumulus cells as COCs. COCs were cultured in 500 µl of Medium 199 with 25 mM NaHCO3, and further supplemented with 0.4% fatty acid free BSA, 0.68 mM L-glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, 10^{-4} IU/ml of recombinant human follicle-stimulating hormone (r-hFSH; Gonal F; Serono S.p.A.) and 10 µM cilostamide, for 24 h or for different times intervals according to the experimental design (see below), in four-well dishes (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) at 38.5°C under 5% CO_2 in humidified air. According to the experimental design, IVCO medium was supplemented with 6.6 µg/mL zinc sulphate, which corresponds to 1.5 µg/µl of Zn^{2+}. This concentration is within the range of follicular fluid content and has been proved to be beneficial during bovine in vitro maturation (Picco, Anchordoquy et al. 2010, Anchordoquy,

Anchordoquy et al. 2014). In a preliminary set of experiments, the IVCO medium was supplemented with 10 µM *N*,*N*,*N′*,*N′*-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN), a cell permeable zinc chelator (Kim, Vogt et al. 2010). To assess the effect of zinc modulation on meiotic competence, after IVCO, COCs were washed in M199-D and cultured for 24h under standard serum free IVM conditions. IVM medium was M199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO3, 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, and 0.1 IU/ml of r-hFSH (Luciano, Franciosi et al. 2011). To assess meiotic progression, at the end of IVM, cumulus cells were mechanically removed and oocytes were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, stained with DAPI, and analyzed by fluorescence microscopy to assess meiotic progression. Oocytes from the GV breakdown to the metaphase I stage were classified as intermediate; oocytes at the anaphase I, telophase I, and metaphase II stages as mature; and oocytes that could not be identified as being at any of the previous stages as degenerate (Luciano, Franciosi et al. 2011).

Assessment of labile zinc content in the oocytes

Labile zinc content in oocytes with different degree of chromatin compaction at the time of collection was assessed using the cell permeant zinc indicator FluoZin™3-AM dye (Molecular Probes, Thermo Fisher Scientific), which had previously been used for spatial and temporal resolution of labile zinc concentrations in both mouse and bovine oocytes (Kim, Bernhardt et al. 2011, Lisle, Anthony et al. 2013, Que, Duncan et al. 2019). Further validation of the FluoZin™3-AM using the zinc chelator TPEN is reported in supplemental Figure 1. After collection, COCs from early

and medium antral follicles were incubated in 2 μM of FluoZin-3 and mixed to an equal volume of Pluronic® F-127 20% solution in DMSO (Invitrogen, Molecular Probes, Thermo Fisher Scientific) dissolved in 500 μL of M199 with 25 mM $NaHCO_3$ and supplemented with 0.68 mM L-glutamine, 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 μg/mL kanamycin and 0.5 mM IBMX, in 4-well dishes at 38.5 °C under 5% CO_2 in humidified air for 90 min. After 1-hour incubation 1 μg/mL of Hoechst 33342 was added to each well. Subsequently, COCs were denuded mechanically by pipetting to remove cumulus cells in M199D supplemented with IBMX and 1 μg/mL of Hoechst 33342. Oocytes were placed on slides in 10 μL drops of the same medium and covered with a coverslip using secure-seal spacer (Invitrogen, Thermo Fisher Scientific). Digital images of each samples were immediately acquired using an epifluorescence microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan) equipped with a digital camera (Nikon DS-F12) maintaining identical acquisition settings for all samples. All the images were acquired at the focal plane in which the chromatin was more focused. The oocyte labile zinc content was estimated by assessing the mean fluorescent intensity value emitted by the probe using ImageJ software (1.49v). The Total Fluorescent intensity emitted by the oocyte area (integrated density) was subtracted by the background and then normalized by the oocyte area. Mean Fluorescent intensity values were Log2 transformed. GV stages were classified according to the degree of chromatin compaction, as previously described (Lodde, Modina et al. 2007). The GV0 stage is characterized by a diffuse filamentous pattern of chromatin in the whole nuclear area; the GV1 stage shows a few points of chromatin condensation; the GV2 stage has the chromatin condensed in distinct clumps; the GV3 stage is the highest levels of compaction with chromatin organized in a single clump within the nuclear envelope (Luciano and Lodde 2013).

Assessment of global transcriptional activity

Transcription was assessed in growing oocytes cultured as COCs in IVCO medium as described above, in the presence or absence of zinc sulphate for 17h. In a second set of experiments, transcription was evaluated in oocytes treated with 10 µM zinc chelator TPEN, in the absence or presence of zinc sulphate for 5 h to prove TPEN specificity as previously described (Kim, Vogt et al. 2010) . Global transcriptional activity was evaluated using the Click-iT® RNA Imaging Kit (Invitrogen, Thermo Fisher Scientific), as previously described in mice (Sanchez, Romero et al. 2015) and following the manufacture's guide.

Briefly, after treatment, COCs were incubated with 2 mM 5-ethynyl uridine (EU) diluted in IVCO medium for 1 h at 38.5°C 5%CO_2. Note that zinc and/or TPEN were included in the medium during EU incorporation, according to the experimental design. After EU incorporation, cumulus cells were mechanically removed, oocytes were briefly washed in warm PBS/PVA, fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, and washed again in PBS/PVA. For detection of EU incorporation into nascent RNA, samples were permeabilized in 0.5% Triton-X 100 in PBS for 15 min at room temperature, briefly washed in PBS/PVA and incubated in Click-iT® reaction cocktail for 30 min at RT protected from light, further washed once in Click-iT® reaction rinse buffer and once more in PBS/PVA. Oocytes were finally mounted and stained in mounting media Vectashield antifade mounting media with DAPI (Vector, Burlingame, CA, USA), using double-sticky tape between the slide and the cover glass. Samples were analyzed and imaged under an epifluorescence microscope as described above, maintaining identical acquisition settings for all the samples. Chromatin configuration

was evaluated under the fluorescence microscope. The GV stage was classified according to the degree of chromatin compaction, as previously described (Lodde, Modina et al. 2007). The level of global transcriptional activity was estimated by assessing the mean fluorescent intensity value emitted by the incorporated EU using NIH ImageJ software (1.49v) (Schneider, Rasband et al. 2012). The mean Fluorescent intensity emitted by the nuclear area was subtracted by the mean background intensity of the oocyte's cytoplasm. Note that transcription was quantified only in oocytes that retained a GV0 configuration during culture.

Assessment of global DNA methylation

Levels of global DNA methylation were assessed by indirect immunofluorescence as previously described (Lodde, Modina et al. 2009) in oocytes subjected to IVCO for 17 h in the presence or absence of zinc sulphate. Briefly, after culture as COC, the oocytes were freed of cumulus cells and the zona pellucida was digested with 0.5% of pronase. Samples were then fixed in 4% paraformaldehyde in PBS for 1 h at 4°C, washed in 0.05% Tween 20 in PBS, permeabilized with 0.2% Triton X100, 0.05% Tween 20 in PBS for 30 min at room temperature, treated in 2 M HCl for 30 min at room temperature to obtain DNA denaturation and then neutralized in 100 mM Tris HCl buffer (pH 8.5) for 10 min. Non-specific binding was blocked by incubating the samples in 20% normal donkey serum, 1% BSA in PBS for 1 h at room temperature before primary antibody incubation. The samples were incubated overnight at 4°C with a mouse anti-5-methylcytosine (Eurogentec SA, Seraing Belgium) 1:500 in PBS containing 1% BSA and 0.05% Tween 20. Samples were extensively washed in 0.05% Tween 20 in PBS and incubated for 1 h at RT with a donkey anti mouse conjugated

with Alexa Fluor 488 (Molecular Probes, Thermo Fisher Scientific, 1:500) in PBS containing 1% BSA and 0.05% Tween 20. Samples were mounted in Vecta Shield supplemented with DAPI, incubated for 5 minutes at room temperature and immediately observed under epifluorescence microscopy. Images were captured under the same exposure conditions. The level of global DNA methylation was estimated by assessing the mean fluorescent intensity value emitted by the incorporated EU using NIH ImageJ software (1.49v) (Schneider, Rasband et al. 2012). The mean Fluorescent intensity emitted by the nuclear area was subtracted by the mean background intensity of the oocyte's cytoplasm. Note that DNA methylation was quantified only in oocytes that retained a GV0 configuration.

Statistical analysis

Experiments were run in 3 independent experiments, unless otherwise specified. Statistical analysis was performed using GraphPad Prism software (GraphPad Prism v. 8, La Jolla, CA, USA). Data were pooled and expressed as mean ± SEM. Data were firstly analyzed by D'Agostino & Pearson test, in order assess whether data distribution was normal. Parametric tests (Student's t test and one-way ANOVA followed by Tukey's Multiple Comparison test) were used to determine differences when data where normally distributed. A non-parametric test (Mann Whitney test) was chosen when the data were not normally distributed or assumption on distribution could not be made. In the experiments where the labile zinc content was evaluated, values were Log2 transformed. Details on the statistical analysis are indicated in the figure caption. $P<0.05$ were considered statistically significant and

indicated as *. Further differences, P<0.01, P<0.001, P<0.0001 are noted as **, *** and ****, respectively.

Results

Zinc transporters and metallothionein's encoding mRNAs are differentially expressed during oocyte growth and differentiation

To gain insights into the possible role of zinc during oocyte growth and differentiation that occur within the antral follicle before meiotic resumption, we mined the EmbryoGENE Profiler to assess the expression profiles of genes that encode for proteins that modulate zinc homeostasis in mammalian cells (Kimura and Kambe 2016, Maret 2017). Specifically, two major classes of ion transporters participate in this process: the SLC39A family members, which encode for ZIP proteins that transport zinc into the cytosol from either the extracellular space or intracellular stores such as the endoplasmic reticulum, and the SLC30A family members, which encode for the ZnT proteins that mediate zinc efflux from the cytosol (Kimura and Kambe 2016). Moreover, MTs bind metals intracellularly, thus lowering their concentration at critical sites, or function as buffering molecules providing labile zinc for use by target proteins or enzymes when zinc is limited (Kimura and Kambe 2016). Thus, the EmbryoGene profiler was mined to assess the expression profile of the known members of the *SLC39A*, *SLC30A* and *MT* gene families in oocytes collected from follicle of different size (Labrecque, Fournier et al. 2016) as well as in oocytes with different degree of chromatin compaction and in their surrounding cumulus cells (Labrecque, Lodde et al. 2015, Dieci, Lodde et al. 2016).

As summarized in **Table 1**, the expression profiles of 21, 14 and 6 probes (representing constitutive, alternative 3'UTR and splicing variants) of the *SLC39A*, *SLC30A* and *MT* gene families were considered, respectively. Of these, several transcripts were differentially expressed in oocytes (Figure 1A and B). Specifically, *SLC39A7*/ZIP7, *SLC39A8*/ZIP8, *SLC39A10/*ZIP10 (both constitutive and alternative 3'UTR forms), *SLC39A12*/ZIP12, *SLC39A14/*ZIP14 (alternative 3'UTR), *SLC30A1/*ZnT1, *SLC30A5/*ZnT5 were differentially expressed according to follicle size and the degree of chromatin compaction. Furthermore *SLC39A6/*ZIP6, *SLC39A10/*ZIP10 (Alternative 3'UTR) and *SLC30A9/*ZnT9 were differentially expressed uniquely in oocytes derived from follicle of different size (**Figure 1A**), while *SLC39A9/*ZIP9 (alternative 3'UTR), *SLC30A3*/ZnT3, *SLC30A6*/ZnT6 and *SLC30A7*/ZnT7 were differentially expressed uniquely in oocytes with different degree of chromatin compaction (**Figure 1B**). A lower number of genes, namely *SLC39A7*/ZIP7, *SLC39A8*/ZIP8, *SLC39A14*/ZnT14 alternative 3'UTR and *SLC30A4*/ZnT4*,* were differentially expressed in cumulus cells according to chromatin configuration (**Figure 1C**).

Zinc supplementation improves the meiotic competence of growing oocytes

Unlike fully-grown oocytes isolated from middle antral follicles, bovine oocytes collected from early antral follicles are not capable of spontaneously resuming meiosis once isolated from the follicular compartment and progressing to the MII stage. However, their ability to undergo meiosis and early embryonic development is, at least partially, enhanced when oocytes are cultured for 24 h before in vitro maturation in a low FSH concentration-based culture system that sustains oocyte-cumulus cells

coupling (Luciano, Franciosi et al. 2011). To the best of our knowledge this is one of the few available culture systems for this class of oocytes. In the present study we used this culture system as experimental model to further assess the role of zinc during oogenesis. Preliminary experiments showed a detrimental/toxic effect of long-term treatment with the zinc chelator TPEN, as none of the oocytes exposed to IVCO supplemented with TPEN for 24 h was able to progress through meiosis when further subjected to standard IVM and, instead appeared degenerated. Therefore, we focused our attention on the effects of supplementing the IVCO medium with zinc and evaluating the effect on meiotic progression after standard IVM.

As shown in **Figure 2**, zinc supplementation induced a significantly higher percentage of mature oocytes and a lower percentage of oocytes at intermediated stages of meiotic progression, when compared to the control group. No differences were observed in the percentage of oocytes that did not resume meiosis nor in the degenerated class.

Labile zinc content decreases during physiological transcriptional silencing

The increased meiotic competence in response to zinc supplementation during IVCO suggests that zinc supports some of the processes that play a role in oocyte competence acquisition. Typically, the high majority (around 80%) of growing oocytes have their chromatin organized in a GV0 configuration and are transcriptionally active (Fair, Hyttel et al. 1995, Fair, Hyttel et al. 1996, Lodde, Modina et al. 2007, Lodde, Modina et al. 2008, Luciano, Franciosi et al. 2011). Therefore, we hypothesized that zinc supplementation might support transcription in GV0 oocytes. We tested this hypothesis using two experimental approaches.

First, we evaluated the labile zinc content in oocytes with different degrees of chromatin compaction at the time of isolation form early and middle antral follicles, since a major transcriptional silencing occurs during the transition from GV0 to GV1 stages and GV1, GV2 and GV3 oocytes are typically collected from middle antral follicles (Lodde, Modina et al. 2007, Lodde, Modina et al. 2008). As shown in **Figure 3**, quantification of the FluoZin™3-AM dye signal fluorescent intensity revealed a significant decrease of labile zinc content during the transition from GV0 to GV1 oocytes. Labile zinc remained low in GV2 oocytes, while tended to increase again in GV3 oocytes (**Figure 3B**).

Modulation of zinc availability affects global transcriptional activity

To more directly evaluate the role of zinc in the modulation of oocyte's transcription, in a second set of experiments, we supplemented the IVCO medium with zinc and/or with the zinc chelator TPEN and assessed global transcription in GV0 oocytes by means of EU incorporation into nascent RNA. As shown in **Figure 4A** this method clearly allowed the detection of RNA transcription in bovine oocytes. At the time of isolation, a positive signal was detectable only in GV0 oocytes and not in the negative control or fully-grown GV1 oocytes. Zinc supplementation for 17 hours did not change the distribution of GV stages, and the percentage of GV0 oocytes remained high in both control and zinc supplemented group (**Figure 4C**). Importantly, quantification of the fluorescent signal revealed that global transcription was enhanced in the zinc treated group (**Figure 4D, E**).

When oocytes were exposed to the cell permeant zinc chelator TPEN, the level of global transcription significantly decreased (**Figure 5**, further supporting the

hypothesis of a role of zinc in sustaining the transcriptional activity in growing oocytes. Notably, and as a control of the specificity of TPEN activity in acting as a zinc chelator, the TPEN effect was rescued by zinc supplementation, that restored the global transcription at levels comparable with the control group (**Figure 5C**).

Zinc supplementation affects global DNA methylation

Notably, we did not observe gross morphological changes of the chromatin configuration after zinc supplementation under epifluorescence microscopy. Therefore, to further assess the effects of zinc supplementation on chromatin structure, we assessed the level of global DNA methylation in oocytes subjected to IVCO in the presence or absence of zinc, as DNA methylation well correlates with transcriptional activity (reviewed in(Sendzikaite and Kelsey 2019)). As shown in **Figure 6**, the level of global DNA methylation is significantly lower in GV0 oocytes cultured in the presence of zinc when compared to that observed in oocytes cultured in control IVCO condition. This results well correlates with higher levels of transcription in response to zinc supplementation.

Discussion

The major finding of the present study is that altering zinc availability, by means of culture medium supplementation during the final phase of their growth, supports global transcriptional activity, modulates global DNA methylation and ultimately enhances acquisition of meiotic competence. Specifically, the present data indicate that the population of bovine oocytes that benefit from zinc supplementation during

culture are the ones still engaged in accumulating substances for further development that are collected from early antral follicles. The final period of growth and differentiation is a crucial phase for the oocyte, as the epigenome is profoundly remodeled. Remodeling includes large-scale morphological re-organization of the chromatin to form the karyosphere (Bogolyubov 2018), global transcriptional silencing as well as epigenetic modifications of DNA, such as DNA methylation, and histones proteins (De La Fuente 2006, O'Doherty, O'Shea et al. 2012, Tomizawa, Nowacka-Woszuk et al. 2012, Luciano and Lodde 2013). These modifications ultimately confer the oocyte with an appropriate developmental programme.

Preliminary indication of the importance of zinc homeostasis, which in mammalian cells is performed by carrying zinc across cell compartments, during oocyte growth, was revealed by the assessment of gene expression profiles of zinc transporters and methallotionein in the EmbryoGENE transcriptomic platform. To the best of our knowledge, excluding data on *SLC39A6/*ZIP6 and *SLC39A10/*ZIP10 (Kong, Duncan et al. 2014) during murine oocyte maturation, no data are available on the function of other zinc transporters or MTs during mammalian oogenesis. An interesting hypothesis in the context of ovarian function is that different cellular compartments of the follicle cooperate to modulate zinc content in the follicular fluid, and ultimately in the germ cell. In this view, the EmbryoGENE profiler provides interesting targets to be further functionally characterized. For instance, *SLC39A8,* which encodes for ZIP8, would deserve further attention since it is differentially expressed also in granulosa cells at different stages of follicle development as reported by the EmbryoGENE Profiler, (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/cgi-bin/DoProfile.cgi?gene=slc39a8&tissue=granulosa, July 28, 2019).

Even though the characterization of zinc transporters and MTs mRNA expression profiles is not sufficient to predict the overall zinc balance in growing oocytes, the evidences that several zinc related transcripts are differentially expressed during this period, in both the oocyte and the surrounding cumulus cells, suggests that COCs actively control the abundance of these mRNAs, probably to modulate zinc signaling even long before meiotic resumption. This would lead to the statement that zinc availability in in vitro systems aimed to support oocyte growth has been underestimated so far. To the best of our knowledge, apart from the one developed in our laboratory and used as a base in the present study (Luciano, Franciosi et al. 2011), just few other studies focused on the in vitro culture of growing oocytes isolated from early antral follicles (Hirao, Itoh et al. 2004, Alm, Katska-Ksiazkiewicz et al. 2006, Endo, Kawahara-Miki et al. 2013, Makita and Miyano 2014, Alam, Lee et al. 2018), while others had focused on culture of preantral oocytes. None of the published protocols can fully support embryonic developmental competence of growing oocytes. However, with the exception of serum-supplemented media, attempts to grow oocytes in vitro have been generally performed in the absence of zinc. In this study, we demonstrate that zinc supplementation to the IVCO system described by (Luciano, Franciosi et al. 2011) benefits the acquisition of meiotic competence. This result is highly relevant in the context of modern cattle farming, which is increasingly relying on in vitro embryo production (Blondin 2015, Perry 2017). In the practice, only fully-grown oocytes, which are directly subjected to IVM, are exploited. Conversely, growing oocytes contained in early antral follicles are generally discarded due to the lack of vitro culture systems able to support their development thus reducing the possibility of exploiting the ovarian reserve more efficiently (Lussier, Matton et al. 1987, Silva-Santos, Santos et al. 2011, Monget, Bobe et al. 2012). Hence, our findings provide insights on how to improve

culture media and strategies of assisted reproduction in livestock. Moreover, given the relevance of the bovine model to human ovarian physiology (Langbeen, De Porte et al. 2015, Polejaeva, Rutigliano et al. 2016), our findings would likely aid studies aimed at treating human infertility.

Defining the mechanism(s) by which zinc supports the acquisition of meiotic competence is not an easy task, due to the vast number of biological processes in which it is implicated (Beyersmann and Haase 2001). Indeed, it has been estimated that around 2800 human proteins potentially bind zinc in vivo, which account for 10% of the human proteome (Andreini, Banci et al. 2006). Therefore, we focused our attention on one of the most important process occurring in the growing oocytes: transcription, which must be active to allow accumulation of large amounts of RNAs and proteins during growth and must be silenced timely in preparation for meiotic resumption. Zinc is a well-known regulator, acting both as activator and repressor, of gene expression machinery (Andrews 2001, Jackson, Valentine et al. 2008) and epigenetic modification, as many DNA modifiers enzymes bear zinc fingers motifs (Hudson and Buck-Koehntop 2018, Blanquart, Linot et al. 2019, Rausch, Hastert et al. 2019). Its role in controlling transcription has been considered also in the context of development of model organisms (Falchuk 1998) as well as in the mouse preimplantation embryo, where it has been shown that TPEN-induced zinc insufficiency during in vitro culture was associated with altered chromatin structure in the blastomere nuclei and decreased global transcription (Kong, Duncan et al. 2015). Moreover, in the mouse model, it has been shown that transcriptionally active oocytes react to changes of zinc availability by modulating the localization of the zinc sensor MTF-1(Kong, Duncan et al. 2014) presumably to control zinc homeostasis (Andrews 2001). The present study expands these observations by showing that major

transcriptional silencing in bovine oocytes, which in vivo occurs during the transition from GV0 to GV1 configuration, is associated to a decrease of labile zinc content. Accordingly, a short-term treatment with zinc chelator TPEN induces a sudden reduction of transcription which is rescued by zinc supplementation. This might suggest that the signals that lead to transcriptional silencing in oocytes involves, at least in part, a decrease in labile zinc. Interestingly, labile zinc remains relatively low in GV2 oocytes, while it increases in GV3 oocytes, in which transcription is completely silenced (Lodde, Modina et al. 2008). This agrees with the previously postulated hypothesis that increased chromatin condensation may reinforce transcriptional repression once it is initiated (De La Fuente 2006, Luciano and Lodde 2013). If so, we can speculate that GV3 oocytes would not be sensitive to increased labile zinc content, which in turn might correlate with increase of total zinc required during maturation in both cow and mice (Kim, Vogt et al. 2010, Que, Duncan et al. 2019). This hypothesis needs to be tested. Moreover, it will be of utmost importance to assess whether oocytes with different chromatin configurations vary in terms of total zinc content, as described in (Que, Duncan et al. 2019)

Importantly we provide evidences that zinc supplementation enhances transcription in cultured GV0 oocytes, which well correlates with the maintenance of global level of DNA methylation that is known to characterize GV0 oocytes at the time of collection (Lodde, Modina et al. 2009). This likely accounts, at least in part, for the increased meiotic competence of zinc treated oocytes in our experimental model. Future mechanistic studies should be conducted to further clarify whether zinc supplementation affects the amount of labile and total zinc in the oocyte and, importantly, in the surrounding cumulus cells and thus clarify the molecular mechanisms through which zinc enhance oocyte capacity to undergo maturation.

Nevertheless, our finding could be exploited to further ameliorate the cultural system, aiming at prolonging the period in which the oocytes remain transcriptionally active while allowing timely transcriptional silencing when the oocytes have accumulated the critical molecules for subsequent development.

Conclusion

A relationship between zinc deficiency and infertility in dairy cows has been known from many years; zinc deficiency results in increased embryonic mortality and reduced conception rates (Hidiroglou 1979). This might be due, at least in part, to the role that zinc plays in oocyte development, although the mechanisms are not completely elucidated. Here we provide evidences that zinc modulates transcription during a critical step of oocyte development, which is when its developmental program is largely determined. Our results agree with previous findings in mice, where feeding a zinc deficient diet during preconception (3–5 days before ovulation) alters oocyte chromatin methylation (histone H3K4 trimethylation and global DNA methylation) and expression of specific genes (Tian and Diaz 2013).

Clearly, it is likely that zinc participate in many other biological functions in the growing oocytes such as mitochondrial function and metabolism regulation, and might have Antioxidant/Prooxidant activity (Lee 2018). Further studied are required to assess this hypothesis. Finally, of outmost importance will be the assessment of which are the relative contributions of the cumulus cells and the oocytes to the maintenance of zinc homeostasis during oogenesis as well as the characterization of specific biomarkers that primarily control importance of zinc transport and storage during oogenesis.

Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

V.L. acknowledges the receipt of a fellowship from the OECD Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems in 2016 (contract n. JA00091594), which supported part of this study. Part of the experiments were funded by the “Piano Strategico Linea B” of the University of Milan (PSR2019_DIP_AMLUC to A.M.L, PSR2018_DIP_027_VLODD to V.L., and PSR2018_DIP_027_FFRAN to F.F.). P.C.D.A was supported by Scholarship from Capes foundation /PDSE/88881.134900/2016-01. EmbryoGENE Strategic Network was funded by Natural Sciences and Engineering Research Council of Canada (NSERC), Canada. C.R. acknowledges the Natural Sciences and Engineering Research Council of Canada (RGPIN-2017-04775)

Author’s contribution

VL and AML conceived the study. CD and VL participated in EmbryoGENE data analysis. MAS contributed to the study design and EmbryoGene data Analysis. RGB, VL and AML performed experiments on the assessment of the effect of zinc supplementation on meiotic competence and DNA methylation. PCDA, AB CR and VL performed experiments on the assessment on labile zinc content in oocytes. PCDA and FF performed experiments on the assessment of transcriptional activity. VL wrote the manuscript. All authors reviewed and approved the manuscript

Acknowledgements

Authors are grateful to INALCA S.p.A. (Ospedaletto Lodigiano, LO) and support of the Veterinary staff. We would like to thank all the scientists of the EmbryoGENE Network that contributed in creating the EmbryoGENE profiler.

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Figures/ tables

Table 1:

List of zinc related targets identified within the EmbyoGENE profiler web site. The genes whose expression level is significantly affected by the follicular size in the oocyte (Labrecque, Fournier et al. 2016), and by the chromatin status in the oocyte and the corresponding cumulus cells (Labrecque, Lodde et al. 2015, Dieci, Lodde et al. 2016), are marked in dark grey. Expression profiles for each of the indicated probe are shown in Figure 1 and can be assessed in the original dataset (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/). The dataset was last mined on June 4th 2019.

Gene of interest					**Cell type and variable considered in the microarray study**		
					Oocyte from follicle of different size	**Oocyte with different chromatin configuration**	**Cumulus cells isolated from oocytes with different chromatin configuration**
EmbryoGene Probe	**Gene symbol**	**Gene Name**	**Encoded protein Symbol**	**Type**	*Labrecque et al, MRD 2016*	*Labrecque et al, MRD 2015*	*Dieci et al, MHR 2016*

EMBV3_32972	***SLC39A1***	solute carrier family 39 member 1	**ZIP 1**	Constitutive			
EMBV3_18524	***SLC39A2***	solute carrier family 39 member 2	**ZIP 2**	Constitutive			
EMBV3_02694	***SLC39A3***	solute carrier family 39 member 3	**ZIP 3**	Constitutive			
EMBV3_12697	***SLC39A4***	solute carrier family 39 member 4	**ZIP 4**	Constitutive			
EMBV3_01886	***SLC39A5***	solute carrier family 39 member 5	**ZIP 5**	Constitutive			
EMBV3_09740	***SLC39A5***	solute carrier family 39 member 5	**ZIP 5**	Splicing variant			
EMBV3_02273	***SLC39A6***	solute carrier family 39 member 6	**ZIP 6**	Constitutive	*		
EMBV3_34613	***SLC39A7***	solute carrier family 39 member 7	**ZIP 7**	Constitutive	*	*	*
EMBV3_42354	***SLC39A8***	solute carrier family 39 member 8	**ZIP 8**	Constitutive	*	*	*
EMBV3_16082	***SLC39A9***	solute carrier family 39 member 9	**ZIP 9**	Alternative 3'UTR			
EMBV3_17943	***SLC39A9***	solute carrier family 39 member 9	**ZIP 9**	Constitutive			
EMBV3_24626	***SLC39A9***	solute carrier family 39 member 9	**ZIP 9**	Alternative 3'UTR		*	
EMBV3_15122	***SLC39A10***	solute carrier family 39 member 10	**ZIP 10**	Alternative 3'UTR	*		
EMBV3_29633	***SLC39A10***	solute carrier family 39 member 10	**ZIP 10**	Constitutive	*	*	
EMBV3_31415	***SLC39A10***	solute carrier family 39 member 10	**ZIP 10**	Alternative 3'UTR	*	*	
EMBV3_10318	***SLC39A11***	solute carrier family 39 member 11	**ZIP 11**	Constitutive			
EMBV3_21073	***SLC39A12***	solute carrier family 39 member 12	**ZIP 12**	Constitutive	*	*	
EMBV3_19564	***SLC39A13***	solute carrier family 39 member 13	**ZIP13**	Constitutive			

EMBV3_01984	***SLC39A14***	solute carrier family 39 member 14	**ZIP 14**	Constitutive			
EMBV3_03243	***SLC39A14***	solute carrier family 39 member 14	**ZIP 14**	Alternative 3'UTR	*	*	
EMBV3_12779	***SLC39A14***	solute carrier family 39 member 14	**ZIP 14**	Alternative 3'UTR			*
EMBV3_38125	***SLC30A1***	solute carrier family 30 member 1	**ZnT 1**	Alternative 3'UTR			
EMBV3_41937	***SLC30A1***	solute carrier family 30 member 1	**ZnT 1**	Alternative 3'UTR			
EMBV3_21137	***SLC30A1***	solute carrier family 30 member 1	**ZnT 1**	Constitutive	*	*	
EMBV3_40498	***SLC30A2***	solute carrier family 30 member 2	**ZnT 2**	Constitutive			
EMBV3_41101	***SLC30A2***	solute carrier family 30 member 2	**ZnT 2**	Splicing variant			
EMBV3_42173	***SLC30A3***	solute carrier family 30 member 3	**ZnT 3**	Constitutive		*	
EMBV3_18378	***SLC30A4***	solute carrier family 30 member 4	**ZnT 4**	Constitutive			*
EMBV3_16827	***SLC30A5***	solute carrier family 30 member 5	**ZnT 5**	Constitutive	*	*	
EMBV3_09102	***SLC30A6***	solute carrier family 30 member 6	**ZnT 6**	Constitutive		*	
EMBV3_12614	***SLC30A6***	solute carrier family 30 member 6	**ZnT 6**	Splicing variant			
EMBV3_36463	***SLC30A6***	solute carrier family 30 member 6	**ZnT 6**	Splicing variant			
EMBV3_18316	***SLC30A7***	solute carrier family 30 member 7	**ZnT 7**	Constitutive		*	
EMBV3_39734	***SLC30A9***	solute carrier family 30 member 9	**ZnT 9**	Constitutive	*		
EMBV3_02033	***SLC30A10***	solute carrier family 30 member 10	**ZnT 10**	Constitutive			
EMBV3_08028	***MT1A***	metallothionein-1A	**MT1A**	Constitutive			

EMBV3_07532	***MT1E***	metallothionein-1E	**MT1E**	Constitutive			
EMBV3_34220	***MT1E***	metallothionein-1E	**MT1E**	Constitutive			
EMBV3_06636	***MT2A***	metallothionein-2E	**MT2E**	Constitutive			
EMBV3_40027	***MT3***	metallothionein-3	**MT3**	Constitutive	*	*	
EMBV3_30913	***MT4***	metallothionein-4	**MT4**	Constitutive			

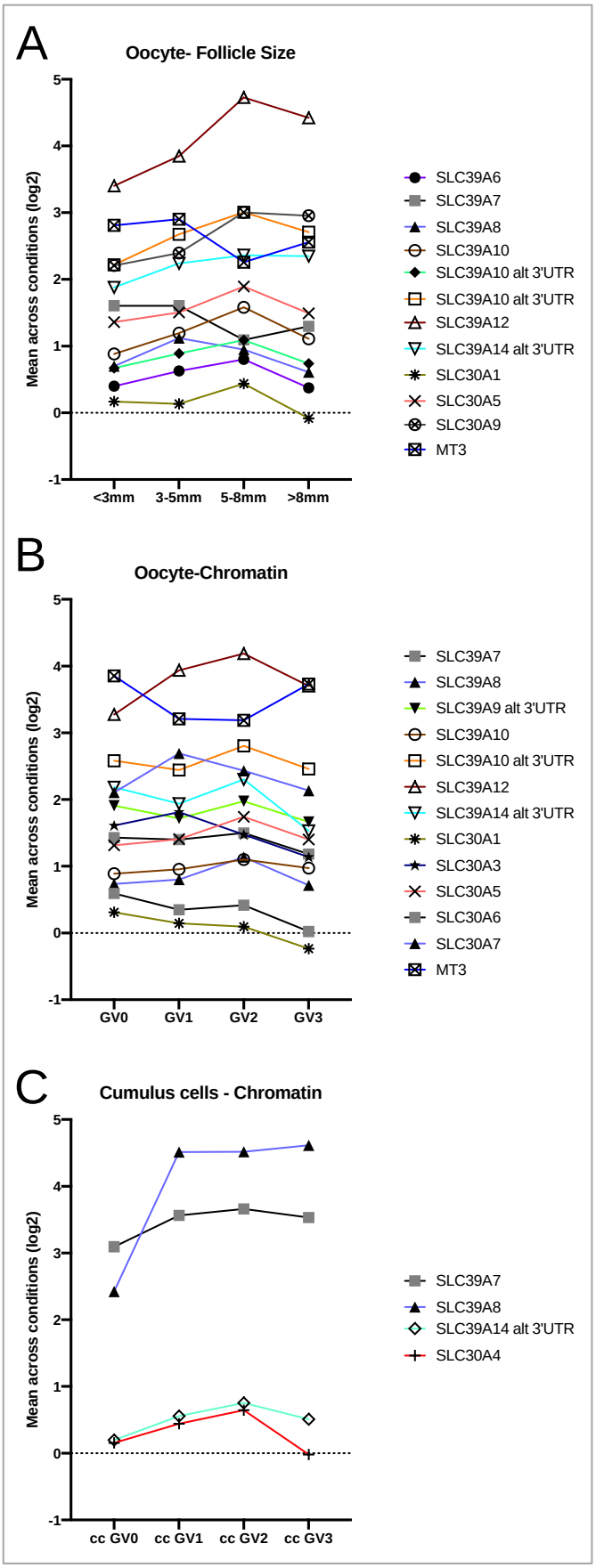


Fig. 1 Levels of differentially expressed mRNAs encoding zinc transporters (ZIP and ZnT) and metallothioneins (MT) during oocyte growth and differentiation

Graphs showing changes of mRNA levels encoding the differentially expressed members of the SLC39A, SLC30A and MT gene families in oocytes collected from follicles of different sizes (**A**) and in oocytes and corresponding cumulus cells with different degrees of chromatin compaction (**B and C**). The data represent the profiles in the original microarray analysis and are obtained/imported from the EmbryoGENE profiler website (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/) on June 4th 2019. Data were generated by (Labrecque, Fournier et al. 2016) and (Labrecque, Lodde et al. 2015, Dieci, Lodde et al. 2016).

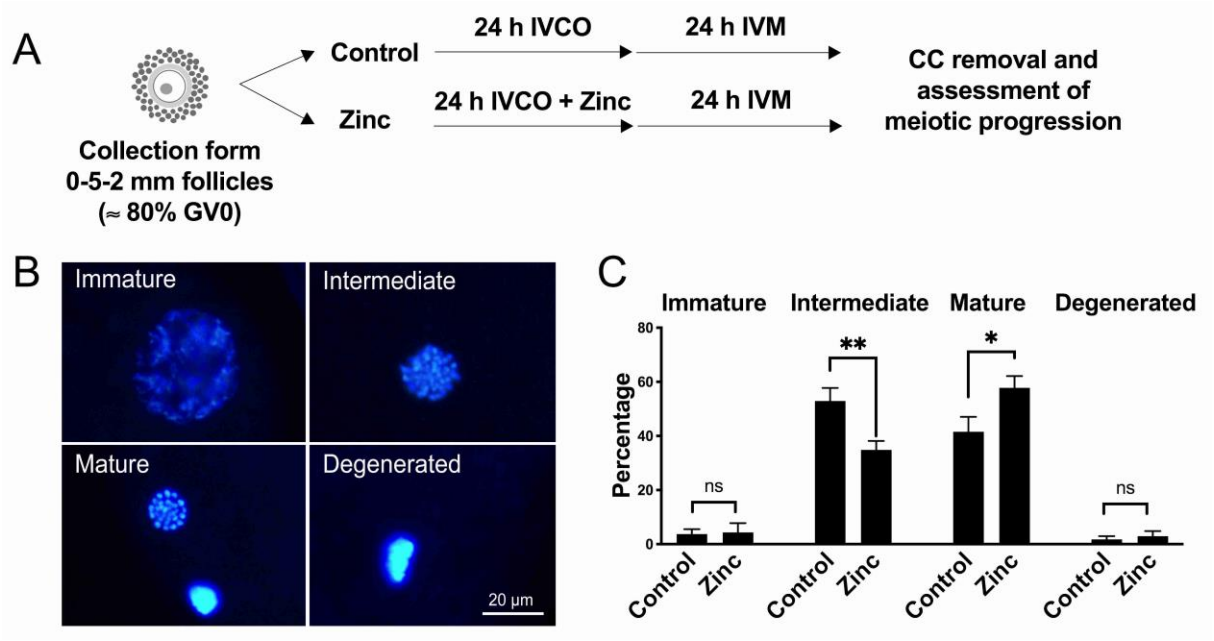


Figure 2: Effect of zinc supplementation during IVCO on meiotic competence acquisition

(**A**) Schematic representation of the experimental design (**B**) Representative epifluorescent images showing immature (GV), intermediate (MI), Mature (MII, polar view and first polar body) and Degenerated oocytes as assessed by DNA staining with DAPI (**C)** Graphs showing the percentage of immature, intermediate, mature and degenerated oocytes in the Control (IVCO in the absence of zinc followed by standard IVM) and zinc (IVCO in the presence of zinc followed by standard IVM) groups. Data were analyzed by Mann-Whithney Test. Values are means ± SEM (n=6). * and ** indicates significant differences between groups (*P<0.05; ** P<0.01). A total of 98 and 102 oocytes were analyzed for Control and zinc groups, respectively in three independent experiments with two technical replicates each.

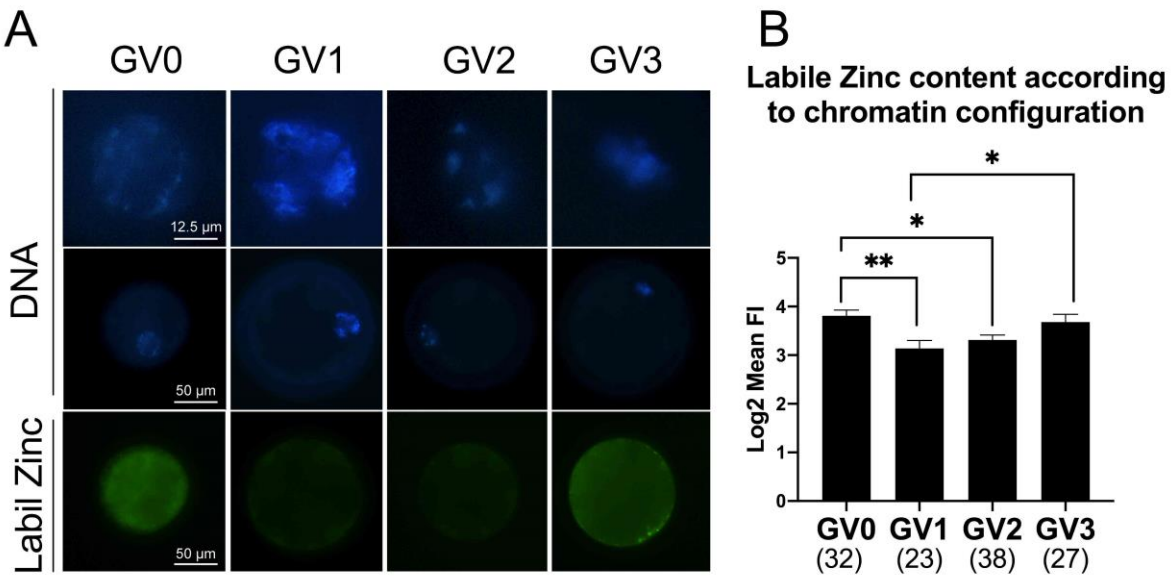


Figure 3: Relationship between large-scale chromatin configuration of the oocyte and labile zinc content

(A) Representative images showing chromatin staining by Hoechst33342 (upper and middle panels) and Labile zinc staining by Fluo Zin (Lower panel) in oocytes with GV0, GV1, GV2 and GV3 chromatin configuration. All Images were captured at the GV focal plane **(B)** Graph showing quantification of labile zinc content as assessed by Fluo Zin loading ad fluorescent image analysis in growing oocytes collected from small antral follicle with a GV0 chromatin configuration, and fully-grown oocytes collected from middle antral follicle with GV1, GV2 and GV3 chromatin configuration as assessed by Hoechst 33342 staining. Total numbers of oocytes analyzed are shown in brackets. Data were pooled together and analyzed by 1-way ANOVA followed by Tukey's multiple comparison test. Values are means ± SEM. * and ** indicate significant differences between groups (*P<0.05; **P<0.01).

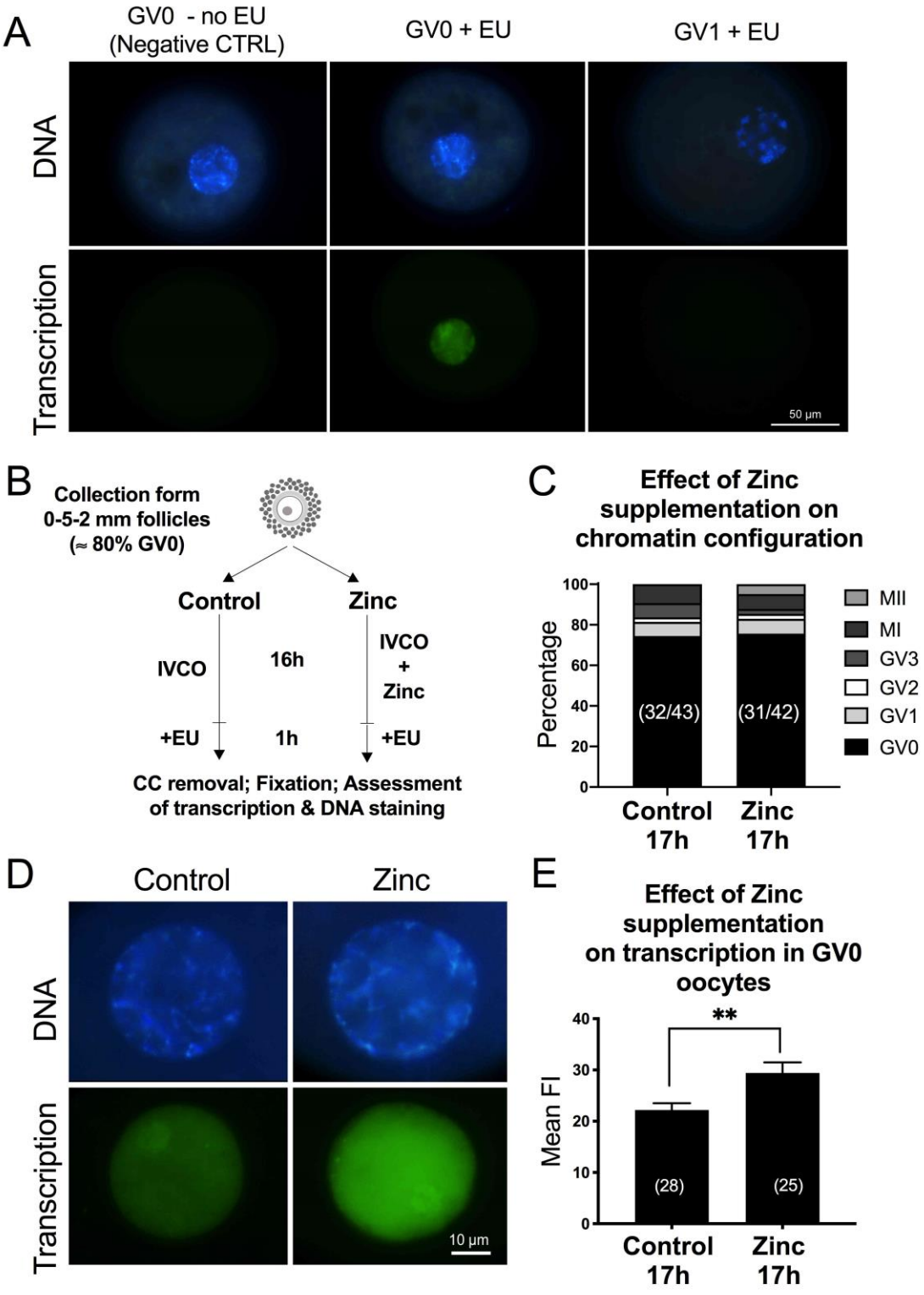


Figure 4: Effect of zinc supplementation during IVCO on global transcriptional activity in GV0 oocytes

(**A**) Representative images showing chromatin staining by DAPI (upper panel) and EU incorporation (lower panel) in GV0 oocytes collected from small antral follicles in the absence (Negative CTRL, left panel) and presence of EU (positive CTRL, middle panel), as well as in a fully-grown oocyte collected

from middle antral follicle with a GV1 chromatin configuration (right panel). Note that a positive signal is detected only in GV0 oocytes in the presence of EU. (**B**) Schematic representation of the experimental design (**C**) Graph showing the effect of zinc supplementation during 17h of culture on chromatin configuration and meiotic progression. Total numbers of oocytes analyzed in two independent experiments are indicated in the graph. Note that no differences were observed (**D**) Representative images showing chromatin staining by DAPI (upper panel) and transcription (lower panel) in GV0 oocytes after culture for 17 h under control IVCO condition or in the presence of zinc (**E**) Graph showing quantification of transcriptional activity as assessed by EU incorporation into nascent RNA and fluorescent image analysis in GV0 oocytes cultured in IVCO medium in the absence or presence of zinc. Data were analyzed by unpaired t-test; total number of oocytes analyzed are indicated in parentheses. Values are means ± SEM. ** indicate significant differences between groups (**P<0.01).

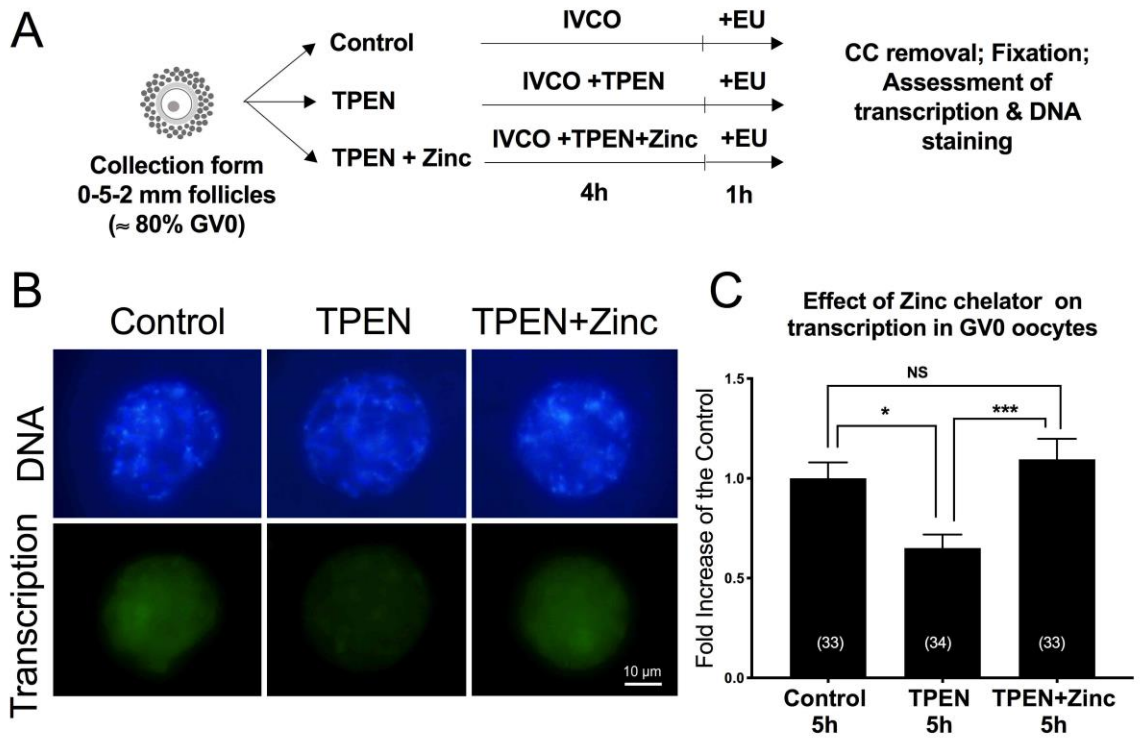


Figure 5: Effect of zinc chelation during 5h IVCO on global transcriptional activity in GV0 oocytes

(**A**) Schematic representation of the experimental design (**B**) Representative images of showing chromatin staining by DAPI (upper panel) and transcription (lower panel) in GV0 oocytes after culture for 5 h under control IVCO condition, in the presence of the zinc chelator TPEN or in the presence of TPEN + zinc (**B**) Graph showing quantification of transcriptional activity in GV0 oocytes cultured in IVCO medium for 5h (CTRL) and in IVCO medium supplemented by TPEN or TPEN + zinc. Data were analyzed by 1 way-ANOVA followed by Tukey's multiple comparison test; total number of oocytes analyzed in three independent experiments are indicated in parentheses. Values are means ± SEM. * and ** indicate significant differences between groups (*P<0.05; **P<0.01).

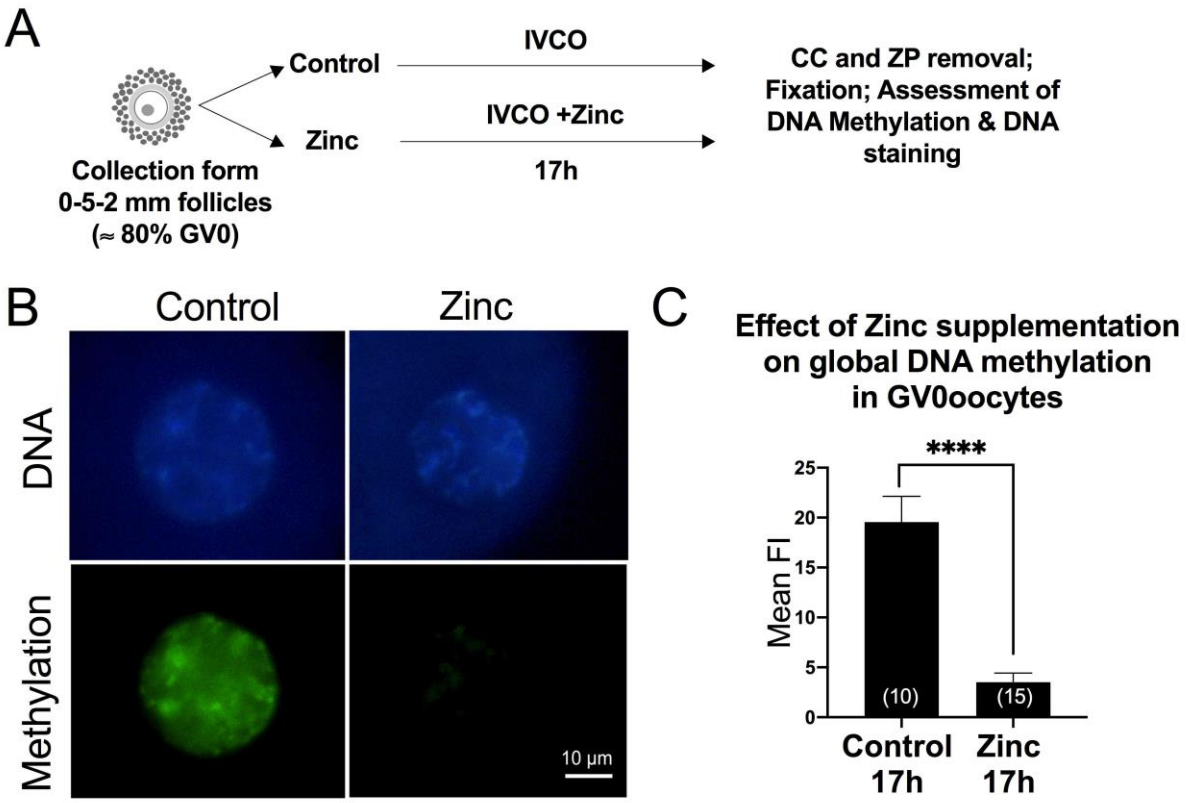


Figure 6: Effect of zinc supplementation during IVCO on global DNA methylation in GV0 oocytes

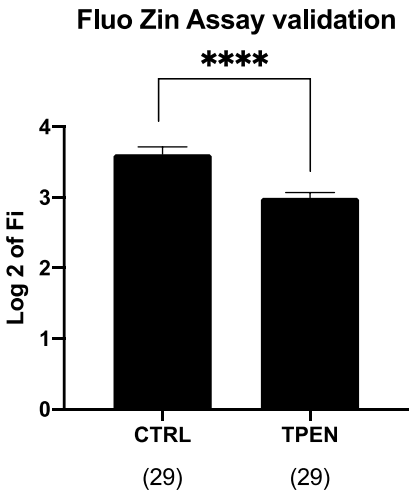
(**A**) Schematic representation of the experimental groups. (**B**) Representative images showing chromatin staining by DAPI (upper panel) and global DNA methylation (lower panel) in GV0 oocytes after culture in IVCO medium in the Control (IVCO medium) and Zinc (IVCO medium in the presence of zinc groups. (**C**) Graph showing quantification of global DNA methylation as assessed by indirect immunofluorescence and image analysis in GV0 oocytes cultured in IVCO medium in the absence or presence of zinc. Data were analyzed by Mann-Whitney; total number of oocytes analyzed in two independent experiments are indicated in parentheses. Values are means ± SEM. **** indicate significant differences between groups

($P<0.0001$).

Supplemental File

Figure 1: FluoZin 3 AM validation

Cumulus oocytes complexes (COC) were collected from middle antral follicles. COCs were loaded with FluoZin™3-AM for 30 minutes as described in the material and methods section. Fluorescent intensity was assessed as described in the method section soon after removal of cumulus cells (Control group, CTRL) or after incubation for 5 minutes in the presence of 100 µM TPEN (TPEN group). The graph shows quantification of labile zinc content in the CTRL and TPEN treated groups. Total numbers of oocytes analyzed in three independent experiments are shown in brackets. Data were pooled together and analyzed by 1-way ANOVA followed by unpaired t-tets. Values are means ± SEM. **** indicate significant differences between groups (P<0.0001).



CHAPTER 3

ADVANCED PHYSIOLOGICAL APPROACHES FOR IN VITRO OOCYTE DEVELOPMENT AIMED AT EXPLOITING THE REPRODUCTIVE POTENTIAL OF THE OVARIAN RESERVE

Manuscript in preparation

Unpublished data – confidential data

Advanced physiological approaches for in vitro oocyte development aimed at exploiting the reproductive potential of the ovarian reserve

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Short Title:

Principle and potential of in vitro culture of growing oocytes

Key Words

Oogenesis, growing oocyte, early antral follicle, oocyte differentiation, bovine species, cattle

Abstract

The limited reserve of mature, fertilizable oocytes represents a major barrier to the success of assisted reproduction in mammals. Considering that during the reproductive life span only a limited fraction of the oocytes in an ovary mature and ovulate, several techniques have been developed to increase the exploitation of the ovarian reserve to the growing population of non-ovulatory follicles. Such technologies have allowed interventions of fertility preservation, selection programs in livestock, and conservation of endangered species. However, the vast potential of the ovarian reserve is still largely unexploited. In cows, for instance, some attempts have been made to support in vitro culture of oocytes at specific developmental stages, but efficient and reliable protocols have not yet been developed. Here we describe a culture system that reproduces the physiological conditions of the corresponding follicular stage and is able to assist oocyte differentiation.

Our results demonstrated that the 5-days L-IVCO protocol promotes growing oocyte differentiation until the acquisition of meiotic and embryonic developmental competencies in a significantly higher proportion of the published protocols. This study demonstrated that a physiological medium could support a gradual transition of the oocyte from immature to mature stage, thus generating suitably quality blastocysts after fertilization.

In conclusion, our study provides an improved protocol that can increase the source of fertilizable gametes in preservation programs and gives a prospective approach in human clinics, animal breeding programs, and salvage intervention of threatened species. Moreover, our studies defined a model to perform in-depth studies of the cellular and molecular processes that regulate the acquisition of meiotic and developmental competence during oocyte differentiation.

Introduction

During the reproductive life span, only a minimal fraction of the oocytes that are present in an ovary cyclically mature, is released in the fallopian tubes upon ovulation and is available for being fertilized and develop into a viable embryo [1]. In cattle, as in humans, where reproductive technologies are largely used to produce embryos and where the number of exploitable oocytes is directly related to the overall efficiency of the technologies, the development of systems that facilitate the production of large numbers of bovine embryos from animals of high genetic merit would be of great commercial value [2].

In several species, in vitro embryo production (IVP) has been developed for some time. In cattle, current standard procedures rely on the collection of fully-grown oocytes from middle-sized antral follicle 2-8 mm in diameter and in vitro maturation (IVM) of oocytes followed by in vitro fertilization (IVF) and the in vitro culture (IVC) of embryos [1]. In the last years, the number of embryos produced in vitro by means of IVP has increased, and in some cases is similar to the number produced in vivo using the multiple ovulation and embryo transfer (MOET), a technique that is still widely used in the field, in which oocyte maturation, fertilization and early embryonic development occur in vivo [3]. However, the developmental competence of oocytes matured in vitro is generally lower than that of oocytes matured in vivo [4]. Moreover, the number of fully-grown oocytes within an ovary at the time of collection still limits the number of oocytes that can be subjected to IVP. In addition, a high number of oocytes that are enclosed in smaller follicle, which are still in their growing phase and have not yet reached the fully-grown stage, are normally discarded, which represent a waste of genetic resources. For this reason, the definition of in vitro culture protocols of growing oocytes from early antral follicles may offer a solution to obtaining a larger number of

oocytes that could be subjected to IVP. However, these oocytes need a dynamic and variable culture system to support all stages of oocyte growth, up to the stage at which oocytes can resume meiosis and undergo meiotic maturation, reaching the Metaphase II stage [5]. The lack of knowledge of the mechanisms involved in the process of oocyte development, specifically during its growth phase, and the lack of standard protocols for preserving this genetic material makes this technology still experimental [6].

By definition, immature oocytes are arrested at the prophase I (diplotene) stage of the first meiotic division, and the chromatin is still enclosed in the oocyte nucleus, called germinal vesicle (GV). As such, they reveal their nuclear and cytoplasmic immaturity [7, 8]. This observation led to important adaptations of the in vitro culture methodology with the aim to improve the developmental potential of oocytes from small follicles in bovines [9, 10] and humans [11]. To date no fully reliable protocols have been developed and only limited success has been reported. This is mostly due to the complex transition that the oocyte undertakes from its growth to the fully-grown state, which in cattle occurs when the follicle develops from the early antral to the medium antral stage. This is, indeed, one of the most critical steps during oocyte development [12].

One of the obstacles in the set-up of an efficient culture system for growing oocyte is that the cumulus cells that should stay functionally coupled with the oocyte tend to separate from the oocyte [10, 13, 14]. In sheep, for example, the cultured cumulus-oocyte complexes (COC) lost their spherical shape and the cumulus cells (CC) migrated away from the oocyte [13]. These considerations lead to the pursuit of a physiological environment where a proper association between the oocyte and granulosa cells could be maintained. This was partially achieved [10] in bovine species through the addition of 4% of polyvinylpyrrolidone (PVP) in the medium together with the use of the basement membrane matrix medium coated culture surface, creating a

3D-like culture. This system was proven to be effective in keeping the oocyte architecture, maintaining the physical association between the oocyte and cumulus granulosa cells [10]. The addition of PVP was useful to prevent loss of oocyte due to stick to the plastic surface and it may be involved in health and function of individual cells, changing the reorganization of the complexes [10]. However, despite this system supported oocyte growth after culture meiotic and developmental capabilities were very limited [10].

Besides paracrine mechanisms, oocytes enclosed in the follicle are connected to their surrounding CC through gap junction communications (GJC) which supports oocyte growth and differentiation [7, 9, 15]. In the follicle, the closure of GJC occurs when the oocyte resume meiosis [16]. Mechanistically, the closure of GJC prevents the transfer of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) from cumulus cells to the oocyte, altering phosphodiesterase (PDE) 3 activity to further decrease cAMP levels [17]. This causes the release of maturation promoting factor (MPF) and the resumption of meiosis (reviewed by [18]. When immature (GV stage) oocyte are isolated from antral follicles to be subjected to IVM, they are spontaneously “forced” to resume meiosis. Meiotic resumption which occurs after removal of the oocyte from its natural environment is indeed called “spontaneous” since it occurs independently from the hormonal stimulation that would physiologically accompany ovulation. Nevertheless, they would not undergo meiotic resumption at that time if they were kept within the ovarian follicle. As a consequence, most of the time oocytes subjected to standard IVM are not ready to resume meiosis. This is particularly true for growing oocytes that are isolated from early antral follicles, which have not even yet acquired the ability to resume and undergo meiotic maturation, since these oocytes are still transcriptionally active with uncondensed chromatin dispersed in the nucleus, accumulating transcripts for the subsequent developmental

steps. Importantly it has been demonstrated that GJC functionality tends to drop soon after oocyte isolation from the follicular environment, which clearly impairs oocyte competence [16, 19]. Therefore, when culturing oocyte in vitro, it is important to ensure the maintenance of the communications, at least until the oocyte becomes ready to resume meiosis and acquires the competence to fully support the subsequent steps of oocyte and embryonic development [9, 20]. Previous studies demonstrate that the supplementation of low concentrations of follicle-stimulating hormone (FSH) in the culture medium was beneficial to oocyte morphology and health status, being able to maintain the GJC functionality and support, at least in part, a proper meiotic resumption [9]. Still, this study demonstrates that the use of the chemical compound cilostamide, a specific PDE3 inhibitor, also affects the GJC, showing the positive effect of the GJC on transcriptional activity and chromatin condensation, probably through cAMP-dependent mechanism [9].

The complex relationship between oocyte and CC is not only mediated by GJC and the CC exert many important functions in the control of oocyte development within the follicular environment. Thus, the knowledge of processes occurring in the CC is important in the field of reproductive biology. Furthermore, it is commonly accepted that the health status of the CC reflects the quality of the enclosed oocyte. In this scenario, the analysis of the transcriptomic profile of both CC and oocytes at different stages of their growth and differentiation, as assessed by the level of chromatin compaction within the GV was evaluated by microarray analysis [21, 22] within the EmbryoGENE program (http://emb-bioinfo.fsaa.ulaval.ca/Home/index.html). This platform was implemented by collecting and storing transcriptomic data of bovine oocytes, cumulus and granulosa cells under different physiological conditions using the same transcriptomic platform and bioinformatics tools. All the collected transcriptomic data sets were subjected to meta-analysis as described in [23] and

made publicly available through an interactive web interface called EmbryoGENE profiler, which thus provides dynamic expression profiles of any gene of interest in the selected sample (http://emb-bioinfo.fsaa.ulaval.ca/IMAGE/index.html). Undoubtedly, this database can be a significant resource to understand the basic oocyte biology and develop a robust protocol for oocytes' culture.

Knowledge about the follicular growth rate is important for basic biology and to recapitulate oocyte growth in vitro. It has been shown that, in vivo, growing oocytes in bovine early antral follicles take approximately 5 days to complete the transition from the growing to the fully-grown state [24]. This was confirmed by experiments in which oocytes collected from small follicles and cultured for 5 days were able to reach the metaphase II (MII) stage after IVM [25].

Furthermore, in the ovary, steroids hormones are also involved and support follicular and oocyte growth; their intrafollicular concentrations correlate with follicle size [26-28]. The hormonal milieu is also involved in the maintenance of GJC functionality [29]. The use of a wide range of hormones concentration and combination during in vitro culture of oocytes has been described [10, 25, 29, 30]. Especially estradiol (E2) has received much attention by the reproductive biologists since it is known that E2 is involved in several processes such as the support the oocyte growth [25, 29, 30] and acquisition of meiotic competence [31].

Starting from all the above consideration and from experience gained during several years in our own laboratory, in the present study we developed an efficient procedure for the culture of immature growing bovine oocyte as COCs, called long in vitro culture of oocytes (L-IVCO), which support the growth and acquisition of embryonic developmental competence of growing oocytes during a 5-days culture system. Such a system took advantages of the concepts and experiences developed in our laboratory that a combination of follicle stimulating hormone (FSH) and a

phosphodiesterase-3 (PDE3) inhibitor is able to enhance cumulus-oocyte cross-talk [9], to ultimately prevent meiotic resumption [9], and to support oocyte growth and differentiation [9].

The efficiency of the of L-IVCO culture was evaluated by assessing the effect on oocyte growth, chromatin transition, ability to expand after IVM, meiotic competence acquisition, and embryonic developmental capability after IVF. The effect of L-IVCO culture on the expression of marker genes in the CC was also assessed as an indicator of the level of COC differentiation during culture.

A further advancement was introducing in this system zinc supplementation [32] and the pre-maturation approach, aimed at culturing oocytes in a physiological environment [26, 33].

Material and methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company except for those specifically mentioned.

Cumulus-oocyte complexes collection and in vitro culture

Bovine ovaries were recovered at local abattoir (IT 2270M CE; Inalca JBS S.p.A., Ospedaletto Lodigiano, LO, Italy) from non-pregnant females (4-8 years old) subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. The ovaries were transported to the laboratory within 2 hours in a sterile saline solution

maintained at 26°C. All the subsequent procedures, unless otherwise specified, were performed between 35°C and 38°C. COCs were retrieved as previously described [34]. Briefly, COCs were collected from 2 to 8 mm middle antral follicles with a 19-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane QLD, Australia) with a vacuum pressure of -28 mm/Hg. After aspiration, small pieces of the ovarian cortex, between 0.5 to 1.5 mm of thickness, were removed and examined under a dissecting microscope. COCs were isolated from 0.5 to <2 mm early antral follicles by rupturing the follicle wall with a scalpel. COCs were washed in Medium 199 supplemented with HEPES 20 mM, 1,790 units/L Heparin and 0.4% of bovine serum albumin (M199-D) and examined under a stereomicroscope, once isolated the COCs were immediately submitted to a media M119-D supplemented with 5 µM of cilostamide. The collection time of oocytes from early antral follicles did not last over 2 hours.

The diameter of the oocytes, excluding zona pellucida, was measured using an inverted microscope (Olympus IX50, magnification 10X) equipped with a CCD camera (3CCD Color Video Camera JVC, Model KYF55B). Only COCs with the diameter between 100 - 110 µm in diameter, medium brown in color, with compact layers of cumulus cells, homogeneous and with finely granulated ooplasm were selected to culture. Then, the COCs were cultured in the L-IVCO medium, TCM199 supplemented with 2 mM glutamine, 0.4% fatty acid-free bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 25 mM sodium bicarbonate, 0.1 mM cysteamine, 21.3 µg/mL of phenol red, 75 µg/mL of kanamycin and 4% Polyvinylpyrrolidone (PVP; 360 k molecular weight), 0.15 µg/mL zinc sulphate, 10^{-4}IU/mL FSH, 10 ng/mL E2, 50 ng/mL testosterone, 50 ng/mL progesterone and 5 µM Cilostamide. Each COCs were placed individually in a well of 96-well plate with a basement membrane matrix medium coated culture surface (Biocoat Collagen I Cellware; Becton Dickinson Biosciences), containing 200 µL of L-IVCO medium. The COCs were incubated for 5 days at 38.5 °C

under 5% CO_2 in humidified air. Every two days (on day 2 and day 4), half of the volume of the L-IVCO medium was replaced with a freshly prepared medium.

In vitro maturation (IVM), fertilization (IVF) and embryo culture (IVC)

After L-IVCO, COCs were washed in M199-D and cultured in groups of 20 – 25 COCs for 24h under standard IVM conditions. IVM medium was TCM199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO3, 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, and 0.1 IU/ml of rh-FSH [9]. Only COCs classified as Class 1, Class 2 and Class 3 (see below), [34] were submitted to IVM and subsequent steps.

After IVM, oocytes were fertilized as previously described [35]. Briefly, the content of straw of cryopreserved bull spermatozoa was thawed and sperm separated with a 45%–90% Percoll gradient. Sperm were counted and diluted to a final concentration of $0.75X10^6$ spermatozoa/mL in fertilization medium, which was a modified Tyrode solution (Tyrode albumin lactate pyruvate) supplemented with 0.6% (w/v) fatty acid-free BSA, 10 µg/ml of heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine. COCs were incubated in 300 µl of IVF medium for 18 h in four-well dishes at 38.5°C under 5% CO2 in humidified air.

After IVF, COCs were vortexed, to remove cumulus cells, for 2 min in 500 µl of a modified synthetic oviduct fluid (SOF) supplemented with 0.3% (w/v) BSA fraction V, essential and nonessential amino acids, and 0.72 mM sodium pyruvate and buffered with 10 mM HEPES and 5 mM NaHCO3. Presumptive zygotes were rinsed two times in SOF-Hepes buffer and then transferred in 500 µl of embryo culture medium. The embryo culture medium was SOF buffered with 25 mM sodium bicarbonate supplemented with essential and nonessential amino acids, 0.72 mM sodium pyruvate,

2.74 mM myo-inositol, 0.34 mM sodium citrate, and 5% bovine serum. Incubation was performed at 38.58C under 5% CO2, 5% O2, and 90% N2 in humidified air, as previously described [35].

Assessment of COC morphology, oocyte diameter and chromatin configuration after L-IVCO

After L-IVCO for 5 days, COC morphology was assessed under a dissection microscope and the COCs were classified in 4 classes: Class 1, COCs showing a compact cumulus cell investment with no sign of cumulus expansion and cell degeneration (Fig 1A'); Class 2, COCs showing a compact cumulus cell investment with no sign of cumulus expansion and cell degeneration and with antrum-like formation in the cumulus mass (Fig 1B'); Class 3 COCs showing several layers of cumulus cell with no sign of cumulus expansion and some disaggregated cells in the outer layer of cumulus cells and no antrum-like formation (Fig 1C'); and Class 4, COCs showing abundant loss of cumulus cells extending for more than 50% of the oocyte surface, and signs of cell degeneration and cell debris (Fig 1D').

Then, cumulus cells were mechanically removed by gentle pipetting and the oocyte diameter was determined. Bright-field images of denuded oocytes (DOs) were taken with a digital camera (3CCD Color Video Camera; JVC) mounted on an inverted microscope (Nikon Diaphot, Japan), and the oocyte diameter excluding the zona pellucida was measured using NIH ImageJ 1.58 software [36]. Subsequently, oocytes were fixed in paraformaldehyde 4% in phosphate buffer saline (PBS) for 1 hour at room temperature and washed three times in PBS containing 1% PVA, after which all samples were mounted in an antifade medium (Vecta Shield; Vector Laboratories, Inc., Burlingame, CA) supplemented with DAPI dilactate (1 μg/mL, 4′,6-diamidino-2-

phenylindole). Samples were analyzed in a conventional epifluorescence microscope (Eclipse E 600; Nikon Corp, Japan) to assess chromatin configuration as previously described [7]. Briefly, oocytes at the GV stage were classified according to the degree of chromatin condensation as follows: GV0, with a diffuse filamentous pattern of chromatin in the whole nuclear area; GV1, with a few foci of chromatin condensation; GV2, with chromatin further condensed into distinct clumps or strands; and GV3, with chromatin condensed in a single clump.

Assessment of cumulus expansion and meiotic competence after IVM

After IVM, the expansion degree of cumulus cells was visually classified according to a subjective scoring system: (-) no expansion, characterized by no morphological changes; (±) sign of expansion with partially denuded oocytes, (+) partial expansion, characterized by fair expansion and some clusters of not expanded cumulus cells; (++) complete or nearly complete expansion. After expansion scoring, COCs were denuded by gently pipetting and oocytes were fixed in paraformaldehyde 4% in phosphate buffer saline (PBS) for 1 hour at room temperature, washed three times in PBS containing 1% PVA, and then all samples were mounted in an antifade medium (Vecta Shield; Vector Laboratories, Inc., Burlingame, CA) supplemented with DAPI dilactate (1 μg/mL). Samples were analyzed in a conventional epifluorescence microscope (Eclipse E 600; Nikon Corp) to access the nuclear maturation stage. Oocytes were classified as follows: GV, all the oocytes that did not develop beyond the GV stage; oocytes from GV breakdown to the metaphase I (MI) stage were classified as MI stage; oocytes at the anaphase I, telophase I, and metaphase II (MII) stages as MII stage; and oocytes that could not be identified at any of the previous stages as degenerated [9].

Assessment of developmental competence after in vitro fertilization and embryo culture

Blastocyst rates were assessed 186 h post-fertilization. The blastocysts rate was assessed under a stereomicroscope, and blastocysts were morphologically classified as not expanded, expanded, and hatched [37]. Briefly, blastocysts in which the blastocoel has just begun to form, and the cell types were not distinguishable were classified as not expanded. Blastocysts were classified as expanded if the blastocoel was fully formed and the trophectoderm and inner cell mass clearly distinguishable but still contained within a thinned zona pellucida. Hatched blastocysts were those outside the zona pellucida. The embryos were then fixed in 60% methanol in DPBS, and the cell nuclei were counted under a fluorescence microscope after staining with 0.5 mg/ml propidium iodide.

RNA extraction and isolation

The expression level of selected genes was assessed in CC of COCs from early antral follicles at the moment of collection and after L-IVCO, which represent the ideal stage that growing oocyte should achieve. Groups of CC isolated from 25 oocytes were pooled and processed for total RNA extraction as described in [22], with minor modification. Total RNA was extracted and purified with the Pico-Pure RNA Isolation Kit (ARCTURUS®, Thermo Fisher Scientific) following the manufacturer's protocol, with minor modifications. Briefly, 100 µl of extraction buffer was added to each tube containing CC pellet and incubated 30 min at 42°C. Following incubation, an equal volume of EtOH 70% was added to each tube. Then, 200 µl of the RNA sample and

EtOH mixture was loaded into the preconditioned purification columns and centrifuged for 2 min at 1200 rpm. Finally, the columns were centrifuged for 30 seconds at 13400 rpm to remove the flow-through and bind RNA. Following these steps, the procedure was performed according to the manufacturer's instructions and including DNase treatment (Qiagen Italia, Milano, Italy) on the purification columns. Total RNA concentration was evaluated using Qbit Fluorometer 2.0 with the Qubit® RNA HS Assay Kits (Invitrogen—Thermo Fisher Scientific).

cDNA preparation and qRT-PCR

Six genes were selected that can predict whether the culture system is supporting the oocyte differentiation and it is following the in vivo pathways (SLC39a8, EGR1, SPRY2, ANGPT2, RGS2, HAS2). The qRT-PCR was performed using four independent biological replicates (each pool containing CC derived from 25 COC). For each sample, 40 ng of total RNA was reverse transcribed using the SuperScript First-Strand Synthesis for RT-PCR (Invitrogen—Thermo Fisher Scientific) with oligo-dT primers following the manufacturer's recommendations. Primer sequences used for real-time RT-PCR are provided in Table 1. Primers were designed using the Primer3 online tool (http://primer3.ut.ee/) from sequences obtained using the UMD3.1 assembly of the bovine genome. The specificity of each primer pair was confirmed by electrophoresis analysis on a standard 2% agarose gel and sequencing analysis.

Serial dilutions of the cDNA (ranging from 10 to 10^{-4} ng/µl) were then used to create the standard curves for the evaluation of the amplification efficiencies. Quantitative real-time PCR was performed on an iQ5 (Bio-Rad, Laboratories S.r.l., Segrate, Italy) using SYBR incorporation. Each qPCR, in a final volume of 20 µl, contained the cDNA corresponding to 0.5 ng of RNA extracted, 0.1 µM of each primer

and 1× SYBR mix (iTaq Universal SYBR Green Supermix, Bio-Rad). The PCR conditions used for all genes, except EGR1, were as follows: a denaturing step of 30 s at 95°C, followed by 40 PCR cycles (95°C for 30 s; 57°C for 1 min), a melting curve (55°C for 1 min and a step cycle starting at 55°C, up to 94.5°C). For EGR1 was: a denaturing step of 30 s at 95°C, followed by 40 PCR cycles (95°C for 30 s; 55°C for 1 min), a melting curve (55°C for 1 min and a step cycle starting at 55°C, up to 94.5°C). PCR specificity was confirmed by melting curve analysis.

Beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl transferase 1 (HPRT1). Each sample was assayed in triplicate, and the relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as a calibrator. The efficiency values for each primer are provided in Table 1.

Statistical analysis

All experiments were repeated at least four times. Means of oocyte diameter, chromatin configuration, expansion degree, meiotic competence, blastocyst cell number and gene expression were verified for the normality and analyzed using the T-test if they were normally distributed or Mann-Whitney they were not normally distributed. Means of blastocyst formation were analyzed using one-way ANOVA followed by Student's post hoc test (Graph Pad Prism 8 San Diego, CA, USA). Data are expressed as mean ± standard error mean (SEM) of sample (n) of biological replicate are indicated in the figure legends Probabilities with values less than 0.05 were considered statistically significant.

Results

Effects of L-IVCO on COC morphology, oocyte diameter and meiotic progression

To assess the effects of L-IVCO on COC morphology, the COCs were visually assessed under a stereomicroscope and classified into 4 distinct classes (Fig. 1). Class 1, 2 and 3 were judged suitable for further oocyte maturation, while an additional set of experiments were necessary to assess the ability of the class 4 COC to properly undergo meiotic maturation. As shown in supplemental data 1, around 30% of the oocytes classified as class 4, which presented abundant loss of cumulus cells on more than 50% of the oocyte surface, signs of cell degeneration and cell debris after L-IVCO (Fig. 1 D'), were already degenerated (supplemental data 1, Table 1). In addition, only a small percentage could reach the MII stage. Therefore, class 4 COC were discarded from the subsequent analysis (Supplemental data 1, Fig. 1).

To assess the effects of L-IVCO treatment on oocyte growth, the oocyte diameter was measured before and after L-IVCO culture. To this aim, groups of COCs were denuded and the diameter measured immediately after collection (EAF 0h), while other COCs were cultured for five days (L-IVCO 5d), after which oocyte diameter was measured after cumulus cell removal. As reported in Fig. 2, the mean diameter of oocytes submitted to L-IVCO was significantly higher than that of the initial population ($p=0.0002$).

To assess the extent to which the treatment supported oocyte differentiation, the effect of L-IVCO on chromatin compaction was assessed. Chromatin configuration is indeed a marker of oocyte differentiation [7] and chromatin configuration was assessed by nuclear staining with Hoechst 33432 and fluorescent microscopy analysis. As shown in Table 2, GV0 was the predominant class at the time of collection (EAF

0h) and a significant decrease in the GV0 stage was observed after 5 days of culture. This was accompanied by a significant increase in the GV2-like configuration, which was the predominant stage after culture (L-IVCO 5d). These results support the hypothesis that the oocytes had undergone differentiation during L-IVCO without resuming meiosis.

Effects of L-IVCO on cumulus expansion and oocyte meiotic progression

In an additional set of experiments, after L-IVCO, COCs were submitted to a standard IVM protocol and then the degree of cumulus expansion was assessed. Growing COCs were submitted directly to IVM and used as control (EAF IVM). As indicated in Table 3, the expansion degree of the cumulus mass was visually classified according to subjective scoring system grade: (-) poor expansion, characterized by no morphological changes; (±) partially denuded oocytes, (+) partial expansion, characterized by fair expansion but notable clusters lacking expansion; (++) complete or nearly complete expansion (Fig. 3). After IVM, a significantly higher percentage (9.52 ± 4.0% vs 39.64 ± 6.18%) of COCs submitted to L-IVCO prior to IVM reached the maximum expansion degree (L-IVCO IVM) when compared to the control group represented by COC submitted to IVM just after isolation.

Oocyte meiotic competence was assessed in the same oocytes. L-IVCO induced a significant decrease in the percentage of oocyte arrested in GV after IVM (Fig. 4A) and in parallel a significant increase in the percentage of oocytes reaching MII after IVM (Fig. 4B), indicating that the L-IVCO treatment promotes the acquisition of meiotic competence.

Effect of L-IVCO treatment on embryonic developmental competence acquisition

In this set of experiments, we assessed the effect on the acquisition of embryonic developmental competence. To this aim, three experimental groups were considered and submitted to the entire process of in vitro embryo production: (1) COCs from EAF submitted directly to IVP (EAF IVP), (2) COCs from EAF submitted to L-IVCO followed by IVP (L-IVCO IVP) and (3) COCs collected from MAF and submitted directly to a standard IVP protocol (MAF IVP) as a positive control.

As shown in Fig. 5, growing COCs submitted to L-IVCO prior to IVP acquired competence to reach the blastocyst stage, while any growing COCs submitted directly to IVP was able to reach the blastocyst stage. Although the L-IVCO IVP group could not develop to the blastocyst stage at the same rate as the MAF IVP group, when the blastocysts quality parameter was compared no significant differences were observed in the percentages of expanded and hatched blastocysts (Fig 6A), nor in blastocyst cell number (Fig. 6B) between the two experimental group. These results indicate that the L-IVCO supports the acquisition of a full developmental competence in almost 20% of cultured oocytes.

Effect of L-IVCO treatment on gene expression profile

To gain insights into the possible role of L-IVCO during oocyte growth and differentiation that occur before meiotic resumption, we mined the EmbryoGENE profiler to assess the expression profiles of genes that encode for proteins according to their significance changes and their known biological functions. The chosen genes were solute carrier family 39 member 8 (SLC39a8), early growth response 1 (EGR1), Sprouty RTK Signaling Antagonist 2 (SPRY2), angiopoietin 2 (ANGPT2), regulator of

G protein signaling 2 (RGS2) and a (HAS2). According to the Embryogene analysis, the expression of SLC39a8, SPRY2 and ANGPT2 are expected to be upregulated during oocyte growth and differentiation, while downregulation is predicted for EGR1 and RGS2 and no change is expected for HAS2.

Groups of CC from oocytes in different stages of development were collected and groups were compared: (1) CC from oocytes from EAF just after collection (EAF 0h); (2) CC from oocytes from EAF submitted to L-IVCO culture for 5 days (EAF 5d).

As shown in Fig 7, most of the genes were consistent with the results predicted in microarray data of the EmbryoGENE profile. Among the six selected genes, only 2 genes (RGS2 and ANGPT2) did not follow the predicted expression pattern according to the microarray data.

Discussion

In the present study, we provide experimental evidence supporting the hypothesis that recreating an in vitro culture system that closely mimics the in vivo environment supports the growth and differentiation of the population of growing oocytes enclosed in the small antral follicle. Indeed, here we show that culturing growing bovine oocytes with the newly developed L-IVCO system, which aims at recreating the follicular composition and the 3d environment, and the timing of oocyte growth, can support the acquisition of meiotic and embryonic developmental competence. This is the first report of such a high success rate obtained from growing bovine oocytes to the best of our knowledge. Compared to data in the literature, both maturation and developmental competence were substantially improved. As for the maturation rate, the one reported in this study is higher than most of the literature, where COCs from early antral follicles were cultured and then submitted to IVM [30,

38-41]. Considering the developmental competence, measured as the ability of the oocytes to develop up to the blastocyst stage, the present results doubled the percentage of blastocysts previously obtained in our laboratory [9] and by other groups [10, 41]. Our results are relevant both in animal breeding and human medicine since this system will put the basis for the exploitation of a higher number of germ cells from the ovarian stockpile bringing significant improvements of reproductive technologies efficiency.

In ART, the in vitro growth of follicles and the enclosed oocytes from the preantral to the antral stage still represents the biggest challenge for reproductive biologists. Few attempts were inefficient as a low survival rate was recorded, mainly caused by precocious loss of association between oocyte and companion cumulus cells [12], underling once again that recreating in vitro the conditions that ensure proper crosstalk between the somatic and the germinal cells is pivotal to support the oocyte developmental competence acquisition. Previous studies tried to recapitulate these events in vitro [9, 10, 25, 29, 30, 38-41], but to date, no reliable protocols have been developed and only limited success has been reported. In the present study, we have considered many aspects of the follicular environment to develop the L-IVCO system, such as the medium composition, the length of the culture, the medium characteristic, especially the viscosity to recreate a 3D environment and the need to keep the communications between oocyte and CC. Starting from the composition of the culture medium, an important aspect that deserves discussion is the hormonal support. In fact, steroids hormones are involved in the control of follicular and oocyte growth [26-28, 42] and in the maintenance of GJC [29]. In the literature, a wide range of hormones concentration has been tested in vitro [10, 25, 29, 30]. In particular, E2, which exerts a fundamental role during folliculogenesis [10, 29, 43], has been generally used at higher concentrations compared to the one found in the follicular fluid [10, 25, 29, 30, 39].

Therefore, preliminary experiments were conducted to assess the most efficient E2 concentration to be used in our system, i.e in combination with other hormones (FSH, Testosterone and Progesterone), by comparing the physiological concentration (10 ng/ml) with higher doses (100 and 1000 ng/ml, on oocyte growth, on the ability to maintain oocyte under meiotic arrest, on the degree of cumulus expansion and on meiotic competence after IVM. These set of experiments, which are presented in the Supplemental data 2, confirmed that the L-IVCO system with the physiological dose of E2 [27, 28] was already able to promote oocyte growth and prevent meiotic resumption while ensuring acquisition of meiotic competence. Moreover, no significant difference of these parameters were observed when higher doses of E2 were used.

In this study, the L-IVCO system promoted a significant increase of the oocyte diameter (from 109.2 µm to 113 µm). This result is comparable with previous experiments from our laboratory [9]. However, oocytes submitted to the L-IVCO treatment did not reach the final diameter of a fully-grown oocyte, which is typically 120 µm [7], indicating that the system can still be improved. Nevertheless, our data are in accordance with the literature since it has been reported that oocytes acquire a certain degree of competence before reaching their full size [44].

In the present study, the oocytes subjected to the L-IVCO system, not only increased their diameter but also remained meiotically arrested and they undergo chromatin remodeling from the GV0 configuration (which correlates with very poor meiotic and developmental competence) to a more compacted GV2/3 configuration (which denotes the achievement of meiotic and developmental competence). Previous work conducted in our laboratory has demonstrated that chromatin morpho-functional remodeling before meiotic resumption is regulated, at least in part, by GJC through cAMP mediated mechanisms [7, 9], which in turn also regulates meiotic resumption. The L-IVCO system took advantage of this concept. Indeed, the use of physiological

concentration of FSH in combination with cilostamide was meant to promote GJC mediated coupling and chromatin remodeling. Thus, we can speculate that, as in previous studies, the L-IVCO system promotes the maintenance GJC functionality, that would otherwise drop soon after the removal of the COC from the follicle [7]. It will be important, in future studies, to confirm this hypothesis and to assess the extent to which the L-IVCO system is able to prolong GJC functionality. Similarly, since GJC also affect transcriptional activity [9], it will be pivotal to assess if and for how long transcription remains active during the 5 day-long culture period. This will be a fundamental observation since it is known that transcription during oocyte growth is necessary to confer the oocyte with the appropriate storage of molecules and that it has to be gradually silenced before meiotic resumption. This information will be fundamental to tune further and improve the efficiency of the L-IVCO system.

As expected, the changes in oocyte diameter and chromatin compaction observed in the L-IVCO treated oocytes, were reflected in a higher meiotic and embryonic developmental competence. In fact, when the L-IVCO treated oocytes were submitted to standard IVM, IVF and IVC protocols, both the maturation rate as well as the number of blastocyst formation after fertilization were higher, compared to oocytes not submitted to the L-IVCO system. Moreover, the higher maturation rate was accompanied by a higher degree of cumulus expansion, which in vivo is an important process involved in the rupture of the follicle and the release of the mature oocyte into the oviduct [45] and related to a higher developmental competence [46, 47]. Nevertheless, in the present study not all the L-IVCO treated COC could undergo proper cumulus expansion and a small percentage of oocytes were only partially surrounded by CC after IVM. Similar results were observed in humans [14] and we can speculate that these oocytes may have intrinsic insufficient level of cGMP to firmly prevent meiotic resumption for the full culture period. Thus, we can hypothesize that

the oocytes with loosely attached CC after IVM were the ones with poor developmental competence and that prematurely resumed meiosis during IVCO. Unfortunately, this hypothesis could not be tested in the present set of experiments since after L-IVCO, where COC are cultured individually, COC were grouped together and submitted to IVM. Therefore, the impact of poor association between oocyte and CC after IVM in this group of oocytes still remains to be assessed and, nevertheless, we cannot exclude that they have been supported also by the presence of CC in the co-cultured COC. Indeed, it has been shown that, while removal of CC in fully grown oocytes impairs developmental capability, it can be restored by co-culturing these oocytes with intact COC [35].

To further evaluate the extent to which the L-IVCO system promoted differentiation of the cultured COC, we firstly selected molecular markers based on previous transcriptomic analysis in CC isolated from oocytes at different stages of their differentiation (i.e with different degree of chromatin configuration) and then analyzed the expression level of these marker genes before and after L-IVCO, ultimately aiming at identifying possible ways to finely tune and further optimize the culture system. Gene expression analysis revealed that four out of six of the genes analyzed (SLC39a8, EGR1, SPRY2 and HAS2) changed their expression as predicted [21-23].

For example, EmbryoGENE data indicated that SLC39A8 is one of the genes whose expression constantly increases in CC during the GV0-to-GV3 transition, which is in accordance with the results demonstrated in this study. SLC39A8, also known as ZIP8, encodes for a member of the SLC39 family of solute-carrier genes, which shows structural characteristics of zinc transporters [48]. Recently, it has been shown that the oocyte zinc content exerts important roles in oocyte function in mice, especially during meiotic maturation and fertilization [49-54]. Another study suggested that CC regulate the amount of free zinc in the oocyte during maturation [55]. Interestingly, our recent

findings showed that zinc is also involved in several processes during oocyte growth, especially in the transcription activity [32], which is in agreement with another experiment where acute dietary zinc deficiency during preconception disrupts oocyte chromatin methylation and alters transcriptional regulation and it is associated with severe defects of embryonic development [56]. Zinc was added in the L-IVCO media due its role in supporting oocyte differentiation and transcriptional activity of growing bovine oocytes in culture.

EGR1 is a zinc-finger transcription factor that responds rapidly to FGFs in different cell types, including those of the reproductive tissues [57]. Moreover, after the LH surge, an increase in EGR1expression is observed [58-60]. It was demonstrated that overexpression of EGR1 protein increases the relative mRNA level of SPRY2 which regulates signals of receptor tyrosine kinases, including FGF and epidermal growth factor receptors (EGFR [60-62]. This gene acts to reduce cell proliferation and amplify the EGFR pathway [63-65]. In this study, the expression of EGR1 is decreased and SPRY2 gene expression is increased during the oocyte growth, additionally, these genes are in accordance with EmbryoGENE data analysis.

EmbryoGENE data indicate that ANGPT2 is one of the genes whose expression increases in CC during the GV0-to-GV3 transition. Our data suggest that ANGPT2 follow a different path during L-IVCO. This gene is an antagonist of angiogenic factor ANGPT1 that signals through the endothelial cell specific Tie2 receptor tyrosine kinase. ANGPT2 disrupts the vascular remodeling ability of ANGPT1 and may induce endothelial cell apoptosis [66, 67]. ANGPT2 was found to be upregulated in early atretic follicles [68, 69]. Since in this study the ANGPT2 expression was not increased, we can infer that the CC are not prone to apoptosis. Nevertheless, we cannot exclude that different results from the one reported in the transcriptomic analysis [22] may be due to the different source of the CC as in the previous study, we

used a pool of CC coming from oocytes at the same differentiation stage (i.e where the chromatin configuration at the time of collection was analyzed and oocytes and CC were pooled accordingly) while in the present study it was analyzed in pools of CC coming from oocytes in different stages of differentiation with a predominance of oocytes in GV2 stage.

RGS2 gene encodes for GTPase-activating protein that hydrolyses GTP to GDP on the alpha subunit of an activated G protein [70]. RGS proteins can regulate the duration and intensity of the signal transduction process by antagonizing or shortening the interaction between G alpha proteins with effectors such as phospholipase C. It is probably involved in granulosa cells response to gonadotrophins and represents an important marker of luteinization. In addition, it has been reported that its expression in CC is correlated with oocyte developmental capacity and clinical pregnancy [71, 72]. In our study, it differs from EmbryoGENE data, again it can be caused by the pool of CC coming from oocytes in deferent stages of development. Moreover, the cells analyzed in this study are not showing signs of luteinization.

HAS2 expression, which is following the EmbryoGENE data, did not alter its expression during oocyte growth. This gene is involved in mucification and expansion of cumulus cells and it is expressed after the LH surge [63, 73]. In fact, in the present study it was stable during the oocyte growth and comparable with results obtained from experiments in mice [74] and bovine [75] where an increase in the expression of HAS2 mRNA was observed only after the LH surge.

In conclusion, our findings allowed us to achieve a protocol able to further exploit the ovarian reserve by bringing the growing oocytes to achieve meiotic and developmental competence and became a blastocyst. Also, it led us to define a model through which to conduct in-depth studies of the cellular and molecular processes that regulate the acquisition of meiotic and developmental competence during oocyte

growth. Our results demonstrate the necessity of understanding the basic mechanisms of the biological process occurring in the COC during final growth for the tailor of the culture system. Such a culture system should enhance the potential source of oocytes obtained to reach meiotic competence in vitro, thus offering useful and safe fertility preservation options.

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Figures and tables

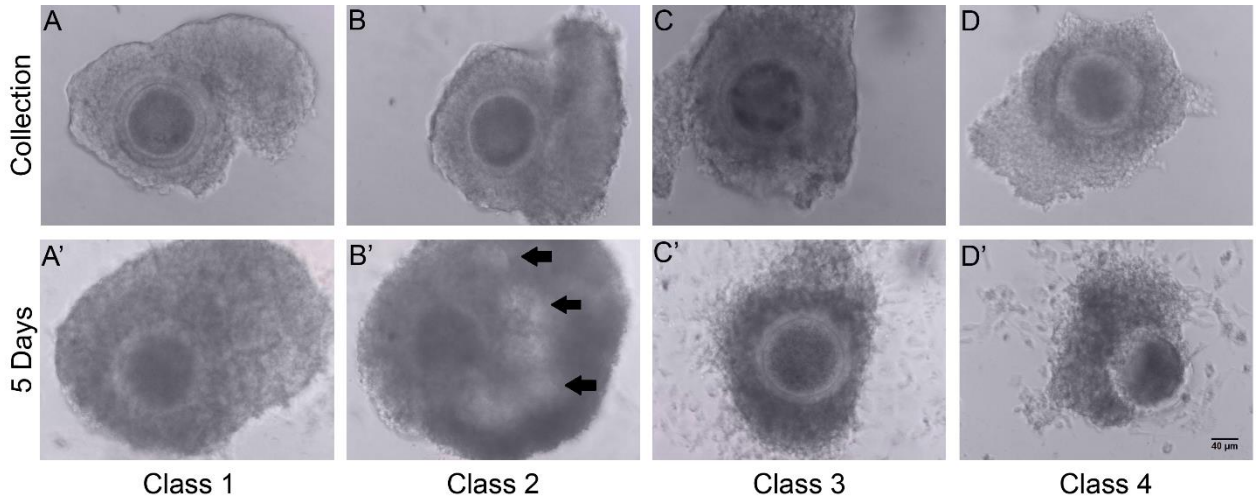

Figure 1: Representative images of COCs at the time of collection and after L-IVCO. (**A, B, C, D**) The upper row (Collection) represents COCs at the time of retrieval. (**A, B, C, D**) The same COC is pictured 5 days later, at the end of L-IVCO and classified according to the COC morphology. The lower row (5 days) represents COCs classified as: Class 1, showing a compact cumulus cell investment with no sign of expansion and cell degeneration (A'); Class 2, showing a compact cumulus cell investment with no sign of expansion and cell degeneration and with antrum-like formation (arrows) in the cumulus mass (B'); Class 3, showing several layers of cumulus cell with no sign of cumulus expansion and some disaggregated cells in the outer layer of cumulus cells (C'); class 4, showing abundant loss of cumulus cells on more than 50% of the oocyte surface and signs of cell degeneration and cell debris (D'). Scale bar 40 µm.

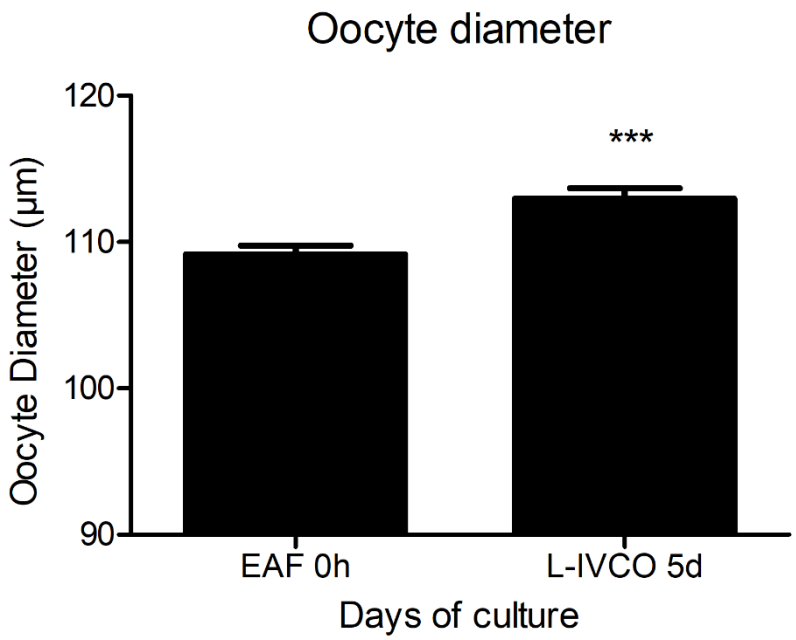

Figure 2. Effect of L-IVCO treatment on oocyte growth. Graphs showing changes in oocyte diameter of growing oocytes collected from EAF at the time of collection (EAF 0h; n=58) and after L-IVCO treatment for five days (L-IVCO 5d; n=73). Data were verified for the normality and analyzed by Mann-Whitney test and values are means ± SEM. *** indicate significant differences between groups (P<0.0001; N=5).

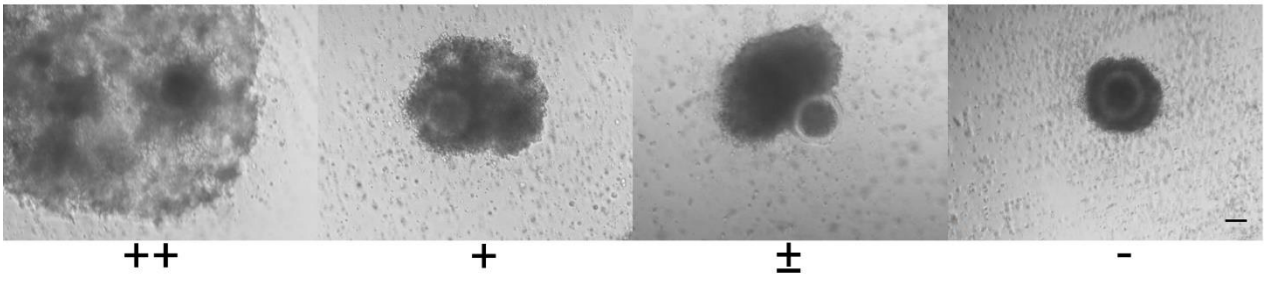

Figure 3. Representative image of expansion degree of the cumulus mass. After IVCO followed by IVM the expansion degree of the cumulus mass was visually classified according to subjective scoring system grade: (-) poor expansion, characterized by no morphological changes; (±) partially denuded oocytes, (+) partial expansion, characterized by fair expansion but notable clusters lacking expansion; (++) complete or nearly complete expansion. Scale bar 40 µm.

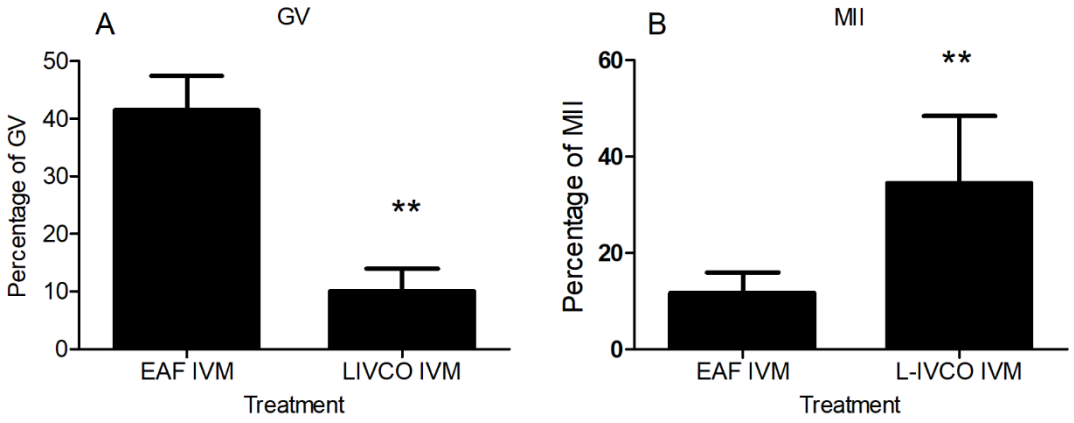

Figure 4. Effect of L-IVCO treatment in the oocyte meiotic competence acquisition. A) Graph showing the percentage of oocytes in the GV stage when growing oocytes were submitted directly to IVM (EAF IVM; n=60) or treated in L-IVCO medium prior to IVM (L-IVCO IVM; n= 55). Data were analyzed by unpaired t-test and values are means ± SEM. ** indicate significant differences between groups (P=0.0013). B) graph showing the percentage of growing oocytes that reach the MII stage when submitted directly to IVM (EAF IVM; n=60) or L-IVCO followed by IVM (L-IVCO IVM; n=55). Data were analyzed by unpaired t-test and values are means ± SEM. ** indicate significant differences between groups (P=0.0065).

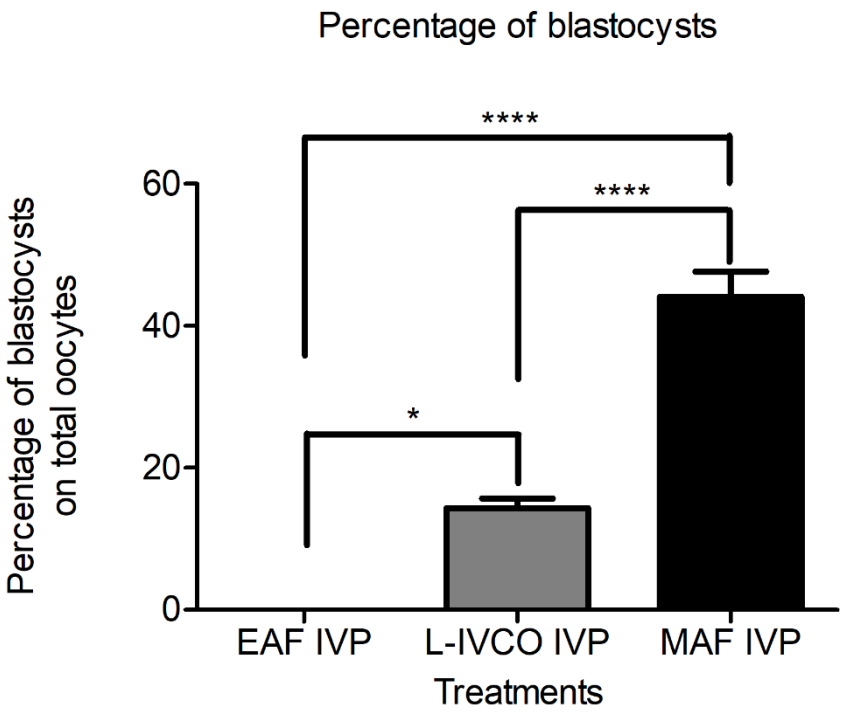

Figure 5. Influence of L-IVCO treatment on embryo production. Graph showing blastocyst production on total number of growing oocytes submitted directly to embryo production (EAF IVP, n=86), total number of oocytes growing treated in L-IVCO medium prior to embryo production (L-IVCO IVP, n=95) and the total number of oocytes from MAF submitted to embryo production (MAF IVP, n=261). Data were analyzed by one-way ANOVA followed by Holm-Sidak's multiple comparison test and values are means ± SEM (*P<0.05 and ****P<0.0001).

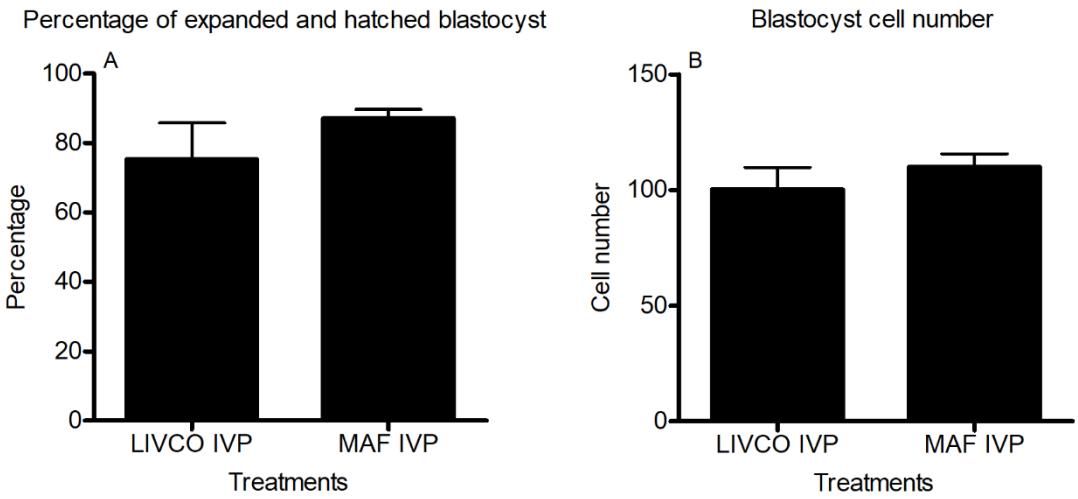

Figure 6. Influence of L-IVCO treatment on the percentage of expanded/hatched blastocysts and blastocysts cell number. (A) Graph showing the percentage of hatched and expanded blastocysts in relation to total blastocysts between L-IVCO IVP (n=13) and MAF IVP (n=115) group. Data were analyzed by Mann-Whitney test and values are means ± SEM. (B) Graph showing blastocyst cell number in L-IVCO IVP (n=13) and MAF IVP group (n=115). Data were analyzed by Mann-Whitney test and values are means ± SEM.

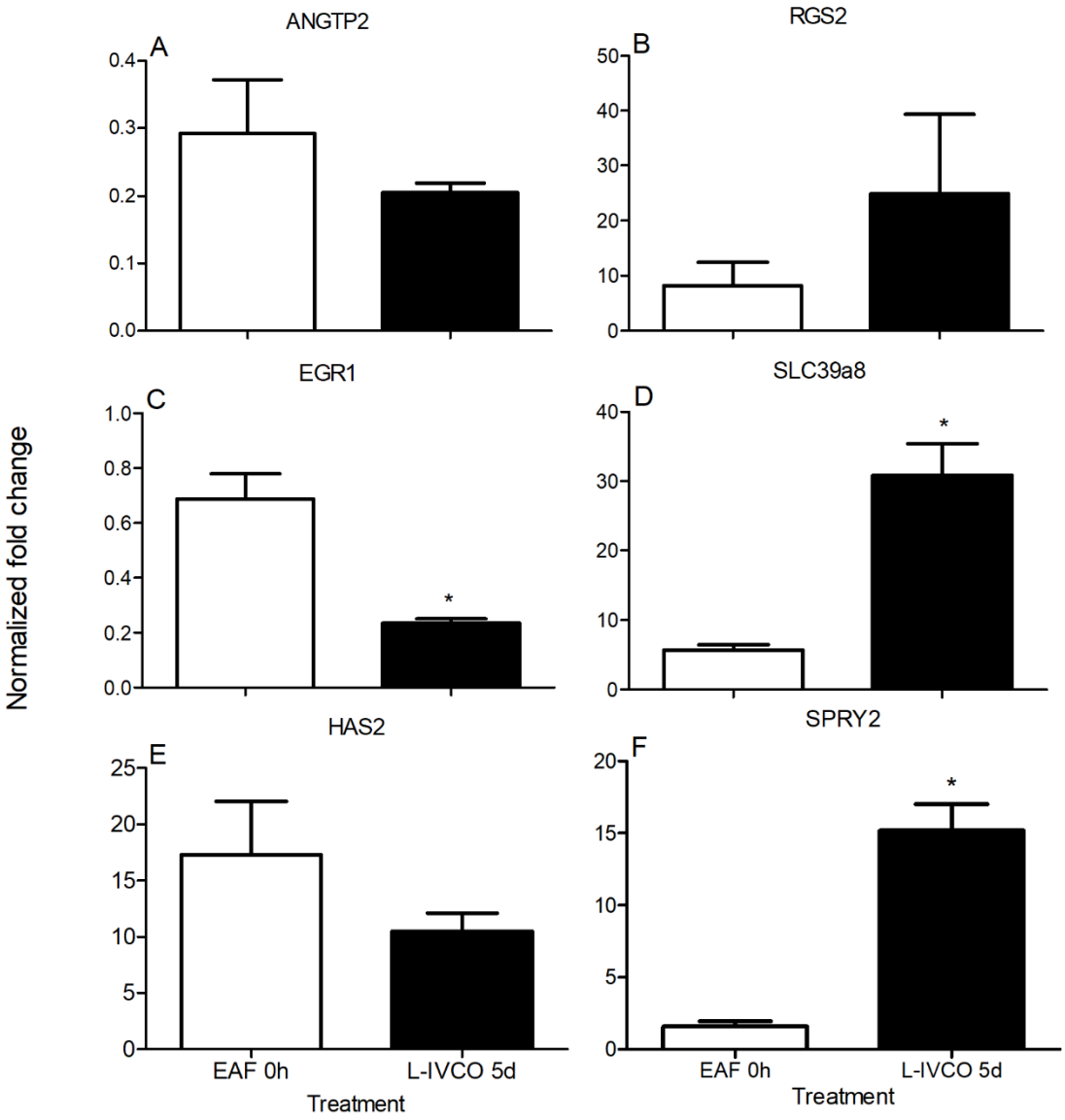

Figure 7. Effects of L-IVCO on mRNA abundance of selected genes. Graphs indicate relative expression levels of selected genes in CC isolated from growing oocytes at the moment of collection (EAF 0h) and after L-IVCO culture for 5 d (L-IVCO 5d); expression levels for each gene were normalized using ACTB, GAPDH and HPRT1 as reference genes. Relative fold changes were calculated using the delta-delta Ct. Data were analyzed by t-test followed by Mann Whitney test and are expressed as means ± SEM. * indicate significant differences between groups ($P < 0.05$).

Table 1. Primer sequences used for real-time RT-PCR.

Gene name	Forward primer sequence	Reverse primer sequence	Fragment size (bp)	Efficiency (%)
SLC39a8	TGAACATCCTAGCCCCAATC	TGACACAATACAAGGTTTCAAAGG	250	80.4
HAS2	GATAACGCCACCAAAGGAGA	TTTTCTTCCATGATAACCCACA	249	75
RGS2	GCCGAAAAGACTGACCTTGA	GAGGCCACATAATCCCAGAC	277	79.8
EGR1	AGAGCCAAGTCCTCCCTCTC	TCCAAAATCCATGCAAATCA	184	77.9
ANGPT2	CCAGAGACTTGCTCCCAAAG	GATGCAGGTTCAAGGGGTAA	102	81.7
ACTB	TGAACCCTAAGGCCAACCGTG	TGTAGCCACGCTCGGTCAGGA	267	85
GAPDH	CCAACGTGTCTGTTGTGGATCTGA	GAGCTTGACAAAGTGGTCGTTGAG	217	92.5
HPRT1	GGCTCGAGATGTGATGAAGG	GCAAAGTCTGCATTGTCTTCC	293	96.8
SPRY 2	CTGGGTGCCGTTGTAAAAAT	CACTGCAAACAGCACAGGAT	236	73.7

Table 2. Effect of IVCO treatment on chromatin configuration

	GV0 (%)	GV1 (%)	GV2 (%)	GV3 (%)	Deg (%)
EAF 0h	78.85 ± 2.58[a]	9.72 ± 4.64	7.38 ± 3.55[a]	0.0 ± 0.0	4.04 ± 2.49
L-IVCO 5d	12.29 ± 4.11[b]	6.67 ± 1.76	46.54 ± 8.08[b]	12.89 ± 6.28	9.28 ± 4.76

Data showing the degree of chromatin compaction of oocytes at the time of collection (EAF 0h; n=58) and after L-IVCO treatment for 5 days (L-IVCO 5d; n=73). Data were analyzed by paired t-test and values are means ± SEM. Different letters in the column indicate significant differences ($P<0.05$; N=5).

Table 3. Expansion degree of cumulus cells after IVM in COCs from EAF.

	++	+	±	-
EAF IVM	11.34 ± 3.66[a]	49.45 ± 1.00	0.0 ± 0.0[a]	39.21 ± 3.88[a]
L-IVCO IVM	29.60 ± 4.32[b]	27.94 ± 8.48	29.25 ± 8.47[b]	13.21 ± 4.18[b]

Data showing the expansion degree of cumulus cells in COCs from EAF submitted direct to IVM (EAF IVM, N=7) or treated in L-IVCO medium prior to IVM (L-IVCO IVM; N=7). Data were analyzed by paired T-test and values are means ± SEM. Different letters indicate significant differences in the columns ($P<0.05$). Expansion degree of cumulus cells was visually classified according to subjective scoring system grade: (-) poor expansion, characterized by no morphological changes; (±) partially denuded oocytes, (+) partial expansion, characterized by fair expansion but notable clusters lacking expansion; (++) complete or nearly complete expansion.

Supplemental data 1

In this study, the Class 4 COCs were analyzed regarding their developmental competence acquisition and the results confirmed that they are not able to acquire developmental competence. Immediately after the 5 days L-IVCO culture, these oocytes showed progression until GV2/3 stage but also a high rate of degeneration as demonstrated in Supplementary Table 2.

Supplemental Table 1. Chromatin configuration according to the oocyte class after 5 days of culture.

Class	GV0	GV1	GV2	GV3	DEG
1	22.22	5.56	50.00	5.56	0.00
2	5.26	10.53	47.37	10.53	5.26
3	13.33	6.67	50.00	10.00	13.33
4	0.00	0.00	42.86	28.57	28.57

Table showing the chromatin configuration of oocytes from EAF treated in L-IVCO medium for 5 days. The oocytes classified as Class 4 present the same rates of GV2/3 as the other classes, but it also demonstrated a higher percentage of oocytes degenerated.

One hypothesis is that during the follicle development, the follicle size used in these experiments is the one that presents a drastic increase in the atretic rates [24], so these cells already started the irreversible process of atresia. Nonetheless, a precocious disconnection between oocyte and CC is not physiological and it is expected to be detrimental for the further processes. In fact, when the Class 4 oocytes were submitted to in vitro maturation although few oocytes could reach MII stage most

of them have degenerated and some of them remained blocked in the MI or GV stage (Supplementary figure 3).

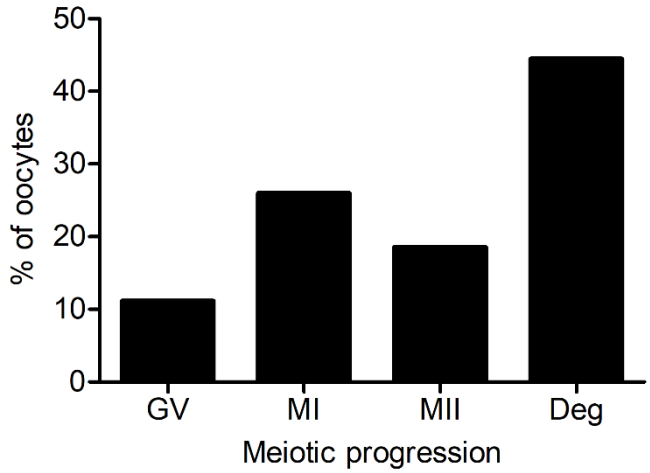

Figure 1. Meiotic progression of partially denuded oocytes. Graph showing the meiotic progression of oocytes Class 4 after IVM. Just a few oocytes could reach MII stage most of them were degenerated and some of them remained blocked in the MI or GV stage.

These observations confirmed that the Class 4 COCs have low competence and show a high percentage of degeneration. Based on this data Class 4 oocytes were excluded from subsequent steps.

Supplemental material 2

Previous studies indicated that the rate of in vitro growth increased incrementally with E2 concentration in the culture media [25, 29, 30, 76]. Although the concentration we selected corresponds to that measured in early antral follicles [27, 28], this set of experiments was designed to verify the effects of different concentrations of E2 (10, 100 and 1000 ng/mL) on oocyte growth and meiotic competence. As indicated, oocyte growth was assessed by measuring oocyte diameter at the time of collection and after 5 days of L-IVCO. E2 induced a significant increase in oocyte diameter compared with the control group. However, no differences were seen between treatments with higher E2 concentrations (Sup Fig 1A). Moreover, all concentrations of E2 were able to arrest oocytes at the GV stage with no differences between treatments (Sup Fig 1B).

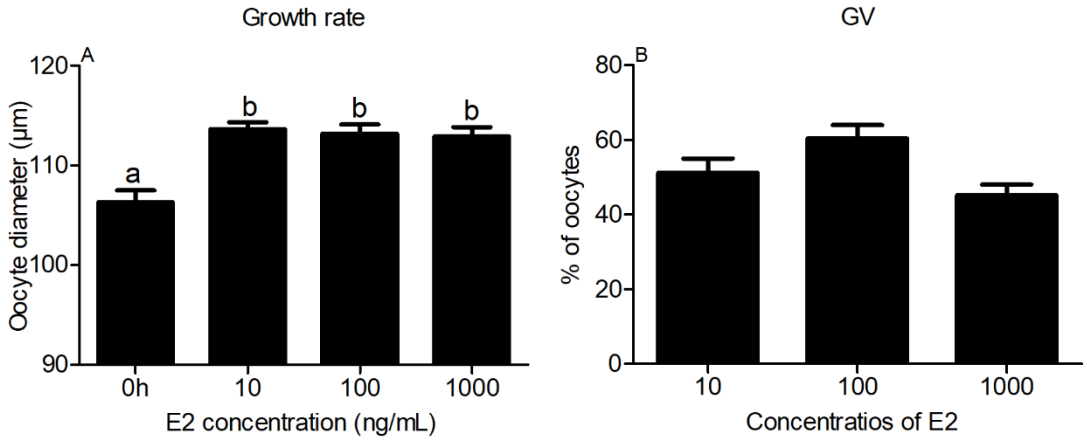

Figure 1. Effect of different E2 concentrations during L-IVCO. (A) Graph showing the effect of different E2 concentrations on oocyte growth measured as diameter at the time of collection (0d, n=24) and after 5 days of L-IVCO with different concentrations of E2: 10 ng/mL (n=58), 100 ng/mL (n=60) and 1000 ng/mL (n=59). Data were analyzed by 1- way ANOVA followed by Kruskal-Wallis test and values are means ± SEM. Different letters indicate significant differences between groups (P<0.0001; N=3).

(B) Graph showing the percentage of oocytes arrested at the GV stage after L-IVCO for 5 days in different concentrations of E2. Data were analyzed by 1-way ANOVA followed by Kruskal-Wallis test and values are means ± SEM (N=3).

After L-IVCO treatment, COCs were submitted to IVM and no significant difference in MII rates was observed between treatments with different concentrations of E2, although the percentage of MII was numerically higher in 10 ng/mL than 100 and 1000 ng/mL of E2 (Sup Fig 2).

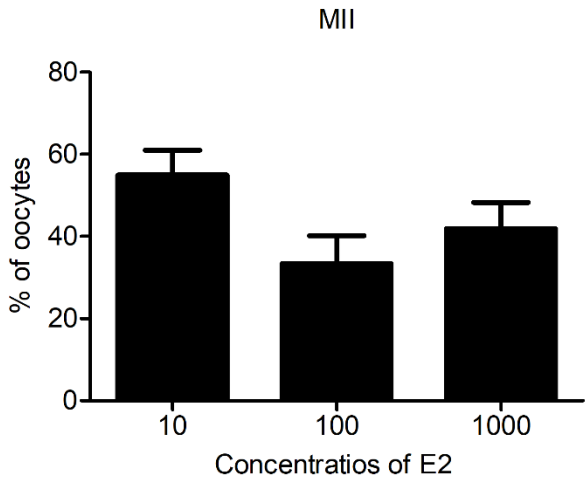

Figure 2. Effect of different E2 concentrations on oocyte meiotic competence acquisition. Graph showing the effect of different E2 concentrations on the percentage of oocytes reaching MII after IVM. Data were analyzed by 1-way ANOVA followed by Kruskal-Wallis and values are means ± SEM (P=0.1431; N=4).

Also, the expansion degree of cumulus cells was assessed after IVM and the concentration that induced the highest number of COCs fully expanded was the lowest (10 ng/mL), although this difference was not statistically significant. In parallel, a significant difference between 100 and 1000 ng/mL in the number of COCs showing no expansion was observed (Sup Table 1).

Table 1. Effect of E2 on cumulus expansion after IVM

(ng/mL)	++	+	±	-
10	29.92 ± 10.61	26.84 ± 9.62	26.28 ± 13.87	16.96 ± 5.56
100	18.25 ± 6.51	27.95 ± 9.42	33.85 ± 11.58	19.96 ± 4.81
1000	12.37 ± 5.26	38.85 ± 9.83	43.35 ± 11.73	6.44 ± 4.02

Table showing the expansion degree of cumulus cells in COCs from EAF treated in L-IVCO medium with different concentrations of E2 (ng/mL) before IVM (N=4). Data were analyzed by 1-way ANOVA followed by Friedman test and values are means ± SEM. Different letters indicate significant differences between the columns (P=0.0185). Expansion degree of cumulus cells was visually classified according to subjective scoring system grade: (-) no expansion, characterized by no morphological changes; (±) sign of expansion with partially denuded oocytes, (+) partial expansion, characterized by fair expansion and some clusters of not expanded cumulus cells; (++) complete or nearly complete expansion.

These observations showed us that although not statistically significant differences could be observed the most suitable concentration of E2 to be used was the one similar to the concentrations found in the follicular fluid in vivo which is the 10 ng/mL.

CHAPTER 4

DEVELOPMENT OF NEW OOCYTE IN VITRO CULTURE STRATEGIES TO ENHANCE THE OUTCOME OF ASSISTED REPRODUCTIVE TECHNOLOGIES

SECTION I

DEVELOPMENT OF NEW OOCYTE IN VITRO CULTURE STRATEGIES TO ENHANCE THE OUTCOME OF ASSISTED REPRODUCTIVE TECHNOLOGIES

Published in:
Journal of visualized experiments (JoVE) 2020 Jul 8; 161

DOI: https://doi.org/10.3791/6125

Title:

In Vitro Culture Strategy for Oocytes from Early Antral Follicle in Cattle

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Keywords:

Growing oocytes, early antral follicles, oocyte differentiation, oocyte isolation, bovine, folliculogenesis in vitro, ovarian reserve, fertility preservation, atresia, in vitro embryo production, IVP, follicle stimulating hormone, FSH

Summary:

We describe the procedures for isolation of growing oocytes from ovarian follicles at early stages of development, as well as the setup of an in vitro culture system which can support the growth and differentiation up to the fully-grown stage.

Abstract

The limited reserve of mature, fertilizable oocytes represents a major barrier for the success of assisted reproduction in mammals. Considering that during the reproductive life span only about 1% of the oocytes in an ovary mature and ovulate, several techniques have been developed to increase the exploitation of the ovarian reserve to the growing population of non-ovulatory follicles. Such technologies have allowed interventions of fertility preservation, selection programs in livestock, and conservation of endangered species. However, the vast potential of the ovarian reserve is still largely unexploited. In cows, for instance, some attempts have been made to support in vitro culture of oocytes at specific developmental stages, but efficient and reliable protocols have not yet been developed. Here we describe a culture system that reproduce the physiological conditions of the corresponding follicular stage, defined to develop in vitro growing oocytes collected from bovine early antral follicles to the fully-grown stage, corresponding to the medium antral follicle in vivo. A combination of hormones and a phosphodiesterase 3 inhibitor was used to prevent untimely meiotic resumption and to guide oocyte's differentiation.

Introduction

During the reproductive life span, only a minimal fraction of the oocytes that are present in an ovary mature, are released in the fallopian tubes upon ovulation, and are available for being fertilized and develop into a viable embryo[1]. On the other hand, most of the oocytes within an ovary undergo atresia and are never ovulated. In vitro embryo production (IVP) technologies have attempted to increase the exploitation of the ovarian reserve[2,3]. Thus far, such technologies have allowed interventions of fertility preservation, selection programs in livestock, and conservation of endangered species. Nevertheless, most protocols use oocytes that have basically completed the growth phase within the antral ovarian follicle, and hence are referred to as fully-grown oocytes. In cattle, where IVP technologies are widely used, fully grown oocytes reach a final diameter of approximately 120 µm and are collected from follicles that span from 2 to 8 mm in diameter (medium antral follicles)[1]. Upon isolation from the follicles, such oocytes are in vitro matured and fertilized. The zygotes are then cultured up to the blastocyst stage and either transferred into a recipient or cryopreserved. In cattle, as well as in many other species, despite the potential offered by IVP, the number of in vitro produced embryo per cow did not largely improve for the last 40 years. This is in part due to the limited number of fully grown oocytes that populate an ovary at a given time which can be retrieved and subjected to standard IVP techniques[4-6].

The oocytes enclosed within early antral follicles, i.e., those follicles that are less than 2 mm in diameter, represent a potential source to be used in fertility preservation programs[7] , as an ovary roughly contains 10 times more early antral follicles than medium antral[8]. However, these oocytes are still in the growth phase and have not yet reached the fully-grown state[9]. As such, they are still transcriptionally active, producing mRNAs that will be stored for later developmental steps, and have

not yet undergone all the differentiation process required to confer the oocytes with the ability of spontaneously resuming and completing meiosis I once isolated from the follicular compartment[10,11]. Therefore, they cannot be directly submitted to standard in vitro maturation (IVM) protocols, but they require an additional period of culture that would allow them to complete the growth phase and properly differentiate.

The transition from the growing to the fully-grown state, which in cattle occurs when the follicle develops from the early antral to the medium antral stage, is one of the critical steps during oocyte development. In cattle, several studies attempted to recapitulate these events in vitro[2,12-19]. However, to date no reliable protocols have been developed and only limited success has been reported. According to previous studies[20], these growing oocytes constitute a homogeneous population. Besides being transcriptionally active, their chromatin is dispersed in the germinal vesicle (GV), in a configuration that is named GV0[2,21]. Conversely, the population of fully-grown oocytes obtained from medium antral follicles is more heterogeneous, a condition that is mirrored by the various degrees of chromatin compaction (GV1, GV2 and GV3) that can be observed[20]. Among these, previous data have shown that GV2 and GV3 oocytes are overall characterized by a better quality and higher embryonic developmental competence[20-24].

Starting from the above observations, here we describe a 5-days long culture system of oocytes (L-IVCO) that allows the differentiation of oocytes isolated as cumulus-oocyte complexes (COCs) from early antral follicles. This culture strategy has evolved from 10 years long studies conducted in our lab and roots its ground on the previously developed 24-48 hours in vitro oocyte culture (IVCO)[2], prematuration systems[23,25] and zinc supplementation during oocyte culture . A combination of follicle stimulating hormone (FSH) and a phosphodiesterase-3 (PDE3) inhibitor, able to

enhance cumulus-oocyte communication[2], prevent untimely meiotic resumption[2], and support oocyte growth[2] was used.

Protocol

The study is approved by the University of Milan's animal use and care committee.

1. Media preparation

NOTE: All media must be prepared at least four hours before use. Sodium bicarbonate buffered media are incubated at 38.5 °C and 5% CO_2 in air, maximum humidity. HEPES-buffered media are maintained at 38.5 °C in thermostatic oven.

1.1. Long in vitro culture of oocytes (L-IVCO) medium

1.1.1. Prepare 15 mL of the basic culture medium (M199-B): Supplement M199 with 2 mM glutamine supplement, 0.4% fatty acid free bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 25 mM sodium bicarbonate, 0.1 mM cysteamine, 21.3 µg/mL of phenol red, 75 µg/mL of kanamycin and 4% Polyvinylpyrrolidone (PVP; 360 k molecular weight).

1.1.2. Prepare 3 mL of the holding medium (M199-H): To M199-B add 5 µM cilostamide and pour it in a 35 mm Petri dish.

1.1.3. Prepare the L-IVCO medium (M199-L): Supplement M199-B with 0.15 µg/mL Zn sulphate, 10^{-4} IU/mL FSH, 10 ng/mL estradiol, 50 ng/mL testosterone, 50 ng/mL

progesterone and 5 µM Cilostamide.

1.1.4. Place 200 µL of M199-L medium in each well of the 96 well coated plate. Fill the wells in the four edges of the plate with sterile culture water to compensate for evaporation and to maintain appropriate humidity during culture.

1.1.5. Incubate the 96 well plate and the M199-H medium in the incubator at 38.5 °C and 5% CO_2 in air, maximum humidity.

1.2. Dissection medium

1.2.1. Prepare the dissection medium (M199-D): Supplement M199 with 0.4% BSA fraction V, 0.164 mM penicillin, 0.048 mM streptomycin, 1790 units/L heparin. M199-D can be prepared in bulk, dispensed in 20 mL aliquots and stored at 4 °C for 6 months. When needed, warm and supplement 1 aliquot.

1.2.2. Prepare 20 mL of M199-D supplemented with 5 µM cilostamide (M199-D cilostamide).

2. Ovary collection and processing

NOTE: All procedures are conducted at room temperature (26 °C) unless otherwise indicated.

2.1. Recover the ovaries at the abattoir from cows (4 to 8 years old).

2.2. Place the ovaries in sterile saline (NaCl, 9 g/L) at 26 – 28 °C added with penicillin 100 U/mL and streptomycin 0.1 mg/mL.

2.3. Transport the organs to the laboratory in warm sterile saline within 4 h.

2.4. Wash the ovaries 4x in sterile saline maintained at 26 °C.

2.5. Remove all mid-to-large antral follicles by aspirating all follicles more than 2 mm in diameter using a 18 G needle connected to an aspiration pump with vacuum pressure set at -28 mmHg and place the aspirated ovaries in a beaker with sterile saline at 26 °C.

NOTE: Removal of the content of follicles > 2 mm is a critical step. Remove fully-grown oocytes as much as possible that would ‘contaminate’ the experiment.

2.6. Under a horizontal laminar flow hood, place one ovary at the time on a sterile polytetrafluoroethylene cutting board. Using a surgical blade #22 mounted on a scalpel handle, cut slices of ovarian cortex (the outer portion of the ovary, which contains the follicles), 1.5 – 2 mm thick and parallel to the major axis of the organ.

2.7. Place the slices of ovarian cortex in a sterile glass Petri dish covered with dissecting medium on a warm plate at 38.5 °C.

NOTE: From now on all the procedures are performed at 38.5 °C using a warm plate.

3. Selection and isolation of the follicles and retrieval of the COCs

3.1. Place one ovarian cortex slice in a 60 mm glass Petri dish with 2-3 mL of M199-D.

3.2. Using a dissection microscope, select the follicles between 0.5 – 2 mm using a micrometer-equipped eyepiece.

3.3. Identify the healthy, non-atretic follicles under the stereomicroscope. Assess follicle atresia by observing morphological parameters, such as very clear translucent appearance, with a dark COC inside. Discard the atretic follicles and process all the others.

3.4. Using a surgical blade #22 mounted on a scalpel handle remove the ovarian tissue surrounding the follicle on one side until the follicle is exposed on one edge.

3.5. Using a 26G needle mounted on a syringe, carefully make a slit in the exposed follicle wall. This action will release the follicular content, comprising the COC, follicular fluid and clumps of cells.

3.6. Identify the COC under the microscope and examine for cumulus integrity, zona pellucida integrity and homogeneity of the cytoplasm. If these criteria are fulfilled, aspirate the COC using a P20 pipette.

3.7. Place the isolated COC in M199-D cilostamide.

3.8. Continue the isolation procedure for 30 min.

4. Selection of COCs to be subjected to in vitro culture

4.1. Under the dissection microscope, select healthy COCs based on the criteria in step 3.6.

4.2. In a 60 mm Petri dish, prepare 16 drops of 20 µL of M199-D cilostamide and place one healthy COC per drop (**Figure 1A**).

4.3. Using an inverted microscope attached to a camera measure the oocyte diameter, excluding the zona pellucida, using the software provided with the camera.

4.4. With a clear visualization of the oocyte, make two perpendicular measurements excluding the zona pellucida (**Figure 1B**).

4.5. Assure whether the mean of the two oocyte's measurement, excluding the zona pellucida, is within a range of 100 – 110 µm. Discard COCs with not-rounded shaped oocyte or with oocytes that are not measurable.

4.6. Transfer the selected COCs in a 35 mm dish containing M199-H medium and keep them in the incubator at 38.5 °C and 5% CO_2 in air, maximum humidity until step 5.1.

4.7. Repeat steps 3 and 4 up to 4x. The overall working time must not exceed 2 h.

5. Long in vitro culture of the oocytes (L-IVCO)

5.1. Transfer one COC per well in the center of a well of the 96 well plate to be prepared in step 1.1.5.

5.2. Incubate the plate for 5 days at 38.5 °C and 5% CO_2 in air, maximum humidity.

5.3. Every other day (day 2 and day 4) prepare fresh M199-L as described in step 1.

5.4. Renew half of the medium by removing 100 µL of medium and replacing with 100 µL of freshly prepared M199-L. Perform the medium renewal under the stereomicroscope and avoid moving the COCs into the well.

6. COC classification after the culture

6.1. At the end of the L-IVCO, analyze the COCs' morphology under the dissection microscope.

6.2. Classify as depicted in **Figure 2**.

6.2.1. Classify as Class 1 if COCs show a compact cumulus cell investment with no sign of cumulus expansion and cell degeneration.

6.2.2. Classify as Class 2 if COCs show a compact cumulus cell investment with no sign of cumulus expansion and cell degeneration and with antrum-like formation in the

cumulus mass.

6.2.3. Classify as Class 3 if COCs show several layers of cumulus cell with no sign of cumulus expansion and some disaggregated cells in the outer layer of cumulus cells and no antrum-like formation.

6.2.4. Classify as Class 4 if COCs shows abundant loss of cumulus cells extending for more than 50% of the oocyte surface, and signs of cell degeneration and cell debris.

7. Evaluation of meiotic progression after culture

7.1. Oocyte denudation

7.1.1. Place each COC in a single well of a four-well plate containing 400 µL of 199D per well.

7.1.2. Under a dissection microscope gently remove the cumulus cells mechanically by repeated pipetting using a pipette set at 130-140 µL.

7.1.3. Once the oocytes are free from the cumulus investment, transfer them to another well containing 199D.

7.1.4. Repeat the process until all the oocytes are completely denuded.

7.2. Oocyte nuclear staining

NOTE: From now on all the procedures are performed at room temperature. Reagents are at room temperature.

7.2.1. Fix the oocytes in paraformaldehyde 4% in phosphate buffer saline (PBS) for 1 h.

CAUTION: Wear personal protective equipment when handling paraformaldehyde and dispose of contaminated materials in accordance with hazardous waste disposal guidelines.

7.2.2. Wash the oocytes 3x for 5 min each in PBS containing 1% polyvinylalcohol (PVA).

NOTE: The samples can be processed right away or stored at 4 °C for maximum one week.

7.2.3. Place the oocytes in PBS containing 0.1% Triton X for 10 min.

7.2.4. Wash the oocytes 3x for 5 min each in PBS containing 1% PVA.

7.2.5. Place the oocytes singularly in drops of 5 µL of antifade medium supplemented with 4',6-diamidino-2-phenylindole (DAPI) dilactate (1 µg/mL) over a slide.

7.2.6. Place two strips of double-sided tape along the long sides of the slide, to avoid excessive flattening of the oocytes when putting the cover slip on top.

7.2.7. Place the cover slip on top, make it adhere to the tape and keep in the dark while processing all the samples.

7.2.8. Analyze the oocytes using a conventional epifluorescence microscope equipped with DAPI filters (Excitation/Emission: 358⁄461) to assess the meiotic progression of the oocytes.

7.2.9. Classify the oocytes according to their meiotic progression: GV - oocytes with different degrees of chromatin condensation within the GV; MI – oocytes from GV breaking down to metaphase I; and degenerate – oocytes that could be not identified as being at any of the previous stages.

Representative results

At the end of the L-IVCO, the gross morphology of the COCs changed and 4 classes were identified based on the appearance of the cumulus cells, as shown in **Figure 2**. Based on the morphological criteria commonly adopted to select healthy COCs[11,26,27], the class 1, 2 and 3 were judged healthy, while the class 4, which showed clear signs of degeneration such as the absence of complete layers of cumulus cells surrounding the oocytes, were considered severely compromised and unsuitable to undergo downstream procedures in a prospective IVP setting. Overall, 74 oocytes in 5 biological replicates were analyzed, of which 9.45% were in class 4 and were discarded from further evaluation.

As shown in **Figure 3** and **Figure 4**, assessment of the meiotic stage at the end of the L-IVCO showed that a significantly higher percentage of the oocytes (78.57 ± 4.43%) remained arrested at the immature stage, with the chromatin still enclosed

within the GV (therefore, also referred to as GV stage), without degenerating. Among them 59.43% were in a GV2/3 configuration. A small percentage resumed meiosis reaching the metaphase I stage (13.76 ± 5.85%) or degenerate (7.67 ± 4.61%). Overall, 67 oocytes in 5 biological replicates were analyzed. Altogether these data indicate that the L-IVCO culture supports the oocyte viability while preventing meiotic resumption for 5 days.

Discussion

Here we describe a culture system for growing oocytes that promotes oocyte development for 5 days by supporting their viability and preventing meiotic resumption. This latter aspect is of the outmost importance to allow the continued growth and differentiation necessary to confer the oocyte with meiotic and embryonic developmental competence[2,20], that would be otherwise blocked by a premature resumption of the meiotic division.

When developing this culture system, we took into consideration several characteristics of the physiological growth and differentiation that occurs in the follicle. In this section we provide an overview of the main aspects that we considered when developing this strategy.

First, growing oocytes in bovine early antral follicles take approximately 5 days to undergo the transition from the growing to the fully grown state in vivo[8,19]. Therefore, the length of the culture was increased to 5 days as opposed to previous attempts made in our lab where the oocytes were cultured for up to 24 h[2].

Another factor that we included in the L-IVCO was the increased viscosity of the medium in which the COCs are cultured to mimic the physiological viscosity of the follicular fluid. This was recreated by adding 4% PVP and, together with the use of

basement membrane matrix medium coated culture surface, it promoted the formation of a 3D like culture, as reported by previous studies[13].

Cilostamide, a PDE3 inhibitor, was added to maintain oocytes meiotically arrested at the GV stage, preventing precocious meiotic resumption by keeping high levels of cyclic nucleotides within the oocytes[2,19,25,28,29]. Our results indicate that a 5-day-long treatment with cilostamide does not have a gross impact on COCs health, as only a small fraction of complexes degenerated, also in agreement with the results obtained by Alam et al.[19].

The inclusion of Zn sulfate, and its concentration, is substantiated by recent results showing that this trace element has a role in supporting the differentiation and transcriptional activity of bovine growing oocytes in culture[30].

Finally, a combination of hormones was introduced to closely mimic the physiological hormonal milieu typical of the early antral follicle[31-33]. For instance estradiol has known activities in supporting the oocyte growth[16,17,19] and the connection among granulosa cells[17], while also promoting the acquisition of meiotic competence[34]. Similarly, testosterone, besides being a precursor of estradiol, also stimulates follicular growth and development[35], while progesterone was mainly added for its antiapoptotic activity[36].

Importantly and in agreement with our previous study[2], the concentration of FSH was kept at a concentration that is physiological for the growing phase. Indeed, a low FSH concentration promotes oocyte development by sustaining gap-junction mediated communication between the oocyte and the companion cumulus cells and promotes transcriptional activity and oocyte differentiation without inducing meiotic resumption[2].

In our experience one of the keys for the success of the L-IVCO is the selection of a homogeneous population of healthy COCs coming from early antral follicles.

According to data in the literature, 80% of the oocytes collected from early antral follicles are characterized by chromatin organized in a configuration termed GV0[20]. This homogeneity represents an advantage for in vitro culture, as in principle it ensures that the cells will behave similarly when exposed to the culture environment. With this in mind, COCs collection must be performed trying to minimize the 'contamination' with COCs coming from less or more advanced stages of differentiation. However, due to the fact, that processing of the cortical slices is quite time consuming and should be carried out in a relatively short time, the collection/selection step probably represents the most critical passage of the L-IVCO. To achieve that, some key considerations should be beard in mind.

For instance, the researcher/technician needs to be trained to recognize and discard follicles with signs of follicular atresia. At this stage, only morphological parameters can be used to recognize atretic follicles, such as very clear translucent appearance, and the presence of a dark COC inside. All the other follicles, in which atretic signs cannot be clearly distinguished, should be opened and further selection based on the morphology of the isolated COCs should be carried out to identify the healthy ones[2,3,37-39]. This is achieved again by morphological observations such as the presence of at least four layers of cumulus cells, grossly spherical shape, intact oolemma and homogeneous and finely granulated ooplasm[11,26,27].

COCs isolation and manipulation represents an additional technical challenge, which requires skilled personnel and proper equipment for micro-dissection under the stereomicroscope and accurate determination of oocyte diameter. This last step is essential to select a uniform population of oocytes, thus excluding any possible source of contamination with COCs coming from other follicular stages. For this reason, it is important to make sure that the oocytes enclosed in the retrieved COCs have a diameter between 100 and 110 μm[2,40].

Besides supporting oocyte viability and preventing meiotic resumption, the L-IVCO promoted the transition of the chromatin configuration from GV0 to the progressively more condensed GV2 and GV3 in 59% of the oocytes. Notably chromatin condensation within GV is a marker of 'gain' of meiotic and developmental competence in basically all the mammalian oocytes studied thus far[20]. This result is very promising, especially when compared to our previous 24 hours IVCO system. In that study, the highest degree of chromatin compaction within the GV were not reached and 22% of oocytes were found with a GV1 configuration[2], a stage associated with full meiotic competence but still scarce developmental competence[20]. Even in those conditions, the otherwise incompetent growing oocytes were able to mature and produce embryos, although in limited amount. The consistent increase in GV2/3 stages observed in the L-IVCO is therefore compatible with a higher potential to produce viable embryos. We are in the process of testing this hypothesis experimentally by submitting COCs derived from L-IVCO to the following steps of IVP (in vitro maturation, fertilization, and embryo culture up to the blastocyst stage). If confirmed, the L-IVCO will unleash some of the yet unexploited potential of the ovarian reserve, with important implications on several areas of interest for female fertility preservation. For instance, it will increase the source of fertilizable gametes to be used in preservation programs of high genetic merit breeders. Another application that we foresee is for the genetic salvage of threatened species of the bovid family as well as of local breeds that are endangered or at risk of genetic erosion due to the widespread diffusion of cosmopolite breeds. Last but not the least, L-IVCO represents a tool for all the scientists that are interested in dissecting the cellular and molecular processes that regulate the formation of a competent gamete.

Acknowledgments

This work was supported by Regione Lombardia PSR INNOVA n.201801061529 and UNIMI n.PSR 2019_DIP_027_ALUCI_01

Disclosures

The authors have nothing to disclose.

Figures and tables

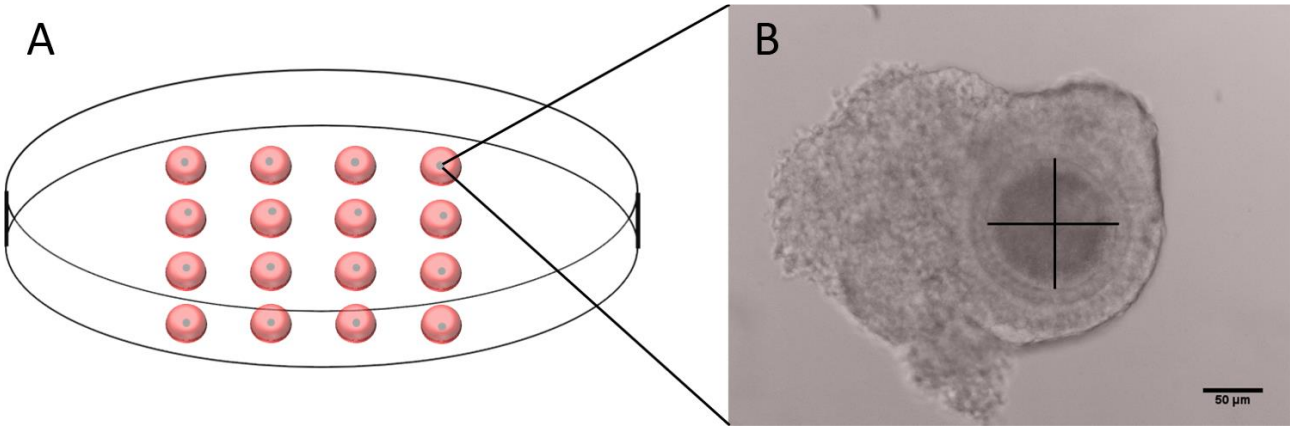

Figure 1: Outline of the dish used for measuring the oocyte diameter and representative image of a COC. (**A**) Schematic representation of a 60 mm Petri dish with 16 drops of 20 µL of M199-D, each one containing a single COC. (**B**) Representative image of a COC with the axis used for measuring the diameter. Note that the zona pellucida is not included. Scale bar 50 µm.

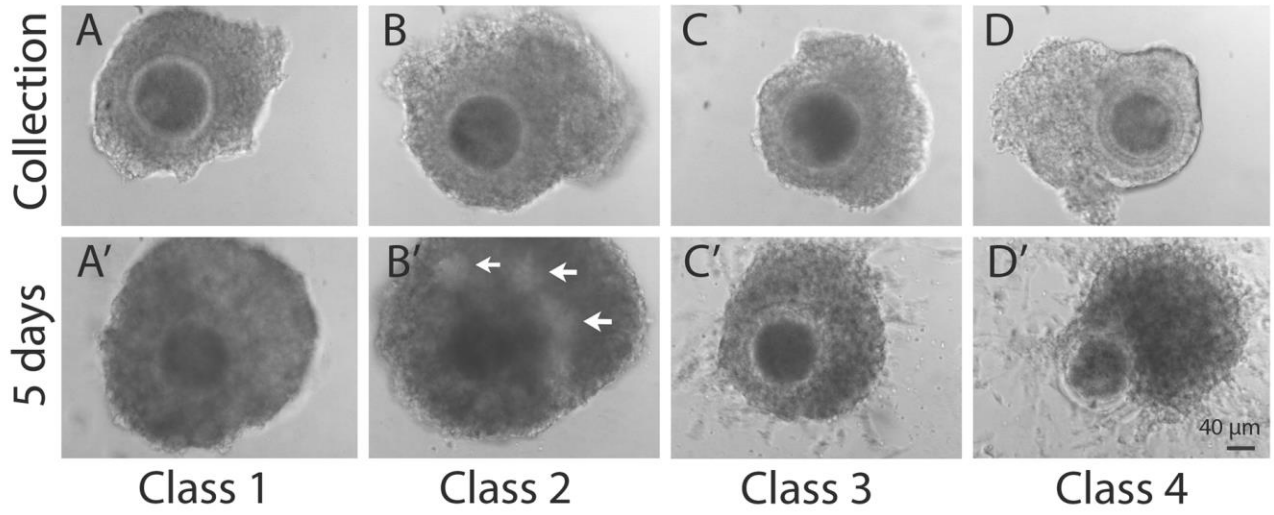

Figure 2: Representative images of COCs at the time of collection and after L-IVCO. (**A, B, C, D**) The upper row (Collection) represents COCs at the time of retrieval. (**A', B', C', D'**) The same COC is pictured 5 days later, at the end of L-IVCO and classified as reported in step 6.1. The lower row (5 days) represents COCs classified as: Class 1, showing a compact cumulus cell investment with no sign of expansion and cell degeneration (A'); Class 2, showing a compact cumulus cell investment with no sign of expansion and cell degeneration and with antrum-like formation (arrows) in the cumulus mass (B'); Class 3, showing several layers of cumulus cell with no sign of cumulus expansion and some disaggregated cells in the outer layer of cumulus cells (C'); class 4, showing abundant loss of cumulus cells on more than 50% of the oocyte surface and signs of cell degeneration and cell debris (D'). Scale bar 40 µm.

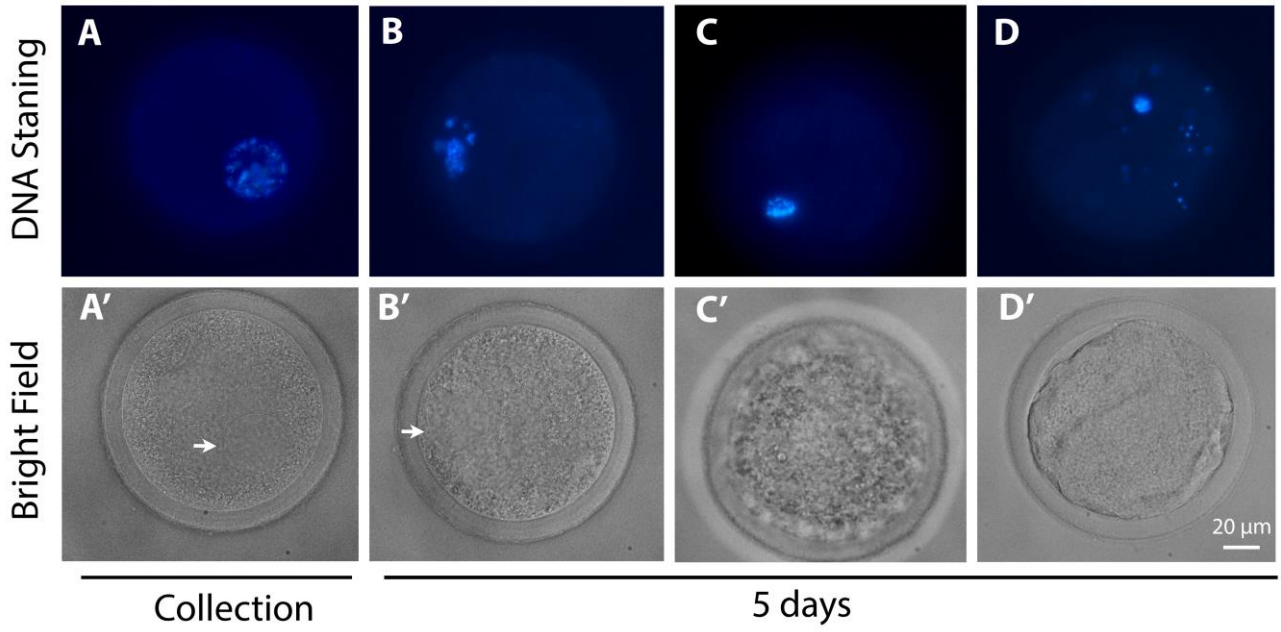

Figure 3: Representative images of the meiotic progression. The upper row (DNA staining) shows the DNA (blue) of representative oocytes at (**A**) the GV0 stage and (**B**) GV2-like configuration, (**C**) MI stage and (**D**) degenerated oocytes, (**A**) at the time of collection and (**B, C, D**) after 5 days of L-IVCO. The lower row is the corresponding image in bright field of the oocyte in the upper row. The arrow indicates the GV. Scale bar 20 µm.

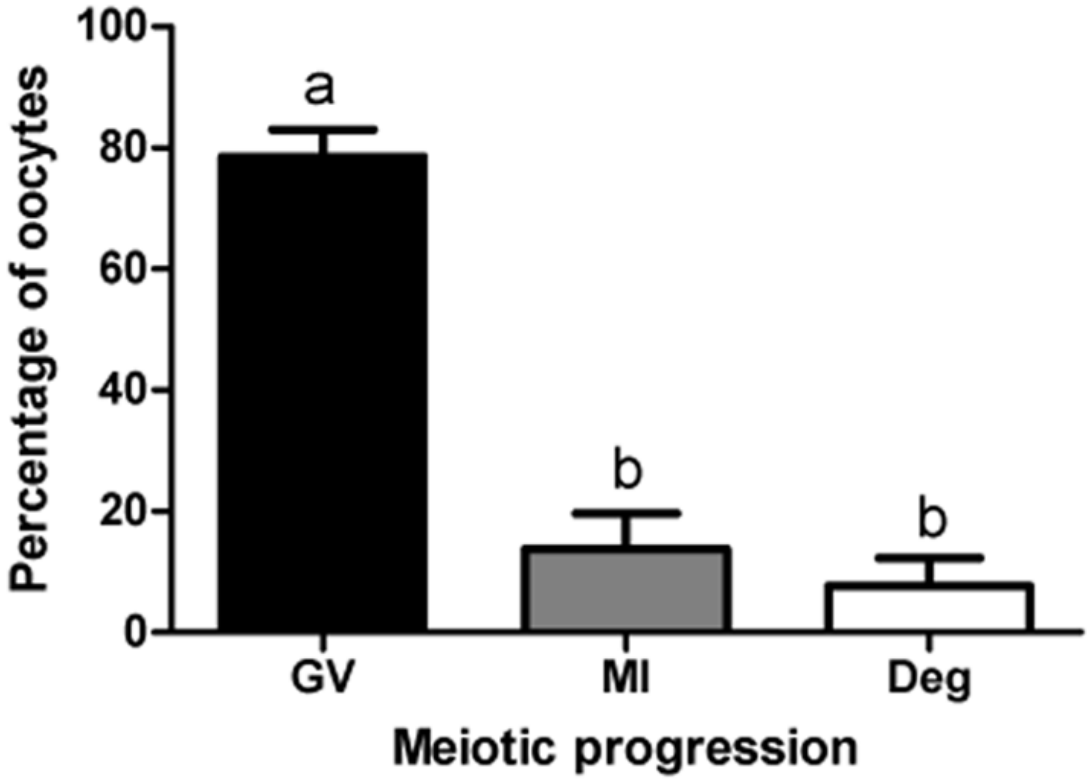

Figure 4: Meiotic progression of the oocytes at the end of culture. The bar graph represents the distribution of oocytes at GV and MI stage and degenerated oocytes at the end of the L-IVCO. The oocytes previously classified in class 4 were excluded. Data were analyzed by 1-way ANOVA followed by Tukey's multiple comparison test and values are means ± SEM (N=5; P<0.05).

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SECTION II

ADVANCED PHYSIOLOGICAL APPROACHES FOR IN VITRO OOCYTE DEVELOPMENT AIMED AT EXPLOITING THE REPRODUCTIVE POTENTIAL OF THE OVARIAN RESERVE

In press:

Methods Molecular Biology, Vol. 2273, TizianaA.L. Brevini et al. (Eds): Next Generation Culture Platforms for Reliable In Vitro Models, 978-1-0716-1245-3, 500220_1_En, (Chapter 1)

DOI: 10.1007/978-1-0716-1246-0

Running title: Strategies for bovine oocytes in vitro culture

Recreating the follicular environment: a customized approach for in vitro culture of bovine oocytes based on the origin and differentiation state

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Summary/Abstract

The mammalian ovary is a large source of oocytes organized into follicles at various stages of folliculogenesis. However, only a limited number of them can be used for in vitro embryo production (IVEP) while most have yet to complete growth and development to attain full meiotic and embryonic developmental competence. While the in vitro growth of primordial follicles in the ovarian cortex has the potential to produce mature oocytes, it is still at an experimental stage. The population of early antral follicles (EAFs), instead, may represent a reserve of oocytes close to completing the growth phase, which might be more easily exploited in vitro and could increase the number of female gametes dedicated to IVEP.

Here we present in vitro culture strategies that have been developed utilizing physiological parameters to support the specific needs of oocytes at distinct stages of differentiation, in order to expand the source of female gametes for IVEP by maximizing the attainment of fertilizable oocytes. Furthermore, these culture systems provide powerful tools to dissect the molecular processes that direct the final differentiation of the mammalian oocyte.

Key Words

Oocyte, cumulus cells, gap-junction, chromatin, germinal vesicle; intercellular communication; meiotic arrest, prematuration, oogenesis, in vitro oocyte growth

Introduction

IVEP technologies developed to allow the transfer of embryos obtained through in vitro maturation (IVM) of immature oocytes, followed by in vitro fertilization (IVF) and in vitro culture (IVC) of early embryos. However, the efficiency is still limited, and the small supply of mature, fertilizable oocytes is one of the main limitations to the success rate of assisted reproduction, in cattle as well as in other mammals.

The mammalian ovary is a potential large source of oocytes that are enclosed into follicles at various stages of development, from the primordial to the ovulatory ones. Nonetheless, only a limited number of such oocytes can be readily submitted to routine IVEP protocols and generate viable embryos. These oocytes are the ones that have reached the so-called fully-grown stage and are enclosed in middle and large antral follicles (MAFs and LAFs, respectively). Yet, not all of them reach the blastocyst stage of embryonic development when subjected to standard IVEP. Furthermore, most of the oocytes in an ovary have yet to reach the fully-grown stage and ultimately acquire full meiotic and embryonic developmental competence [1]. Specifically, these oocytes span from the ones enclosed in primordial follicles to the ones enclosed in EAFs, that are still in the growing phase.

While in vitro growth of primordial follicles enclosing the resting oocyte pool in the ovarian cortex has the potential to produce mature oocytes [2], this technique is still experimental, and an efficient exploitation of this reserve seems far from being applicable on a large scale. By contrast, the population of oocytes enclosed in EAFs may represent a more readily exploitable source that would increase the number of female gametes that can successfully undergo IVEP [3] (**Table 1**). Finally, optimization of culture systems for fully-grown oocytes isolated from MAFs may increase the overall efficiency.

Oocytes enclosed in EAFs and MAFs represent an extremely heterogeneous cell population, with very distinct morphological and functional characteristics [4,5]. In bovine, nearly all of the oocytes isolated from EAFs (0.5-2 mm) are still growing, the chromatin appears mostly uncondensed and dispersed throughout the nucleoplasm, in the so-called GV0 configuration [6]. At this stage, oocytes are still transcriptionally active and are functionally fully coupled through gap junctions (GJs) to the surrounding cumulus cells [6-8]. On the other hand, oocytes from MAFs (2-8 mm), which are the most commonly used for IVEP, are characterized by various degrees of progressive chromatin compaction, named GV1, GV2 and GV3 configurations. The 3 classes are equally represented within the populations of oocytes collected from MAFs [6,9]. The transition from GV0 to the higher classes of chromatin compaction, up to GV3, is accompanied by progressive transcriptional silencing [7], changes in the epigenetic signatures such as global DNA methylation [10] and histone modifications [11,12] and changes in cytoplasmic organelle redistribution and nuclear architecture [7]. More importantly, these changes are also accompanied by a gradual acquisition of meiotic and developmental competence [6,7,3,13,14].

In the present work, we describe cultural strategies customized to better fulfill the physiological needs of oocytes at the distinct stages of differentiation. The described procedures induced an increased chromatin compaction, that was accompanied by a progressive and significant increase in developmental capacity (**Figure 1**). Notably, that the transition to higher degrees of chromatin compaction is associated with an increase in developmental competence has recently been observed also in vivo [15,16]. Specifically, oocytes that were ‘synchronized’ at the GV2 stage using a mild FSH stimulation before ovum pick up (OPU), reached significantly higher blastocyst rates when submitted to IVM/IVF/IVC [15].

Precisely, in this method article we will describe:

- the long in vitro culture of oocytes (L-IVCO), targeted to growing oocytes isolated from EAFs [17];
- the prematuration (pre-IVM), mostly beneficial to the GV1-enriched population of oocytes isolated from MAFs [9];
- the IVM, mostly beneficial to the GV2 and GV3-enriched population of oocytes isolated from MAFs [9];

The rationale adopted in designing and optimizing the above-listed treatments stems from the idea of recreating the follicular environment in which a developmentally competent oocyte grows and differentiates until the first meiotic division is completed.

Each of the proposed protocols has been optimized considering the following main physiological characteristics and experimental evidences.

Once the oocytes have acquired the molecular machinery necessary to resume meiosis, the follicular environment acts to keep the cell cycle arrested by supporting the intra-oocyte content of cyclic nucleotides [18]. In vivo, the protracted arrest ensures that the oocyte undergoes the final steps of differentiation that confer developmental competence, by avoiding untimely meiotic resumption. This condition was recreated in vitro by supplementing the L-IVCO and pre-IVM media with cilostamide [3,14,19-21], an inhibitor of phosphodiesterase-3 (PDE3) which is specific to the oocyte in bovine as in most mammals [22]. As an alternative to pharmacological treatment, also the use of natriuretic peptide precursor C (NPPC) has been shown to be effective [14,23-28]. NPPC is the natural activator of the guanylyl cyclase-coupled natriuretic peptide receptor type-2 (NPR2) [29] that induces the production of 3’-5’ cylic guanosine monophosphate (cGMP), which is then transferred via GJs to the oocyte [30] where it inhibits PDE3A, thus maintaining the meiotic arrest [31,32].

The hormonal milieu, including estradiol (E_2), testosterone (T), progesterone (P_4), and follicle stimulating hormone (FSH), was studied to mimic as much as possible the follicular conditions in EAFs [33-36]. E_2 and T, which also acts as an estrogen precursor, primarily support oocyte and follicle growth [37,21,38], by promoting granulosa cells intercellular communication and meiotic competence acquisition [39]. P_4 has instead a role in inhibiting the apoptosis in granulosa cells [40]. Since a specific hormonal composition was also found in the follicular fluid at the time of selection for dominance [41-44,28], the pre-IVM medium can be similarly supplemented with E_2, T, and P_4 [28].

Studies conducted in several mammalian species show that supplementation of the IVM medium with FSH improves oocyte quality [45-50], prompting the widespread use of FSH as hormonal supplementation in IVM protocols. Even though there is not consensus on the exact signaling mode, FSH at high dosage seems to exert its effects through the activation of the epidermal growth factor (EGF) network [51,45,52,53,50], which is physiologically triggered in vivo by the surge in luteinizing hormone (LH) [54]. However, FSH levels similar to the ones used in IVM proved to be harmful for the culture of growing oocytes, that underwent early closure of the GJ-mediated communications with the cumulus cells, precocious meiotic resumption and insufficient growth [3]. The preservation of cumulus oocyte communication functionality during in vitro growth and prematuration protocols represent a key point and specifically the intracellular cAMP concentration evoked by FSH stimulation plays a crucial role [55-60,3,61-63,20,64,13,19,65]. By conducting dose/response curves, it was established that one thousand times less FSH was instead beneficial during the growing phase, sustained the functionality of the GJs, and promoted transcriptional activity and oocyte growth and differentiation [3].

During pre-IVM, FSH concentrations similar to the ones used in L-IVCO also improved oocyte quality by sustaining GJ coupling with the cumulus cells [13,14,9,28].

Other aspects that are instead peculiar to the L-IVCO aimed at promoting a 3D structure and supporting the transcriptional activity. Specifically, the first was achieved by increasing the viscosity of the medium to mimic the physiological viscosity of the follicular fluid, and by culturing the cumulus oocyte complexes (COCs) on a collagen coated surface (**Figure 2**) [17,66]. Finally the inclusion of zinc sulphate is supported by recent results showing the role of this trace element in promoting the differentiation and transcriptional activity of bovine growing oocytes [67].

The procedures described herein represent multiple culture strategies that have been developed to support the specific oocyte needs in relation to the developmental step, with the aim of expanding the source of oocytes usable for IVEP. Nonetheless, these protocols will provide useful tools to dissect the cellular and molecular process that control the final oocyte development.

Materials

All media are prepared with embryo tested ultra-pure water. Disposable, sterile plasticware is from BD Falcon by Corning, NUNC IVF Line, and Sterilin™ by Thermo Scientific, where duly specified. Final filtration of all stock solutions, as well as preparation of working solutions, is performed using sterile techniques under a biohazard laminar flow cabinet or laminar flow hood to keep sterility. All glassware is exclusively used for embryo culture media preparation and is high pressure steam-sterilized by autoclaving at 121°C for twenty minutes. After use, glassware is immediately washed and rinsed with running tap water for 30 minutes, rinsed three

times with 18.2 mΩ water, then dried completely and covered with aluminum foil until sterilization.

All the procedures are conducted at 26-28 °C unless otherwise specified.

Collection media

1. Prepare HEPES-based manipulation medium (HM199) from Medium 199 with Earle′s salts supplemented with 0.68 mM L-glutamine, 25 mM HEPES, 0.4% Bovine Serum Albumin (BSA) Fraction V, 0.164 mM penicillin, 0.048 mM streptomycin and 1790 u/L heparin. HM199 are aliquoted in 20 mL in Sterilin tube (Thermo Scientific) and stored at 4°C for 6 months.

2. Prepare meiotic arrest holding medium (HM199-Cilo) with HM199 supplemented with 5 µM of the selective PDE3 inhibitor cilostamide [14] (see **Note 1)**.

3. Warm sealed aliquots of HM199 and HM199-Cilo at 38.5°C in a dry thermostatic chamber before use.

Long in vitro oocyte culture (L-IVCO) medium

1. Prepare the basic oocyte culture medium (bM199) from Medium 199 with Earle′s salts supplemented with 25 mM sodium bicarbonate, 21.3 µg/ml phenol red, 75 µg/mL kanamycin, and 4% polyvinylpyrrolidone (PVP; 360k molecular weight) (see **Note 2**).

2. Five hours before culture (see **Note 3**), prepare the L-IVCO medium by supplementing the above bM199 with 2 mM GlutaMAX™, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 0.15 µg/mL zinc sulphate, 10^{-4} IU/mL recombinant human FSH (r-hFSH), 10 ng/mL E_2, 50 ng/mL T, 50 ng/mL P_4 and 5 µM Cilostamide [17].

3. Fill a BioCoat™ Collagen I 96-well plate (Corning) with 200 µl of L-IVCO medium and equilibrate for at least 4 hours before use for oocyte culture at 38.5°C and 5% CO_2 in air, maximum humidity. For all additives' storage, see **Note 4**.

Pre-IVM medium

1. Prepare the oocyte pre-IVM medium from Medium 199 with Earle′s salts supplemented with 25 mM sodium bicarbonate, 2 mM GlutaMAX™, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, 10^{-4} IU/mL r-hFSH and 10 µM Cilostamide [14,9] (see **Note 5**). Equilibrate in the incubator at 38.5°C and 5% CO_2 in air, maximum humidity (see **Note 6**).
2. Fill each well of a NUNC IVF four-well plate (Thermo Scientific) with 500 µl of pre-IVM medium and equilibrate for at least 4 hours before use at 38.5°C and 5% CO_2 in air, maximum humidity.

IVM medium

1. Prepare IVM medium from Medium 199 with Earle′s salts supplemented with 25 mM sodium bicarbonate, 2 mM GlutaMAX™, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, 0.1 IU/ml of r-hFSH.
2. Fill each well of a NUNC IVF four-well plate with 500 µl of IVM medium and equilibrate for at least 4 hours before use for oocyte culture at 38.5 °C and 5% CO2 in air, maximum humidity.

Methods

The recommended operative procedure is to first proceed with the isolation of COCs from MAFs (2-8 mm) by aspiration through a needle connected to a vacuum pump (3.1). These COCs will be divided in subgroups and either subjected directly to IVM (3.2) or to pre-IVM (3.3) followed by IVM as detailed below. COCs from EAFs (0.5-2 mm) are isolated by individual follicle dissection from slices of ovarian cortex (3.4) taken from the ovaries that already underwent MAFs aspiration. These oocytes are submitted to L-IVCO (3.5) and, at the end of L-IVCO, can be transferred to IVM.

All the procedures are conducted at 36-38°C, unless otherwise indicated.

Isolation of COCs from MAFs

1. Collect bovine ovaries from 4-8 years old Holstein dairy cows subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications, at the abattoir. Transport to the laboratory, within 3 h, in sterile saline maintained at 28° C.

2. Retrieve COCs from 2-8 mm follicles with an 18-gauge needle mounted on an aspiration pump (COOK-IVF, Australia) with a vacuum pressure of 28 mm/Hg (see **Note 7**). Once the content of the MAFs has been aspirated, keep the ovaries in warm saline for procedures described in 3.4.

3. Examine the morphology of the COCs under a stereomicroscope and select the complexes medium brown in color, with five or more complete compact layers of cumulus cells [68,69]. These COCs will be further divided in 3 subclasses, according to previously described morphological features [70,71,9]: Class 1, with homogeneous ooplasm and compact cumulus cells; Class 2, with minor granulation of the ooplasm and compact cumulus cells; Class 3, with highly granulated ooplasm and slight

expansion of cumulus cell layers (**Figure 3**). As previously demonstrated, Class1 is GV1-enriched and Class 2/3 are GV2/3-enriched [9].

IVM

Group the Class 2 and Class 3 COCs, wash twice in HM199, then culture in groups of 30-34 in 500 µl of the IVM medium for 24 h, in four-well dishes at 38.5°C under 5% CO_2 in humidified air (see **Note 8**).

Pre-IVM

Wash twice the Class 1 COCs in HM199, then culture in groups of approximately 30 COCs in 500 µl of the pre-IVM medium for 6 h, in four-well dishes (NUNC) at 38.5°C under 5% CO_2 in humidified air. After pre-IVM culture, wash COCs of Class 1 twice in HM199, then transfer them in 500 µl of IVM medium for 24 h, in four-well dishes (NUNC) at 38.5°C under 5% CO_2 in humidified air (see **Note 8**).

Isolation of COCs from EAFs

1. Prepare ovarian cortex slices as previously described [17]. Under a horizontal laminar flow hood, with warm plate and a stereomicroscope equipped with a heating stage, place one ovary at the time on a sterile Teflon cutting board. Using a surgical blade n. 22 mounted on a scalpel handle, cut slices 1.5-2 mm thick of ovarian cortex and parallel to the major axis of the organ. Maintain the slices of ovarian cortex in a sterile glass Petri dish covered with HM199 on a warm plate at 38.5 °C.

2. Place the ovarian cortex slice in a 60 mm glass Petri dish with 2-3 mL of HM199. Under the stereomicroscope select the follicles between 0.5-2 mm by using a micrometer-equipped eyepiece. Remove the ovarian stroma surrounding the follicle to expose it on the edge using a surgical blade n. 22 mounted on a scalpel handle.

Carefully, holding the slice with tweezers, make a slit on the follicle wall using a 26G needle mounted on a syringe to release the follicular content, including the COC, follicular fluid and clumps of cells.

3. Under the stereomicroscope select COCs medium brown in color, with five or more complete compact layers of cumulus cells, intact zona pellucida and homogeneous cytoplasm. The diameter of the oocyte, excluding the zona pellucida, must be within a range of 100 - 110 µm (see **Note 9**). Place the isolated COCs in HM199-Cilo.

Limit each round of COCs isolation to 30 min, then incubate the selected COCs in a 35 mm Petri dish with 2 ml of L-IVCO at 38.5°C under 5% CO2 in humidified air, until the collection procedure is completed and the COCs can be transferred individually into a well of the L-IVCO 96-well plate.

Long in vitro oocyte culture

1. Place each selected COC singularly in the center of a well of the 96-well plate containing the previously prepared L-IVCO medium (see 2.2) and incubate the plate for 5 days at 38.5°C and 5% CO_2 in air, maximum humidity.

2. On day 2 and day 4 renew half of the medium by removing 100 µL of medium and replacing with 100 µl of freshly prepared and incubator-equilibrated L-IVCO medium. Perform the medium renewal gently under the stereomicroscope to avoid detaching of the COCs from the bottom of the well.

3. On day 5, select all COCs with compact cumulus cell investment, with no sign of cumulus expansion and cell degeneration while discard COCs showing abundant loss of cumulus cells extending for more than 50% of the oocyte surface, and signs of cell degeneration and cell debris, as previously described in [17].

4. Wash the selected in vitro grown COCs twice in HM199 then transfer them in 500 µl of IVM medium for 24 h, in four-well dishes (NUNC) at 38.5°C under 5% CO_2 in humidified air (see **Note 8**).

Notes

Note 1: Meiotic arrest during the collection procedure can also be maintained by using the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) at the final concentration 0.5 mM [13].

Note 2: To prepare the 4% PVP basic L-IVCO, first prepare an 8% PVP solution by dissolving 40 gr of PVP in 500 ml of embryo tested cell culture water in a sterile bottle. Seal the cap tightly and keep in a water bath at 100 °C for 40 minutes. Leave to cool. Prepare basic L-IVCO 2X by dissolving the Medium 199 with Earle′s salts powder in 500 ml of embryo tested cell culture water and supplement with 25 mM sodium bicarbonate, 21.3 µg/ml of phenol red, 75 µg/mL of kanamycin. Adjust the pH to 7.4 and filter-sterilize. Mix basic L-IVCO 2X and 8% PVP solution 1:1 to obtain basic L-IVCO medium.

Note 3: All $NaHCO_3$-buffered media must be equilibrated in the incubator for a minimum of 4 h before use. This step allows the gas exchange with the correct CO_2 pressure to obtain a pH of approximately 7.4.

Note 4: Additives such as sodium pyruvate and cysteamine are prepared as stock solutions 100X and stored at -20°C for up to three months. The BSA stock solution is

50X and is stored at +4°C for up to three months. The r-hFSH is prepared as 100 IU/ml stock in PBS containing 0.1% of BSA and stored at -20°C up to three months. Steroids are prepared as stock solutions 1000X in absolute Ethanol and stored at -20°C for up to one year.

Note 5: For pre-IVM, depending on the experimental design, cilostamide can be replaced with 100 nM natriuretic peptide precursor C (NPPC) [14,28,72].

Note 6: In order to further reproduce the follicle environment during the phase of selection for dominance, pre-IVM medium can additionally be supplemented with 500 ng/mL E_2, 50 ng/mL P_4, 50 ng/mL T [28,16].

Note 7: During follicle aspiration, aspirate aliquots of about 0.5 ml of HM199 through the needle into the tube circuit every 2 ml of aspirated liquid to prevent formation of clots. Avoid excessive dilution of follicular fluid with HM199 to effectively maintain oocytes in meiotic arrest [73]. The whole procedure (follicle aspiration and COCs selection) must be performed in approximately 60 min to avoid meiotic resumption of Class 1 oocytes. Alternatively, use the HM199-Cilo as holding medium to pool all Class 1 COCs during selection.

Note 8: After IVM, oocytes can be submitted to IVF and presumptive zygotes cultured for 7 days as previously described [3,14,9,28].

Note 9: Measure the diameter of the oocytes using a micrometer-equipped eyepiece or other appropriate tools such as a microscope-mounted camera and software that allows measurements. The diameter of the oocyte excluding the zona pellucida must be within a range of 100 - 110 µm. Discard COCs with larger or smaller oocytes and with irregular and not-rounded shaped oocyte.

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Acknowledgements

This work was supported by Regione Lombardia PSR 2014/2020 - Operazione 10.2.01 "Conservazione della Biodiversità Animale e Vegetale" - n. 201801061529 - INNOVA to AML and by MIUR, PRIN 2017 Linea b n. 20172N2WL3_002 to FF

Figures and tables

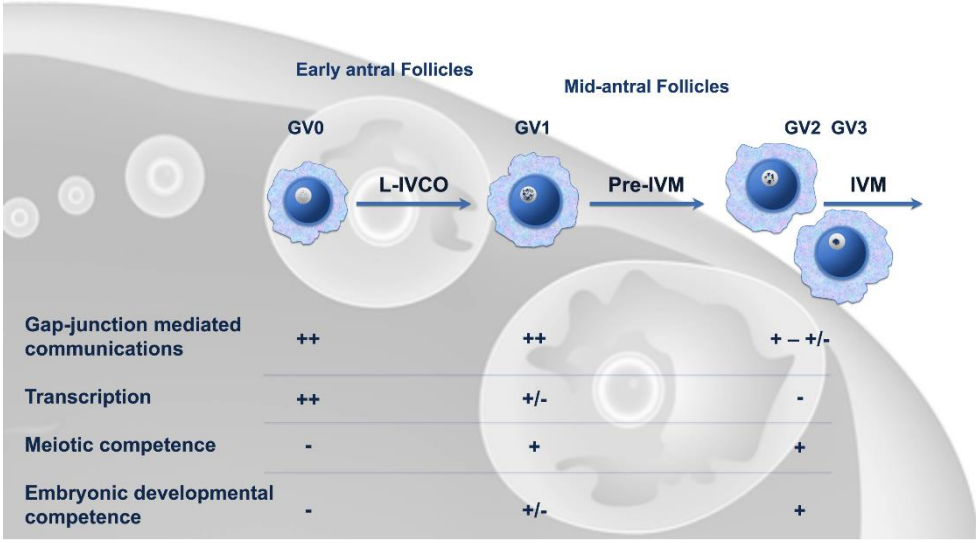

Figure 1. Multiple approach: a customized culture system for each stage specific need.

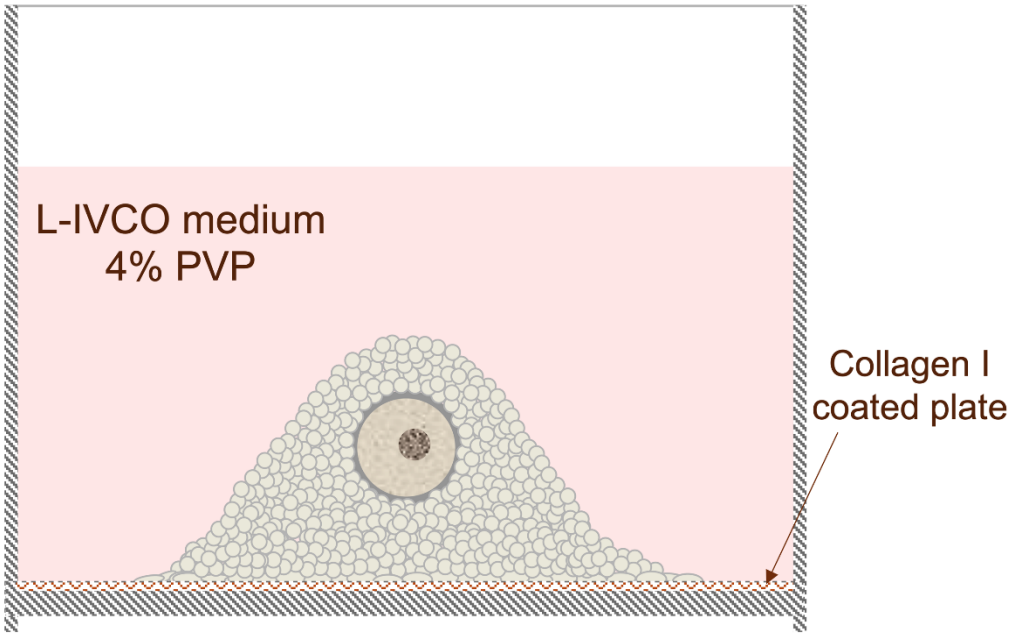

Figure 2. Schematic representation of the COCs growing in the BioCoat™ Collagen I 96-well plate during L-IVCO. To promote the three-dimensional organization of cells, the COCs are cultured in L-IVCO medium where viscosity is increased by adding 4% PVP to mimic the physiological viscosity of the follicular fluid and by culturing the COCs on a collagen coated surface that promote the formation of a 3D-like culture [17,66].The stem of granulosa cells adheres to the coated bottom while the cumulus oophorous protrudes into the medium in the center of the well.

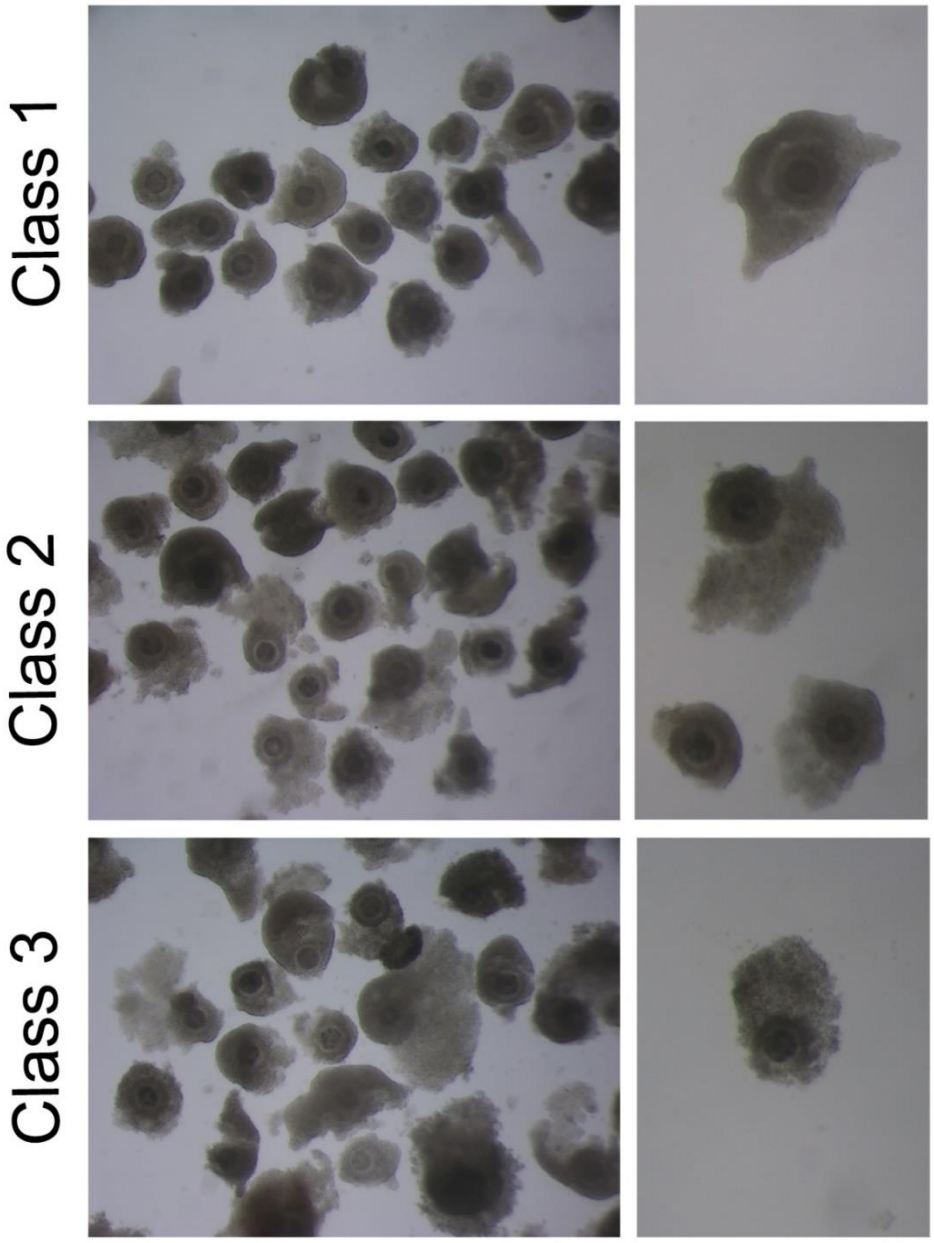

Figure 3. Representative pictures of COCs isolated from MAFs. After collection, COCs are separated according to morphological criteria. Class 1: homogeneous ooplasm and absence of expansion of outer layer of cumulus cells; Class 2: minor granulation of the ooplasm and/or beginning of expansion of outer layer of cumulus cells; Class 3: highly granulated ooplasm and few cumulus cells layers showing expansion.

Table

Table 1. Extent of follicle reserve and follicle categories in 4-8 years old bovine ovaries (data were extrapolated from [74-77,44]). The bottom part of the table are the proposed optimal exploitation strategies.

Follicle category	Primordial (<0.1 mm)	Primary and Secondary >0.1 to <0.5	Early antral (≥0.5 to <2 mm)	Antral (2 to 8 mm)
Number/ovary	65,000	25,000	120	25
Atresia	<5%	5%	30%	60%
Chromatin	-	-	GV0	GV1, GV2, GV3
Optimal exploitation strategies				
Ex-vivo (following culling) ovaries	Freezing/culture [2]	Freezing/culture [2]	L-IVCO [17,3]	Pre-IVM (GV1) and direct IVM (GV2 and GV3) [70,9]
In vivo	-	-	-	Synchronization followed by OPU and direct IVM [15]

CHAPTER 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Oocytes in early stages of development represent a potentially large source of oocytes compared with those in antral follicles [33, 58, 145-147] and this source can be could be 'unlocked' for reproduction, preservation or research purposes. Consequently, the need for a dynamic and variable culture system to support all stages of oocyte development, from activation of the dormant primordial follicle to a stage where oocytes can undergo meiotic maturation, is vital for the achievement of the use of this source [9], but the lack of knowledge in understanding the mechanisms involved in the early development of the oocyte is an important limiting factor. Moreover, the lack of standard protocols makes this technology still in its experimental phase [3].

In this project, we aimed to set the physiological conditions to promote the oocyte's growth and differentiation during the early stages of development by monitoring the state of coordinated differentiation of the oocyte and the surrounding cumulus cells. We recreated the culture conditions that simulate the physiological environment for growing oocytes during follicle development. We achieved this by using relevant biological components to support COC differentiation evaluated through the ability to achieve both meiotic and embryonic developmental competencies.

In the second chapter, studies focused on zinc's role during the late stages of oocyte growth and differentiation, particularly exploring the hypothesis that zinc participates in transcription control during this stage. This hypothesis arises from its biological functions and previous observations that indicated the zinc transporters are differentially expressed in cumulus cells during oocyte differentiation [121].

Our study demonstrated that zinc supplementation improves the meiotic competence of growing oocytes, affects the global transcription activity and the global DNA methylation and ultimately enhances acquisition of meiotic competence. Thus, zinc modulates transcription during a critical step of oocyte development, when its developmental competence is determined. These results agree with previous findings

in mice, where feeding a zinc-deficient diet during preconception alters oocyte chromatin methylation and expression of specific genes [139].

Moreover, this study provided evidence that zinc supplementation enhances transcription in cultured GV0 oocytes, which correlate to the maintenance of the global level of DNA methylation that is known to characterize GV0 oocytes at the time of collection [161]. This result is relevant since increased levels of transcription in GV0 oocytes during culture can be beneficial since the oocyte would have more time to produce and store a large quantity of RNA and proteins that are needed until the embryonic genome activation.

Due to these experiments' positive results, zinc became a natural supplementation in the following studies. Moreover, it gave insights to further studies about the contributions of the CC and oocytes in the maintenance of zinc homeostasis and characterization of specific biomarkers that control zinc transport and storage during oogenesis.

The third chapter provided evidence of a significant 5-days protocol, namely L-IVCO, that supports the differentiation of growing oocytes. This culture strategy that closely mimics the physiological environment for growing oocytes that is based on the physiological concentrations of FSH, E2, P4 and T2 and the zinc supplementation [162], confirm and expand the studies previously conducted in our laboratory [8]. In particular, starting from the principle of applying the follicular conditions through a tuned modulation of the hormonal components that characterize the follicular environment, we created a milieu that resembles the follicular environment at the specific stage [8, 159, 163].

Previous studies tried to recapitulate these events in vitro [8, 151-158], but to date, no reliable protocols have been developed and only limited success has been reported. In this study, a physiological 3D like culture system proved to be suitable for

the culture of COCs from EAF, with an increase in the oocyte diameter, changes in the gross morphology of the oocytes, and a higher percentage of oocytes showing advanced stages of the chromatin compaction.

These results were reflected in the maturation rate that when compared to the literature our study shows a higher maturation rate than most of the literature, where COCs from early antral follicles were cultured and then submitted to IVM [151, 153-155, 157]. Moreover, the present protocol doubled the number of blastocysts previously obtained in our [8] and other laboratories [152, 157]. This protocol can sustain the gradual chromatin transition of the oocyte and promote the progressive achievement of meiotic and embryonic developmental competence.

In ART, the in vitro growth of follicles and the enclosed oocytes from the preantral to the antral stage still represents the biggest challenge for reproductive biologists and the present culture system can represent a robust model for deepening the knowledge on the biological process's basic mechanisms that occur during the oocyte's final growth and lays the foundations for developing customized culture systems dedicated to oocyte at different stages of development.

The previous experiments' positive results generate two published protocols focusing on in vitro culture methods utilizing physiological conditions to support the specific needs of oocytes at distinct stages of differentiation. It increases the source of fertilizable gametes used in preservation programs of high genetic merit breeders. Another application is for the genetic salvage of threatened species of the bovid family and local breeds that are endangered or at risk of genetic erosion due to the widespread diffusion of cosmopolite breeds. Ultimately, it gives an alternative to human female fertility, especially for cancer patients undergoing gonadotoxic treatments.

In conclusion, the conservation of fertility and the possibility of controlling reproductive potential is now a need that extends to many fields, from human medicine

to animal breeding and the genetic rescue of genetic value subjects as species threatened by extinction. Our findings allowed us to achieve a protocol able to further exploit the ovarian reserve by bringing the growing oocytes to achieve meiotic and developmental competence and became a blastocyst, demonstrating the necessity of understanding the basic mechanisms of the biological process occurring in the COC during final growth for the tailor of the culture system. Also, it led us to define a model through which to conduct in-depth studies of the cellular and molecular processes that regulate the acquisition of meiotic and developmental competence during oocyte growth, thus offering useful and safe fertility preservation options.

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APPENDIX: PUBLISHED FULL PAPERS, LIST OF PUBLICATIONS AND COMMUNICATIONS

Papers (Published)

1. **Barros RG**, Lima PF, Soares ACS, Sanches L, Price CA & Buratini J 2019 Fibroblast growth factor 2 regulates cumulus differentiation under the control of the oocyte. J Assist Reprod Genet. 2019; 36(5):905-913
2. **Barros RG,** Lodde V, Franciosi F, Luciano AM. In Vitro Culture Strategy for Oocytes from Early Antral Follicle in Cattle. J Vis Exp 2020:e61625.
3. Lodde V, **Barros RG**, Dall'Acqua PC, Dieci C, Robert C, Bastien A, Sirard MA, Franciosi F, Luciano AM. Zinc supports transcription and improves meiotic competence of growing bovine oocytes. Reproduction 2020; 159:679-691
4. Luciano AM, Franciosi F, **Barros, RG,** Dieci, C, Lodde V. 2018.The variable success of in vitro maturation: can we do better? Anim. Reprod., v.15, (Suppl.1), p.727-736.
5. Luciano AM, **Barros RG**, Soares ACS, Buratini J, Lodde V, Franciosi F. Recreating the follicular environment: a customized approach for in vitro culture of bovine oocytes based on the origin and differentiation state. Methods Mol Biol (Springer Nature) 2020. (accepted, in press)

Manuscript in preparation

6. **Barros RG**, Lodde V, Franciosi F, Luciano AM. Advanced physiological approaches for in vitro oocyte development aimed at exploiting the reproductive potential of the ovarian reserve (In preparation)

7. **Barros RG**, Lodde V, Franciosi F, Luciano AM. The zinc sink: role of zinc during preimplantation embryo development (In preparation)
8. Luciano AM, Franciosi F, **Barros RG**, Lodde V. Progress towards species-tailored approaches in reproductive biotechnologies. Theriogenology 2020. (In preparation)

Abstracts

1. **Barros RG**, Lodde V, Dieci C, Franciosi F, Luciano AM. Study on the effects of zinc supplementation during in vitro embryo production technologies in cattle. In: HAF (ed.) Proceeding of Veterinary and Animal Science Days 2018, 6th-9th June, vol. 5. Milan, Italy: International Journal of Health, Animal Science and Food Safety; 2018: 1-2.
2. Lodde V, **Barros RG**, Franciosi F, Luciano AM. The Emerging Role of Progesterone Receptor Membrane Component 1 in Bovine early embryogenesis: a preliminary study. In: 2018 Gordon Research Conference: Mammalian Reproduction, Poster Presentation. Lucca Barga (Italy); 2018.
3. Soares ACS, de Oliveira JB, **Barros RG**, Sakoda JN, Gama IL, Buratini J. Varying COC density and culture medium volume during IVM: effects on meiosis progression. Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019. Abstract. Animal Reproduction.
4. **Barros RG**, Lodde V, Franciosi F, Buratini J, Luciano AM. New oocyte in vitro culture strategies to enhance the outcome of assisted reproductive technologies (submitted to ICAR2020 conference, postponed to 2021)

5. Lodde V, **Garcia Barros R**, Franciosi F, Luciano AM. The emerging role of progesterone receptor membrane component-1 in bovine early embryogenesis: a preliminary study. In: 19th International Congress on Animal Reproduction (ICAR). Bologna (Italy); 2020. (Postponed to 2021).
6. Luciano AM, Franciosi F, **Barros RG**, Lodde V. Progress towards species-tailored approaches in reproductive biotechnologies. In: Theriogenology (ed.) The 9th Quadrennial International Symposium on Canine and Feline Reproduction in a joint meeting with the 23rd European Veterinary Society for Small Animal Reproduction Congress. Milano, Italy: Theriogenology; 2020 (Postponed to 2021).

Oral communication

1. **Barros RG**, Franciosi F, Lodde V, Luciano AM. Development of new oocyte in vitro maturation strategies to enhance the outcome of assisted reproductive technologies. Fertility Conference and Exhibition 2019, Birmingham, January 3rd to 5th, 2019. (Selected, presented as Speaker).