Universitat Autònoma de Barcelona

Oocyte competence: Study of melatonin and meiotic inhibitors to improve in vitro embryo production in juvenile goats

Sandra Soto Heras

Universitat Autònoma de Barcelona

DOCTORAL THESIS

Oocyte competence:

Study of melatonin and meiotic inhibitors to improve *in vitro* embryo production in juvenile goats

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Under the direction of: Maria Teresa Paramio Nieto

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Universitat Autònoma de Barcelona

La Dra Maria Teresa Paramio Nieto, Catedrática del Departamento de Ciencia Animal y de los Alimentos de la Universidad Autónoma de Barcelona,

CERTIFICA

Que la tesis titulada "**Oocyte competence: Study of melatonin and meiotic inhibitors to improve in vitro embryo production in juvenile goats**" presentada por Sandra Soto Heras para optar al grado de Doctora por la Universidad Autónoma de Barcelona, se realizó bajo mi dirección y con financiamiento del Ministerio de Ciencia, Innovación y Universidades (AGL2014-52408-R y AGL2017-85837-R) y una beca otorgada a Sandra Soto por el Ministerio de Educación y Formación Profesional (FPU2014/00423).

Y para que así conste, firmo la presente en Bellaterra (Cerdanyola del Vallès), el 24 de enero de 2019

Dra. Maria Teresa Paramio Nieto

A mis padres,

A Maria y Sergi,

A Matt,

Abstract

Oocyte *in vitro* maturation (IVM) is a limiting step for *in vitro* embryo production (IVEP). IVM can impair oocyte embryo developmental competence by inducing spontaneous meiotic resumption. IVM culture conditions also induce oxidative stress (OS) that is detrimental for oocyte quality. This is especially relevant in oocytes from juvenile animals which come from small follicles that have not fulfil development and are more sensitive to OS due to low GSH synthesis. We hypothesized that two IVM protocols could improve IVEP in juvenile goats: A) reducing OS during IVM with the addition of melatonin to the culture medium; B) improving oocyte competence before IVM using meiotic inhibitors in a pre-IVM culture (biphasic IVM).

We performed two studies to test the effect of melatonin added to IVM medium on the embryo developmental competence of juvenile-goat oocytes. In the first study, oocytes were *in vitro* matured with different melatonin concentrations (10^{-3}, 10^{-7}, 10^{-9}, 10^{-11} M), fertilized and embryo cultured. Melatonin at 10^{-7} M dose showed a tendency to higher blastocyst rate, although not significant. The antioxidant cysteamine, which was also present in the IVM medium, may have hided melatonin effect. Hence, we performed a further experiment testing the following IVM-treatments: melatonin (10^{-7} M), cysteamine (100 µM), cysteamine plus melatonin, and non-antioxidants. Melatonin increased blastocyst rate compared to non-antioxidants (28.9% vs. 11.7%; $P < 0.05$) and blastocyst quality compared to cysteamine (225 vs. 129 total cell number; $P < 0.05$). Both antioxidants decreased intra-oocyte reactive oxygen species (ROS) after IVM. The higher blastocyst quality compared to cysteamine suggested that melatonin had other effects besides acting as an antioxidant.

In the second study, we assessed melatonin mechanisms of action in juvenile-goat oocytes. First, we immunolocalized melatonin receptor 1 (MT1) in oocytes and cumulus cells (CC) before and after IVM. In a second experiment, we tested the effect of adding 10^{-7} M melatonin to the IVM medium, compared to IVM without antioxidants (control) and IVM with melatonin plus luzindole (10^{-7} M; a melatonin-receptor inhibitor). After IVM, intra-oocyte ROS levels, ATP content and mitochondrial activity were assessed, and oocytes were fertilized and embryo cultured. IVM-oocytes with melatonin showed higher mitochondrial activity and ATP content, and lower ROS levels than oocytes from control group. Melatonin also had a positive effect on blastocyst quality compared to control group (55.8 vs. 30.4 inner cell mass; $P < 0.05$). We could not determine if these effects were mediated by MT1 because IVM with melatonin plus luzindole showed no significant differences compared to melatonin and control groups.

In order to improve oocyte competence before IVM we developed two studies in which oocytes were pre-matured with two known meiotic inhibitors: C-type natriuretic peptide (CNP) and 3-isobutyl-1-methylxanthine (IBMX). The first study was performed at the University of Adelaide (Australia) with bovine oocytes to test the biphasic IVM system in a simpler model than the future experiment in juvenile-goat oocytes. First, oocytes were cultured in pre-IVM with CNP (100 nM), IBMX (500 µM), CNP plus IBMX, and non-inhibitors (control) and nuclear stage was assessed after 6 h. CNP plus IBMX sustained higher germinal vesicle (GV) rate than control oocytes (92% vs. 54%; $P < 0.05$) denoting a synergy on the meiotic inhibition. In a second experiment, oocytes were cultured in biphasic IVM (6 h pre-IVM with IBMX plus CNP, followed by 20 h IVM) compared to control IVM (24 h), fertilized and embryo cultured. Transzonal projections (TZPs), mitochondrial activity and GSH levels were assessed at the end of IVM. Biphasic IVM increased blastocyst rate (45.1% vs. 34.5%; $P < 0.05$), prolonged CC-oocyte communication by TZPs and enhanced oocyte mitochondrial activity.

In the second study, a similar biphasic IVM was tested in juvenile-goat IVEP. Oocytes were cultured in pre-IVM with CNP (0, 50, 100, 200 nM) and nuclear stage was assessed after 6 h, but no significant differences were observed among groups. A second experiment was performed to test the same CNP concentrations plus 10 nM estradiol, which can promote the CNP receptor (NPR2). Pre-IVM with 200 nM CNP plus estradiol maintained higher GV rate than control group (74.7% vs. 28.3%; $P < 0.05$). The mRNA relative quantification showed that *NPR2* was down-regulated after 6 h of pre-IVM, although the addition of estradiol showed a tendency to slow the decline. Lastly, oocytes were cultured in biphasic IVM (6 h pre-IVM with CNP plus estradiol, followed by 24 h IVM) compared to control IVM (24 h). TZPs were assessed at different culture time-points. Intra-oocyte GSH and ROS levels, and the expression of target genes in cumulus-oocyte complexes were determined after IVM. Oocytes were fertilized and embryo cultured. Biphasic IVM maintained higher TZP density for 6 h compared to 6 h of IVM. Biphasic IVM also increased GSH levels, decreased ROS, up-regulated *DNA methyltransferase 1* and *TNF-stimulated gene 6 protein*, and improved blastocyst rate (30.2% vs. 17.2%; $P < 0.05$).

In conclusion, we have improved *in vitro* developmental competence of juvenile-goat oocytes by two different IVM procedures. Melatonin reduced ROS and improved mitochondrial activity during IVM, which leaded to better embryo development. Pre-IVM with CNP and estradiol maintained meiotic arrest and cumulus-oocyte communication for 6 h. When this pre-IVM was applied to a biphasic IVM system, it improved oocyte antioxidant defenses and up-regulated maturation-related genes in COCs, leading to higher embryo rate. These are promising methods to improve IVEP with juvenile oocytes in other species.

List of Abbreviations

2PN	Two pronuclei formation
ART	Assisted reproductive technology
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMP15	Bone morphogenetic protein 15
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CC	Cumulus cell
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
COC	Cumulus-oocyte complex
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
Dpf	Days post-fertilization
E2	17β-estradiol
EGF	Epidermal growth factor
FCS	Fetal calf serum
FF	Follicular fluid
FSH	Follicle-stimulating hormone
FSHR	FSH receptor
GC	Granulosa cell
GCL	ϒ-glutamulcysteine ligase
GDF9	Growth-differentiation factor 9
GJ	Gap junction
GJC	Gap junction communication
GSH	Glutathione
GPx	Glutathione peroxidase
GRd	Glutathione reductase
GPR	G protein-coupled receptor
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HAS2	Hyaluronan synthase-2
Hpf	Hours post-fertilization
IBMX	3-isobutyl-1-methylxanthine
ICM	Inner cell mass
IVM	*In vitro* maturation
IVF	*In vitro* fertilization
IVC	*In vitro* embryo culture
IVEP	*In vitro* embryo production

JIVET	Juvenile *in vitro* embryo transfer
LH	Luteinizing hormone
LOPU	Laparoscopic ovum pick up
MI	Metaphase I
MII	Metaphase II
MEM	Minimal essential medium
MOET	Multiple ovulation and embryo transfer
MPF	Maturation promoting factor
MT1/2	Melatonin receptor 1/2
mtDNA	Mitochondrial DNA
NPR2	Natriuretic peptide receptor 2
OS	Oxidative stress
OSF	Oocyte secreted factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PKA	Protein kinase A
Pre-IVM	Pre-maturation
PTX3	Pentraxin 3
RNA	Ribonucleic acid
RNS	Radical nitrogen species
ROR	Retinoid orphan receptor
ROS	Reactive oxygen species
RPL19	Ribosomal protein L19
RPS9	Ribosomal protein S9
RZR	Retinoid Z receptor
RT-qPCR	Quantitative reverse transcription PCR
SOD	Superoxide dismutase
SOF	Synthetic oviductal fluid
TALP	Tyrode’s albumin lactate pyruvate
TCM199	Tissue culture medium 199 components
TE	Trophectoderm
TNFAIP6	Tumor necrosis factor alpha induced protein 6
TZP	Transzonal projection

Chapter 1

Introduction

In vitro embryo production (IVEP) is an assisted reproductive technology (ART) which aims to obtain embryos from immature oocytes by simulating the *in vivo* embryo development in the female reproductive tract. The main IVEP procedures are: *in vitro* maturation (IVM) of immature oocytes recovered from the ovarian follicles; *in vitro* fertilization (IVF) of IVM-oocytes by co-incubation with capacitated spermatozoa; and *in vitro* culture (IVC) of zygotes to the blastocyst stage, when they can be either transferred to a recipient female or cryopreserved. IVEP has many potential applications for animal husbandry as it enhances the genetic progress through maternal lineage with females of high commercial values. Moreover, IVEP is an essential research tool for increasing the knowledge on developmental biology and physiology, and it is the cornerstone for technologies such as transgenesis and stem cell reprogramming.

In spite of the amount of research carried out during the past 30 years, IVEP still shows poor and unpredictable results which limits its commercial use (reviewed by Paramio & Izquierdo[1]). In cattle, the blastocyst yield is only 30 to 40% of the immature oocytes starting IVM. This occurs despite higher rates of nuclear maturation (~90%) and fertilization (~80%) (reviewed by Lonergan[2]). In goats blastocyst rate is even lower, ranging from 20 to 30% (reviewed by Paramio & Izquierdo[1]). IVM is a key and limiting step for IVEP. Studies in cattle[3], goat[4] and mice[5] show that IVM-oocytes develop to blastocyst stage at a lower rate than *in vivo* matured ovulated oocytes that are fertilized *in vitro*.

Oocyte competence or quality is the ability to resume meiosis, cleave after fertilization, develop to the blastocyst stage, induce pregnancy and bring healthy offspring to term[6]. During folliculogenesis oocytes grow and become competent to undergo development while the somatic cells differentiate (reviewed by Eppig[7]). Consequently, oocyte competence depends on the follicular fluid (FF) composition that surrounds the oocyte besides other factors: follicular diameter[8,9], oocyte diameter[10], grade of follicular atresia[11], grade of oocyte atresia[12], follicular wave phase[13], hormonal stimulation[6], IVM conditions (reviewed by Sutton et al.[14]), season[15], nutrition[16,17], and donor's age[18–22].

Juvenile *in vitro* embryo transfer (JIVET) is an ART that enables the production of *in vitro* blastocysts from juvenile-female oocytes. JIVET has great interest for breeding programs as it can increase the genetic gain rate by reducing the generation interval (reviewed by Morton[23]). In sheep for instance, collecting oocytes from four week-old females can reduce the generation interval to six months and increase the genetic gain rate in 5%[23]. Juvenile females also provide larger numbers of oocytes per animal compared to adults[24]. Furthermore, oocytes

from juvenile females are interesting candidates for research as a model for low quality oocytes. However, blastocyst rates obtained with juvenile oocytes are lower compared to their adult counterparts (24% vs. 34% in goats[25], 19.9% vs. 51.3% in sheep[18], 21% vs. 34% in pig[26], 1.2 vs. 2.2 blastocyst per animal in cattle[27]). Results in our research group are on the low end of this range: juvenile-goat oocytes (3 to 4 weeks old) produce blastocyst rates from 6% to 20%.

Juvenile animals have a high number of small follicles (< 3 mm) in their ovaries, which are correlated to low-competence oocytes (reviewed by Paramio & Izquierdo[1]). There is a direct relation between follicle size, oocyte diameter and embryo development[28]. Research with juvenile-goat oocytes have shown impairment on: the distribution of cortical granules[29] and mitochondria[30], the organization of microtubules and microfilaments[31], total RNA content and maturation promoting factor (MPF) activity[10]. However when juvenile-goat oocytes are selected from follicles larger than 3 mm, blastocyst rate is similar to adult-female oocytes recovered by laparoscopy ovum pick up (LOPU; 18% vs. 20%)[32]. In adult goats, Crozet et al.[4] also observed an increase in blastocyst rate with follicle size: 6% in small follicles (2-3 mm), 12% in medium (3.1-5 mm), and 26% in large (>5 mm). These results suggest that the follicle size has a greater effect on oocyte competence than the donor's age.

Regardless of oocyte origin, *in vitro* matured oocytes have lower embryo developmental competence than *in vivo* matured oocytes[3]. One of the main factors that can impair oocyte competence on *in vitro* conditions is oxidative stress (OS), induced by an imbalance between reactive oxygen species (ROS) production and elimination (reviewed by Tamura et al.[33]). ROS are free radicals continuously generated by oxidative biochemical reactions in the oocyte and eliminated by its antioxidant defenses (reviewed by Devine et al.[34]). But, intra-oocyte ROS levels are higher on IVEP due to: exposure to O_2 and visible light, among others external factors; and absence of antioxidant mechanisms provided by the follicular environment (reviewed by du Guérin et al.[35]). OS accelerates oocyte aging[36], and impairs fertilisation[37] and embryo development[35]. Thus, IVEP culture mediums conventionally include thiol compounds such as cysteamine (reviewed by Deleuze & Goudet[38]) which increase oocyte glutathione (GSH) levels and protect the oocyte against ROS[39–41]. More recently, melatonin has been tested as a more potential antioxidant for IVM (reviewed by Cruz et al.[42]).

On the other hand, conventional IVM can impair embryo development by inducing precocious nuclear maturation, which interrupts the process of oocyte competence acquisition (reviewed by Gilchrist & Thompson[43]). During folliculogenesis oocytes undergo changes at nuclear and

cytoplasmic levels essential for acquiring oocyte competence[43]. Inside antral follicles, oocytes are arrested at germinal vesicle (GV) stage but spontaneously resume meiosis when released and placed *in vitro*[44]. In juvenile goats, Velilla et al.[31] observed that 50% of oocytes resume meiosis before IVM, during recovery from the follicle and preparation for nuclear assessment. The percentage of oocytes that reach metaphase II stage can be higher than 80% in juvenile goat[40,45,46]. However, embryo development depends on synchronized nuclear and cytoplasmic maturation (reviewed by Conti & Franciosi[47]). Therefore, new strategies for IVM are being developed based on the inhibition of spontaneous meiosis with cyclic AMP (cAMP) modulators during a pre-maturation (pre-IVM) phase (reviewed by Gilchrist et al.[48]). The pre-IVM prolongs cumulus-oocyte communication and increases mRNA and protein accumulation, enabling the oocyte to fully acquire developmental competence before IVM (reviewed by Gilchrist[49]).

In JIVET the high percentage of small antral follicles makes oocyte collection by follicular aspiration difficult (reviewed by Paramio & Izquierdo[50]). Instead, juvenile oocytes are usually obtained by slicing of the ovary surface. Using the slicing method instead of aspiration, one obtains a pool of immature oocytes from small-medium antral follicles with a heterogeneous degree of growth and atresia. Sui et al.[51] observed that 45% of goat oocytes from atretic follicles of 2.0-2.8 mm have resumed meiosis inside the follicle, compared to 9% in healthy follicles. This all implies that juvenile oocytes used for IVM have unknown and variable nuclear stage and cytoplasmic competence which limits IVEP success. Moreover, juvenile oocytes are particularly sensitive to ROS. Oocytes from juvenile mice have higher ROS levels compared to adults due to lower GSH synthesis[52]. Similarly, IVM-oocytes from juvenile goats show lower GSH levels than ovulated oocytes from adults[40]. We hypothesized that juvenile-goat oocytes could benefit from the use of a pre-IVM phase to homogenize the oocyte pool before IVM and from the addition of a powerful antioxidant during IVM.

The aim of the present study is to improve IVEP success in juvenile goats by implementing new IVM procedures that could enhance oocyte developmental competence. Considering previous research above mentioned, we will focus on: A) supplementing the IVM medium with melatonin to reduce oocyte exposure to oxidative stress, and B) developing a pre-IVM culture with cAMP modulators to improve oocyte competence prior to standard IVM.

Chapter 2

Literature review

2.1. Current situation of the *in vitro* embryo production in goats

In vitro embryo production (IVEP) involves different procedures from the recovery of immature oocytes to the production of 8-day embryos that can be either cryopreserved or directly transferred to a recipient female. The main steps are: 1) *in vitro* maturation (IVM) of oocytes directly recovered from the ovarian follicles; 2) *in vitro* fertilization (IVF) or co-incubation of matured oocytes with previously capacitated spermatozoa; and 3) *in vitro* culture (IVC) of presumptive zygotes up to the blastocyst stage.

IVEP is an interesting assisted reproductive technology (ART) for increasing genetic diffusion and productivity in livestock species. Data from the Association of Embryo Technology in Europe[53] shows that in 2016 embryo commercial activity in goats was much lower than in sheep and cattle: 358 goat-embryos were *in vivo* and *in vitro* produced and 85 embryo transfers were performed; compared to 12,239 produced embryos and 1,282 transfers in sheep, and 148,851 produced embryos and 130,635 transfers in cattle. There are also fewer research studies about IVEP in small ruminant compared to other livestock species. Yet goat production is economically and socially important in Spain, with 3,088,040 total registered goats and 1,253,737 lactating females in 2016 according to data from the Spanish Ministry of Agriculture, Fishing and Nutrition[54].

In goats, *in vivo* embryo production by multiple ovulation and embryo transfer (MOET) technology has presented important limitations due to (reviewed by Paramio & Izqueirdo[1]): a) high ovulation variability in response to hormonal treatments; b) early regression of the *corpora lutea;* and c) traumatic surgical procedure for embryo recovery. The IVEP overcomes these problems because oocytes are directly recovered from the follicles by laparoscopic ovum pick-up (LOPU) without previous superovulation. Moreover, IVEP enables obtaining embryos from non-fertile, pregnant, lactating, juvenile and even dead females. This is valuable for research purposes as slaughterhouses are a cheap and abundant oocyte source.

2.1.1. *In vitro* maturation

Oocyte IVM includes two main procedures: the artificial removal of immature cumulus-oocyte complexes (COCs) from antral follicles of unstimulated or FSH-primed ovaries; and the oocyte culture during 24 h until they reach metaphase II (MII) stage, when they are ready to be fertilized[44]. In goats, more than 80 % of oocytes reach MII[40,45,46,55], but they also need to mature at cytoplasmic levels to be able to undergo early embryo development after IVF.

The most conventional culture medium for IVM in small ruminants is tissue culture medium 199 (TCM199) bicarbonate-buffered with Earle's salts, which contains minerals, energy sources (glucose, glutamine), vitamins and amino acids, among other components (reviewed by Mermillod et al.[56]). Some research groups use synthetic oviduct fluid (SOF) medium instead of TCM199[57–59]. In goats, TCM199 is usually supplemented with different agents that have proved to stimulate oocyte cytoplasmic and nuclear maturation: 17β-estradiol (E2) and gonadotropin hormones (luteinizing hormone, LH; follicle-stimulating hormone, FSH) that induce cumulus cell expansion among other actions[60]; epidermal growth factor (EGF)[55,58,61]; cysteamine and other thiol compounds as antioxidants[39]; and complex fluids of unknown composition such as fetal calf serum (FCS)[61,62], steer serum[8,45,63], estrous sheep serum[64] and estrous goat serum[65].

According to our group previous research, the following IVM medium is used for goat oocytes: TCM199 supplemented with 5 µg/mL FSH, 5 µg/mL LH, 1 µg/mL E2, 1 mM glutamine, 0.2 mM sodium pyruvate, 10 ng/mL EGF, 100 mM cysteamine and 10% (v/v) FCS[66–68]. COCs are cultured in 100-µl drops covered with mineral oil (1 COC/2-5 µL IVM medium) for 24 h at 38.5ºC in humidified air with 5% CO_2[66,69].

2.1.2. *In vitro* fertilization (IVF)

IVF consists in the co-culture of IVM-oocytes and capacitated spermatozoa with the conditions and during the time that enable spermatozoa to penetrate the oocyte. IVF success depends on previous oocyte IVM, sperm selection, sperm capacitation and IVF medium (reviewed by Paramio & Izquierdo[1]). The sperm (either fresh or frozen-thawed) must be prepared prior to IVF in two phases: 1) selection of the most motile spermatozoa; 2) capacitation or acquiring the ability to undergo acrosome reaction.

There are two methods for selecting spermatozoa: swim-up and percoll gradient. Swim-up is generally used for buck fresh semen[10,12,32,65,68,70]. In this procedure, the semen is placed at the bottom of a tube and layered with medium. After 30-60 min at 38.5ºC, the top layer is recovered in which highly motile spermatozoa are located. Percoll gradient is also used for fresh semen[67] but specially for frozen-thawed semen[58,59,71]. Blastocyst rate is higher when buck frozen-thawed semen is selected by density-gradient than by swim-up[72]. The gradient is formed with two density phases (45% and 90%) of colloidal silica particles. The semen is placed at the top of the 45% layer and centrifuged. The resulting pellet contains the best spermatozoa. Regarding sperm capacitation, incubating sperm with heparin (50 mg/mL) for 15-60 min at 38.5ºC has reported good results in goat IVEP[32,67,68].

In our group, IVF is usually performed in Tyrode's albumin lactate pyruvate (TALP) medium with hypotaurine[67,68]. Groups of 15-30 COCs are cultured with 1-4 x 10^6 spermatozoa/mL in micro-drops (50-100 µL) covered with mineral oil for 17-24 h at 38.5ºC with humidified air and 5 % CO_2. These are the same atmospheric conditions than for IVM, although Leoni et al.[73] observed that lower oxygen during IVF improves blastocyst development in sheep.

2.1.3. Embryo culture

After IVF presumptive zygotes are placed in embryo culture medium that enhances cell division and development until blastocyst stage (6-8 days post-fertilization, dpf)[74]. Blastocyst quality mainly depends on embryo culture conditions[3]. The main developmental events during embryo culture are: first cleavage division, activation of the embryonic genome in 8-16-cells embryos, morula compaction, and blastocyst formation. Blastocyst embryonic cells differentiate into inner cell mass (ICM), which will become the fetus, and trophectoderm (TE), which will form the placenta (reviewed by Watson[75]). For the activation of the embryonic genome there is an increase in metabolic activity[76] and consumption of carbohydrates and oxygen[77]. This is the most sensitive time-point when embryonic arrest can take place if the medium does not supply all required nutrients.

For goat embryo culture SOF medium is conventionally used, which was first described by Tervit et al.[78] based on the composition of ovine oviduct fluid. SOF is usually supplemented with 10% FCS[67,69] because it stimulates mitosis, but it can induce chromosomal abnormalities[79]. For instance, Romaguera et al.[32] observed that 90% of *in vitro* produced goat blastocysts present mixoploidy. Another embryo culture method is the co-culture with oviductal epithelial cells. In goat, Rodríguez-Dorta et al.[80] showed that this system could lead to higher embryo survival rates after vitrification and transfer to a recipient female, compared to embryos cultured with SOF. But co-culture with somatic cells have risk of contamination, unpredictable results depending on cell state and require a long preparation, hence defined medium is preferred. Finally, sequential media can be used in order to match specific embryo phases demands (reviewed by Thompson[81]). The sequential G1.2/G2.2 supplemented with BSA has been tested in goats with good embryo results[24,57,58].

In our group, goat embryos are routinely cultured in 10-µL drops (1 embryo/1-2 µL SOF) under paraffin oil at 38.5ºC in humidified air with 5% CO_2, 5% O_2 and 90% N_2. Embryos are cultured in groups as they reciprocally stimulate each other leading to higher blastocyst rate and quality.[74]

2.2. Oocyte competence

Oocyte competence is the ability to resume meiosis, cleave after fertilization, develop to the blastocyst stage, induce pregnancy and bring healthy offspring to term[6]. This includes complex biological transitions such as intra-oocyte remodeling that prepares the oocyte to be fertilized and integrate the male genome, nuclear reprograming of the zygote to acquire totipotency, and activation of the embryonic genome (reviewed by Conti & Franciosi[47]). Oocyte competence is the key factor for IVEP success[82]. Understanding the biological pathways involved in the acquisition of oocyte competence, as well as the *in vitro* factors that can impair these processes, can help us develop new IVM systems to improve IVEP.

2.2.1. Acquisition of oocyte developmental competence

Following the LH surge *in vivo* or after *in vitro* release from the follicle, the oocyte needs to undergo both nuclear and cytoplasmic maturation. Yet the meiotic and developmental competence for correctly maturing is acquired during antral follicular development[9,83]. In order to achieve full developmental competence nuclear and cytoplasmic processes must be coordinated (reviewed by Eppig[7]).

Oocyte meiotic division begins during fetal development, but oocytes get arrested at prophase I and do not resume meiosis until puberty, after the LH surge prior to ovulation (reviewed by Rimon-Dahari[84]). The oocyte is first surrounded by a layer of pre-granulosa cells forming a primordial follicle, and its nucleus is at germinal vesicle (GV) stage enclosed by the nuclear membrane. During folliculogenesis oocytes grow and follicular cells proliferate progressing into primary and secondary follicles. At the beginning of development, oocyte chromatin is decondensed with high transcriptional activity (reviewed by Mattson & Albertini[85]). Meiotic competence, or the ability to resume meiosis I, is acquired after follicle antrum formation (reviewed by Fair[86]). It involves progressive chromatin condensation related to a decrease in transcriptional activity (reviewed by Luciano and Lodde[87]). The oocyte also assembles different structures for forming and maintaining the meiotic spindle during nuclear maturation: centrosomal proteins, ϒ-tubulin and other regulatory proteins (reviewed by Bennabi et al.[88]). In a later phase of follicular growth, oocytes undergo a process known as *oocyte capacitation* during which they acquire the molecular and cytoplasmic machinery needed for fertilization and early embryo development[6,89]. This includes reorganization of organelles and storage of proteins, lipids, mRNA and transcription factors (reviewed by Krisher[90]).

Oocyte competence depends on the follicular environment (reviewed by Gilchrist & Thompson[43]). Oocyte and follicular development are inter-dependent and coordinated by

gonadotropins and paracrine factors (reviewed by Sugimura et al.[91]). One such gonadotropins is FSH which promotes LH and EGF receptors on granulosa cells[92], and gap junctional communication (GJC) between oocyte and cumulus cells (CCs)[93]. CCs have cytoplasmic extensions called transzonal projections (TZPs) which go through the zona pellucida into the oocyte plasma membrane forming gap junctions (GJs) at their end. These connections generate the COC functional unit (reviewed by Albertini et al.[94]). GJs are transmembrane proteins that allow the transfer of hydrophilic molecules with low molecular weight, essential for oocyte development and meiotic arrest (reviewed by Kidder and Mhawi[95]). Vesicles and mRNA can also be transferred via TZPs[96,97]. At the same time, oocyte secreted factors (OSFs) control the differentiation and function of CCs, and cooperate with FSH for the maturation of the EGF network (reviewed by Sugimura et al.[91]).

After acquiring full developmental competence, the oocyte is able to resume meiosis until MII and undergo cytoplasmic maturation after ovulation. Cytoplasmic maturation mainly involves three processes (reviewed by Ferreira et al.[98]):

- Redistribution of cytoplasmic organelles (mitochondria, ribosomes, Golgi complex, endoplasmic reticulum and cortical granules): Mitochondria are essential for supplying the required ATP during maturation, hence they move to areas of high energy demand[99]. Ribosomes are responsible for protein synthesis and are especially active during metaphase I (MI) stage[100,101]. The endoplasmic reticulum, which regulates Ca^{2+} among other functions, is uniformly distributed at GV stage and progresses to cortical distribution for MII (reviewed by Kline[102]). Cortical granules also change from cytoplasmic distribution at GV stage to peripheral distribution at the end of MII in order to do exocytosis right after fertilization which prevents polyspermy[29,103].
- Dynamics of the cytoskeletal filaments: The cytoskeleton continually changes during oocyte maturation since it controls the movement of organelles and the segregation of chromosomes by forming the meiotic spindle (reviewed by Albertini[104]).
- Molecular maturation: It is the process of mRNA transcription and storage, which stops after meiosis resumption. The stored mRNA will translate into proteins at the appropriate time of embryogenesis (reviewed by Sirard[105]).

2.2.2. Factors affecting oocyte competence on *in vitro* embryo production

There are many factors on IVEP that can impair oocyte competence, some related to the oocyte intrinsic characteristics and some to the *in vitro* conditions. In fact oocytes matured *in vitro* are less competent than *in vivo* matured oocytes, regardless of its origin[3].

- **Age of the female donor**: Oocytes from juvenile females are less capable of producing embryos and offspring to term compared to their adult counterparts (reviewed by Armstrong[21]). Blastocyst rate of oocytes from FSH-stimulated females was 24 % in juvenile goats and 34 % in adults[25]. There are structural and metabolic differences between adult and juvenile oocytes. Compared to adult cows, oocytes from calves are smaller, metabolize less glutamine and pyruvate, have lower protein synthesis[22], less mitochondria[106], and store less maternal mRNA and proteins[107]. In addition, adult-cow oocytes present up-regulation of genes related to mitochondrial activity, cell differentiation and transcription control, whereas calf oocytes have up-regulation of genes related to apoptosis[108]. Oocytes from juvenile goats also show ultrastructural and functional deficiencies such as impaired distribution of cortical granules[29] and mitochondria[30], disorganized microtubules and microfilaments[31], altered total RNA content, p34 and cyclin B1 expression, and activity of the maturation promoting factor (MPF)[10]. Overcoming this low development potential is of great interest for breeding programs as juvenile *in vitro* embryo transfer (JIVET) can further increase genetic gain in livestock species[23].
- **Follicle size**: Various authors have observed a correlation between follicle size, oocyte size and oocyte competence[9,109–112]. In adult goats, Crozet et al.[4] obtained higher blastocyst rate with oocytes from larger follicles: 6%, 12% and 26% blastocyst rate, from small (2-3 mm), medium (3.1-5 mm) and large (> 5 mm) follicles, respectively. In juvenile goats, oocytes from follicles larger than 3 mm have higher oocyte size (128 µm), cleavage (48%) and blastocyst rate (20%) than oocytes from smaller follicles (125 µm, 23% and 4%, respectively)[8]. Moreover, oocytes from follicles larger than 3 mm produce similar blastocyst rates in adult and juvenile goats (21% vs. 18%)[32]. Similarly, Kauffold et al.[113] reported higher blastocyst yield in calf oocytes from follicles larger than 8 mm (47%) than from smaller follicles (< 15%), but no differences between calf and cow oocytes from large follicles (~ 59%). This all suggests that IVEP success depends more on the follicle size than the donor's age. However, juvenile-goat ovaries present high number of follicles between 2.5 to 3 mm[109] which prevents oocyte selection by aspiration of large antral follicles. Instead, oocytes are obtained by slicing of the ovary surface recovering a pool with high heterogeneity in growth and grade of development (reviewed by Paramio & Izquierdo[1]). As reviewed by Cognié et al.[62] and Tibary et al.[114], heterogeneous oocytes respond different to same IVM culture conditions.
- ***In vitro* maturation environment**: Conventional IVM atmospheric conditions present 3-4 times more O_2 than the oviduct[115]. This leads to increase production of reactive oxygen species (ROS) which are toxic for the oocyte (reviewed by Agarwal et al.[116]). Moreover, the

lack of follicular antioxidant mechanisms impairs the oocyte ability of preventing oxidative stress (reviewed by du Plessis et al.[117] and Combelles et al.[118]). In chapter 2.4. *Improvement of oocyte competence by reducing oxidative stress during IVM*, we will discuss the negative effects of ROS on oocyte competence and methods to prevent oxidative stress during IVM.

- **Premature nuclear maturation induced by conventional IVM**: Edwards[44] first observed that oocytes spontaneously resume meiosis when retrieved from follicles and placed *in vitro*. The follicle environment maintains the oocyte on meiotic arrest which ensures full competence acquisition prior to maturation (reviewed by Gilchrist and Thompson[43]). Once ovulated, oocytes mature thanks to an orchestrated process induced by gonadotropin cascade. Whereas on *in vitro* conditions oocytes are forced to mature at nuclear level interrupting the oocyte capacitation process. In chapter 2.5. *Improvement of oocyte competence by biphasic IVM*, we will talk about the physiological control of oocyte meiosis and new biphasic IVM systems, which include a meiotic blocking phase prior to IVM (pre-IVM) to improve oocyte competence.

2.3. Study of the oocyte: assessment of oocyte competence

The assessment of oocyte competence is valuable for research purposes to predict IVEP results and improve IVEP success. As developmental competence ultimately indicates the ability to bring a healthy offspring to term, embryo transfer to a receptor female is the only completely reliable method for determining it (reviewed by Sirard et al.[6]). Due to practical reasons we conventionally use earlier indicators of embryo development such as blastocyst rate and quality at 7-8 dpf. Embryo quality can be determined by analysis of gene expression, morphology and timing of first cleavage division (reviewed by Lonergan[119]). Yet blastocyst differential staining of TE and ICM is probably the most common method. ICM number is correlated to blastocyst quality, implantation and fetal developmental potential[120]. However, it is of great interest to determine oocyte quality after IVM and evaluate specific pathways involved in competence acquisition.

2.3.1. Nuclear stage

Oocyte nuclear stage can be visualized by orcein staining combined with phase-contrast microscopy, which reveals chromatin configuration and integrity of the nuclear membrane[121]. Nuclear stage can be assessed at different time-points of development in order to determine:

- **Meiotic competence** of GV-oocytes prior to IVM: GVs progressively change from disperse to condense chromatin configurations, which is correlated to gradual transcription

silencing and acquisition of meiotic and developmental competence (reviewed by Luciano et al.[122]). Chromatin condensation is also related to different phases of follicle development. Sui et al.[51] suggested a GV classification for goat oocytes (GV1, GV2n, GV3n, GV2c, GV2n and GV4) that differs from other mammal species. This classification is based on the degree of chromatin condensation (diffuse, net-like, and condensed in clumps), the nucleolus size and the nuclear membrane integrity. In chapter 7, figure 1 (p. 79) shows goat GV classification in orcein-stained oocytes.

- **Nuclear maturation** after IVM (figure 1): MII stage is characterized by the presence of chromosomes aligned at the meiotic spindle with haploid number and an extruded polar body. Nuclear immature oocytes can be at: GV (intact nuclear membrane), GVBD (broken nuclear membrane and chromosomes condensing at prophase I) and MI (chromosomes aligned at the meiotic spindle with diploid number). Timings of GVBD and MII arrest vary among species (reviewed by Conti and Franciosi[47]). In goats, 72.7% of oocytes from follicles larger than 3 mm reach MII after 24 h of IVM[51].
- **Pronuclei formation** after fertilization (figure 1): The oocyte completes meiosis and extrudes the second polar body. Female and male chromatin decondense and get surrounded by nuclear membranes, forming the pronuclei. At 17-20 h post-IVF, correctly fertilized oocytes with two pronuclei of similar size (2PN stage) can be observed. Not correctly fertilized oocytes include: polyspermic oocytes (more than two pronuclei which come from multiple fertilizations) and asynchronical oocytes (one female PN and a condense spermatozoa head).

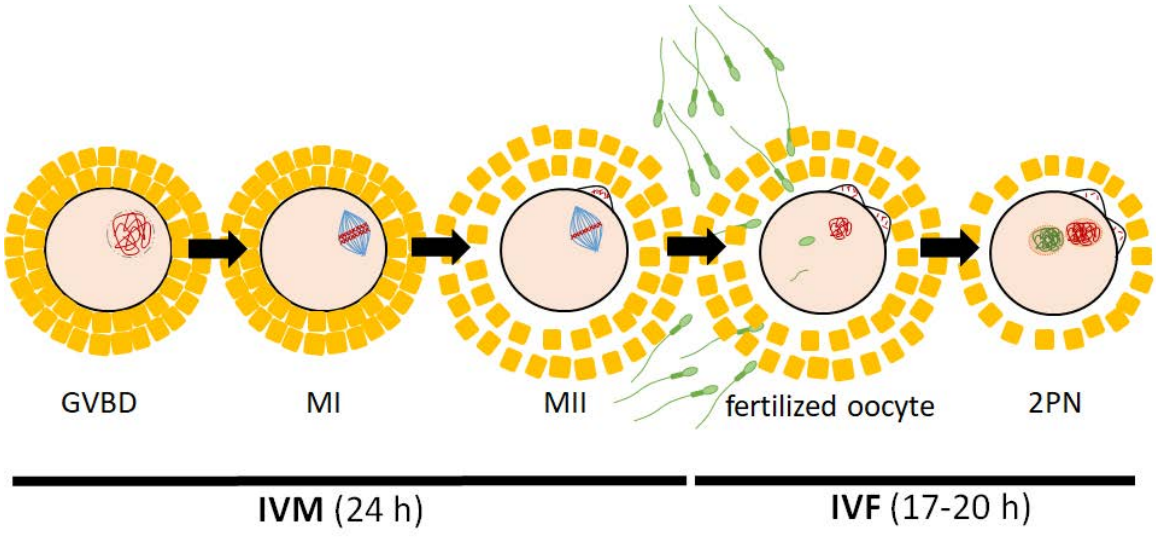

Figure 1: Schematic representation of oocyte nuclear maturation through *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). GVBD (germinal vesicle breakdown); MI (metaphase I); MII (metaphase II); 2PN (two pronuclei).

2.3.2. Reactive oxygen species (ROS) and glutathione (GSH) levels

ROS induces oxidative stress which impairs oocyte maturation and embryo development (reviewed by Tamura et al.[123]). ROS levels can increase due to high exposure to oxidative factors such as heat stress[124] and oocyte aging[36]. But high intra-oocyte ROS are also indicative of reduced antioxidant capacity in low quality oocytes such as oocytes from juvenile animals[52]. It is interesting to study ROS together with GSH because they are usually inversely proportional, since GSH is the main non-enzymatic antioxidant system in the oocyte (reviewed by Guérin et al.[35]). GSH also plays a role in decondensing the sperm head and forming the male pronucleus[125]. GSH high levels are related to better embryo development[39,40].

ROS and GSH intra-oocyte levels can be measured with fluorescent staining. For ROS levels, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is conventionally used[126]. H_2DCF-DA is oxidized by hydrogen peroxide (H_2O_2), forming 2',7'-dichlorofluorescein (DCF) and emitting light. Hence fluorescence intensity is linearly related to the amount of H_2O_2. For GSH levels, monochlorobimane[127] and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (cell tracker blue)[126] can be used. They have a chloromethyl group that reacts with thiol compounds (-SH) via glutathione S-transferase–mediated reaction, emitting light. Thus, fluorescence intensity is linearly related to the GSH content.

2.3.3. Gene expression analysis

Oocytes have high transcriptional activity during follicular growth and store mRNA transcripts (reviewed by Conti & Franciosi[47]). Transcription ceases in fully grown oocytes. Hence fertilization and early embryo development depends on stored mRNA which translate into proteins at the appropriate time. As reviewed by Labrecque and Sirard[128], transcriptomic differences have been found among different quality oocytes. But variability in transcriptomic profiles and low correlation between mRNA and translated proteins limit the interpretation. On the other hand, transcriptomic analysis of CCs can provide information about oocyte quality. As above mentioned, CCs play a pivotal role in the acquisition of oocyte competence. Oocyte competence is impaired by inhibiting CC metabolic, transcriptional and translational activity, as well as by interrupting CC-oocyte communication (reviewed by Sirard and Assidi[129]).

Expression of target genes can be analyzed by quantifying mRNA with quantitative reverse transcription polymerase chain reaction (RT-qPCR) and the $2^{-\Delta\Delta C_T}$ method described by Livak & Schmittgen[130]. RT-qPCR includes different procedures to extract total RNA from a sample, convert it into cDNA by retro-transcription, and amplify the transcripts with specific primers in multiple thermo-cycling periods. DNA amplification is performed by a DNA polymerase

combined with SYBR green, which interacts with the DNA secondary structure and emits lights proportionally to the amount of amplified DNA. The RT-PCR machinery records the cycle number at which emitted light is detected for each sample and gene (Ct value). Hence Ct value is indirectly correlated to the amount of transcript in the sample. Lastly, the $2^{-\Delta\Delta C_T}$ method calculates the mRNA amount relative to the Ct values of the reference gene and the reference group. The reference gene is a cell housekeeping expressed at a constant level in all cells. Thus, this method prevents obtaining variability due to differences in mRNA concentration.

2.3.4. Mitochondrial number and activity

Mitochondria are maternally inherited organelles involved in essential oocyte functions (reviewed by Cecchino et al.[131]): energy synthesis in form of adenosine triphosphate (ATP) via oxidative phosphorylation, control of ROS and regulation of apoptosis. During oogenesis and folliculogenesis mitochondria number increases[132], but it remains constant during oocyte maturation and early embryo development until embryo implantation[133]. After the LH surge, there is a reorganization of oocyte mitochondria. They move to the perinuclear region for the MI spindle formation and acquire a disperse distribution after the extrusion of the first polar body[134]. In juvenile goats, mitochondria shift from peripheral distribution in immature oocytes to semi-peripheral and homogenous distributions after 24 h of IVM[67].

As reviewed by Van Blerkom[133], ATP production is vital for acquisition of oocyte competence, oocyte maturation, fertilization and early embryo development. The number of mitochondria is also related to oocyte competence (reviewed by Fragouli and Wells[135]). Lamas-Toranzo et al.[136] observed that competent bovine oocytes, assessed by brilliant cresyl blue test, have more mitochondria. Moreover, oocytes with normal mitochondria distribution[137] and activity[138] sustain higher embryo development. Therefore, the following methods are used for studying oocyte mitochondria:

- **Mitochondria distribution and activity** with MitoTracker® Orange CMTMRos[66] and MitoTracker™ deep red[127]: These two molecular probes emit fluorescence depending on the mitochondria membrane potential. Hence mitochondrial activity can be quantified by calculating the mean fluorescence intensity and mitochondrial distribution is also revealed.
- **ATP content** with adenosine 5'-thriphosphate bioluminescent somatic cell assay kit[67]: ATP is hydrolyzed and light is emitted when firefly luciferase catalyzes the oxidation of d-luciferin. A luminometer is used to measure the emitted light and ATP concentration per oocyte can be determined comparing with the results of a standard curve.

- **Mitochondrial DNA (mtDNA) copy number** with qPCR[136]: As each mitochondria has 1 to 2 DNA copies, mtDNA copy number is correlated to the number of mitochondria present in the oocyte (reviewed by Fragouli and Wells[135]).

2.3.5. Cumulus-oocyte communication

Cumulus-oocyte communication is bidirectional: CCs support oocyte growth and competence acquisition, and oocytes orchestrate CC activity (reviewed by Russel et al.[139]). Maintaining CC-oocyte communication *in vitro* has proved to reduce oocyte oxidation and enhance embryo developmental competence[140,141]. As above reviewed, there are two main components of CC-oocyte communication:

- **TZPs**: During follicle development TZPs dynamically change as the play a role in coordinating the exchange of information, but they begin to retract after the onset of oocyte maturation (reviewed by Albertini[94]). TZPs can be evaluated by staining with fluorescein isiothiocynate (FITC) conjugated phalloidin which marks actin filaments[142]. Integrity of TZPs can be determined: filaments going from CCs to the oocyte or filaments disrupting and detaching from the oocyte. TZP density can also be quantified by determining the mean fluorescence intensity in the zona area.
- **GJ connections**: GJs rapidly closed after the beginning of oocyte maturation[143]. GJs can be evaluated by microinjecting lucifer yellow into the oocyte and assessing the spreading of the dye into the surrounding CCs[144]. If GJs are opened CCs get stained in a few minutes. However, this technic requires a micro-injector and an inverted fluorescence microscope with a thermo-heated plate in order to monitor the dye spreading in life COCs.

2.4. Improvement of oocyte competence by reducing oxidative stress during IVM

2.4.1. Reactive oxygen species and oxidative stress in the oocyte

ROS are highly reactive molecules derived from oxygen which one or more unpaired electrons (reviewed by Halliwell[145]). The most common are superoxide ion radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$) and hydrogen peroxide (H_2O_2). ROS are generated by cell metabolism, mainly by mitochondria due to electron leakage during oxidative phosphorylation, and continuously eliminated by the cell antioxidant defense mechanisms (reviewed by Haderland[146]). A low physiological level is indispensable since ROS act as second messengers in many cellular signaling cascades (reviewed by Dennery[147]). However an imbalance in ROS

production and elimination leads to oxidative stress (OS; reviewed by du Plessis et al.[117]). ROS induce cell damage at different levels: lipid peroxidation[148] which cause disruption of the cell membrane[149], protein damage like aggregation and degradation[150], and DNA damage such as deamination of nucleotide bases and strand breaks (reviewed by Evans et al.[151]).

Regarding the oocyte, OS can impair oocyte maturation at different levels. First, OS affects nuclear maturation. Tamura et al.[105] observed that inducing OS with H_2O_2 inhibits meiotic progression in mice oocytes. OS causes aneuploidy, errors in the chromosome alignment and changes in the spindle morphology[152]. The impairment of the meiotic spindle formation has been related to alterations in microtubule dynamics[153] and a reduced ATP production due to mitochondria damage[154]. Second, OS induces apoptotic cell death orchestrated by mitochondria[155]. Apoptosis has also been observed in pig oocytes following DNA fragmentation[156] and human oocytes[157]. Third, OS modifies the gene expression of mature oocytes (reviewed by Combelles et al.[118]). Lastly, OS leads to oocyte aging[36]. On the other hand, OS negatively affects fertilization by disrupting the membrane fluidity which impairs the sperm-oolemma fusion (reviewed by Tarín[152]) and by interfering with calcium homeostasis which alters calcium oscillations after fertilization[37].

2.4.2. Oxidative stress on *in vitro* embryo production: ROS and antioxidants

On IVEP oocyte ROS levels are higher than on *in vivo* conditions due to exposure to external deleterious factors and lack of follicle antioxidants (reviewed by du Plessis et al.[117]), consequently, *in vitro* maturation, fertilization and ultimately embryo development are impaired (figure 2).

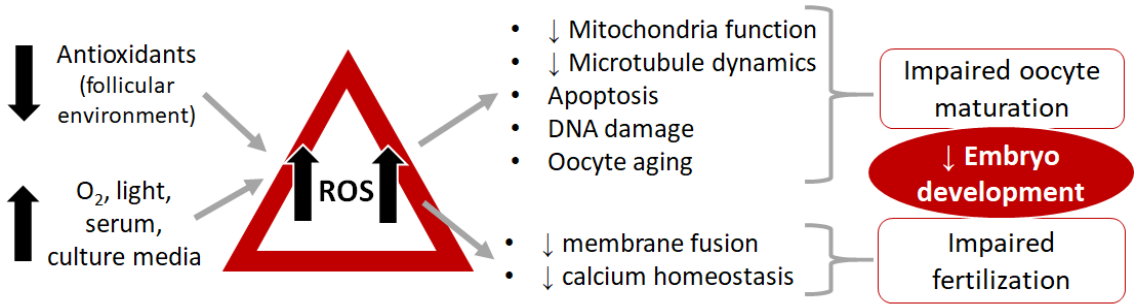

Figure 2: Sources and consequences of reactive oxygen species (ROS) on in vitro embryo production.

Some of the external factors that promote ROS production *in vitro* are reviewed by Guérin et al.[35] and du Plessis et al.[117]: oxygen concentration, in conventional IVM culture conditions is 3-4 times higher than in the oviduct[115]; metallic cations such as Fe^{2+} and Cu^{2+}, usually present in chemical products used for culture media[158]; visible light[159]; and amine oxidases, present in serums used for culture media and released by dead spermatozoa[160,161].

Follicles contain enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), as well as non-enzymatic antioxidants, such as vitamin E, vitamin C, GSH, uric acid and albumin (reviewed by Tamura et al.[33]). SOD converts $O_2^{\cdot -}$ into H_2O_2 in a first reaction, and GPx and CAT further transform it into water and oxygen (reviewed by Combelles et al.[118]). GSH is the main non-enzymatic defense system against ROS in oocytes (reviewed by Guérin et al.[35]) that works as a substrate for GPx to neutralize H_2O_2[162]. Oocytes from juvenile females are particularly sensitive to ROS due to its low ability to synthesize GSH, as shown by Jiao et al.[52]. This study suggested that reduced GSH is responsible for the lower developmental competence compared to adults because it impairs male pronuclear formation and resulting high ROS levels affect fertilization (Ca^{2+} homeostasis and cortical granules migration). A previous study in our group also reported a lower GSH level on juvenile-goat oocytes compared to ovulated oocytes from adults (5.59 vs. 23.73 pmol/oocyte)[40].

Different antioxidants are conventionally used in oocyte IVM to prevent *in vitro* ROS imbalance and OS. Vitamins such as ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) neutralize free radicals and have positive effects for IVM (reviewed by Combelles et al.[118]). Thiol compounds, such as cysteamine, 2-mercaptoethanol, cysteine, cystine and GSH, increase intra-oocyte GSH concentration and protect the oocyte from ROS (reviewed by Deleuze and Goudet[38]). Cysteamine is probably the most commonly added antioxidant to IVM medium with a dose of 100 µM. Cysteamine has proved to increase GSH content and blastocyst rate of sheep[39] and cow oocytes[163]. In juvenile-goat IVM, a previous study from our group showed that cysteamine, cysteine, cysteine and b-mercaptoethanol increase intra-oocyte GSH, but only cysteamine enhances blastocyst yield[40]. Recently, other potentially more powerful antioxidants have been tested for IVM, such as melatonin.

2.4.3. Melatonin: a multitasking molecule and powerful antioxidant

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine hormone synthesized by the pineal gland, firstly isolated in 1958 by Lerner et al.[164]. Its production is regulated by day-light, with a maximum level at night[165]. Yet research in the last 60 years has shown that melatonin is a multifunctional molecule produced by most of mammalian organs in a non-circadian manner. Melatonin acts as an antioxidant, and as an autocoid and paracoid regulating intracellular events (reviewed by Reiter et al.[166]).

Melatonin acts by different pathways including receptor-mediated and receptor-independent actions (figure 3). Melatonin is an amphiphilic molecule; hence it can pass through cellular membranes and directly act in the cell cytoplasm. Melatonin receptor 1 (MT1) and melatonin

receptor 2 (MT2) are members of the G protein-coupled receptor (GPR) family, localized in many cells (reviewed by Slomisnki et al.[167]). Both receptors activate many signaling pathways through the inhibition of cAMP formation (reviewed by Dubocovich and Markowska[168]). Melatonin can also bind to nuclear receptors (retinoid orphan receptors, ROR, and retinoid Z receptors, RZR) to regulate the gene expression (reviewed by Korkmaz et al.[169]). On the other hand, receptor-independent mechanisms include direct scavenging of ROS and radical nitrogen species (RNS) and interaction with cytosolic molecules such as calmodulin, which is a calcium-binding protein that activates protein kinases and other enzymes (reviewed by Reiter et al.[170]).

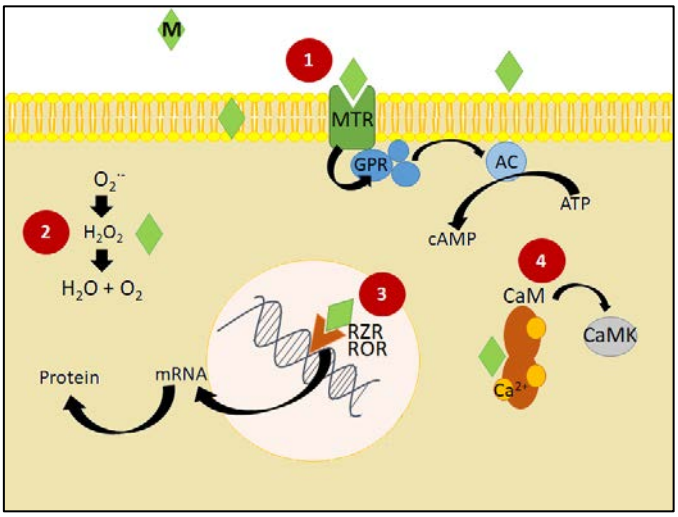

Figure 3: Melatonin mechanisms of action in mammalian cells. 1) Melatonin receptors (MTR): coupled to G-proteins (GPR) which inhibit adenylyl cyclase (AC) decreasing cAMP levels. 2) Scavenger of ROS into non-harmful molecules: melatonin transforms superoxide anion radical ($O2^{\cdot-}$) into hydrogen peroxide (H_2O_2) which further converts into water and oxygen. 3) Retinoid-related orphan nuclear hormone receptor family (RZR/ROR): regulate gene transcription through binding to DNA sequences located in the promoter region of target genes. 4) Calmodulin (CaM): activated by calcium, as part of the calcium signaling-pathway, and induces calmodulin kinase (CaK).

Melatonin is a powerful antioxidant as it can reduce and prevent oxidation at different levels (figure 4). First, it acts as a free radical scavenger by transferring electrons and hydrogens from ROS and RNS to create less harmful molecules. It is able to neutralize the toxic ·OH as well as many other free radicals like peroxynitrite ($ONOO^-$), nitric oxide (NO·), $O_2^{\cdot-}$ and H_2O_2 (reviewed by Reiter et al.[171]). More interestingly, melatonin metabolites that result from these interactions (N1-acetyl-N2-formyl-5-methoxykynuramine, AFMK, and N1-acetyl-5-methoxykynuramine, AMK) are also scavengers and generate a potent antioxidant cascade (reviewed by Reiter et al.[170]). Second, melatonin stimulates antioxidant enzymes such as intracellular superoxide dismutases (CuZnSOD and MnSOD), selenium-containing glutathione

peroxidases (GPx1, GPx2 and GPx3) and CAT, and inhibit pro-oxidative enzymes such as nitric oxide synthase, myeloperoxidase and eosinophil peroxidase (reviewed by Reiter et al.[166]). Melatonin also promotes the recovery of GSH by promoting glutathione reductase (GRd) and y-glutamylcysteine ligase (GCL). Lastly, melatonin can directly prevent the production of free radicals by electron leakage in mitochondria, consequently improving the activity of the respiratory chain. This process is known as radical avoidance (reviewed by Hardeland[146]).

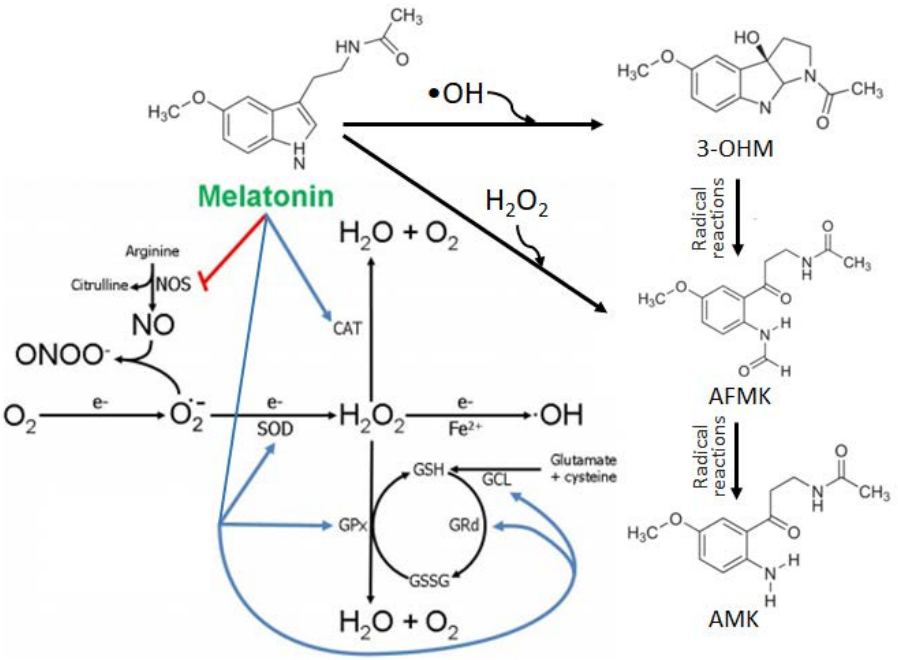

Figure 4: Melatonin mechanisms of action at neutralizing free radicals (modified from Reiter et al.[172] and Hardeland[146]). Melatonin stimulates (blue arrows) various antioxidant enzymes (SOD, superoxide dismutase; GPx, glutathione peroxidase; GRd, glutathione reductase; GCL, y-glutamylcysteine ligase) and inhibits (red arrow) the pro-oxidative enzyme NOS (nitric oxide). Melatonin directly scavenges free radicals (including ROS and RNS), while transforms into other scavengers generating a powerful antioxidant cascade (3-OHM, cyclic 3-hydroxymelatonin; AMFK, N^1-acetyl-N^2-formyl-5-methoxykynuramine; AMK, N^1-acetyl-5-methoxykynuramine). Some of the scavenged free radicals are represented and other are included in *radical reactions*.

2.4.4. *In vitro* maturation with melatonin

Melatonin is essential for maintaining ovarian function (figure 5). It has been detected in the follicular fluid (FF) of different species[173,174]. In humans, melatonin concentration in FF is higher than in blood[175] and increasing with follicular size[176], which suggest a role in follicular development (reviewed by Tamura et al.[177]). In goats, melatonin combined with FSH stimulates the follicular development of *in vitro*-cultured preantral follicles[178,179]. Melatonin receptors have also been localized by immunocytochemistry and PCR in oocytes, CCs and granulosa cells

of multiple species (mice[180], human[181], cow[174,182], pig[183,184] and sheep[185]). Barros et al.[178] localized MT1 by immunohistochemistry in cumulus and granulosa cells of goat antral follicles, although it was not detected in primordial follicles, primary follicles or oocytes. Tamura et al.[186] summarized all the presumed melatonin functions at maintaining a healthy follicle (figure 5), which include regulation of steroid hormones production, stimulation of follicular development, antioxidant and antiapoptotic role in follicular cells, and promotion of oocyte maturation. All this suggests that melatonin could improve oocyte competence during IVM.

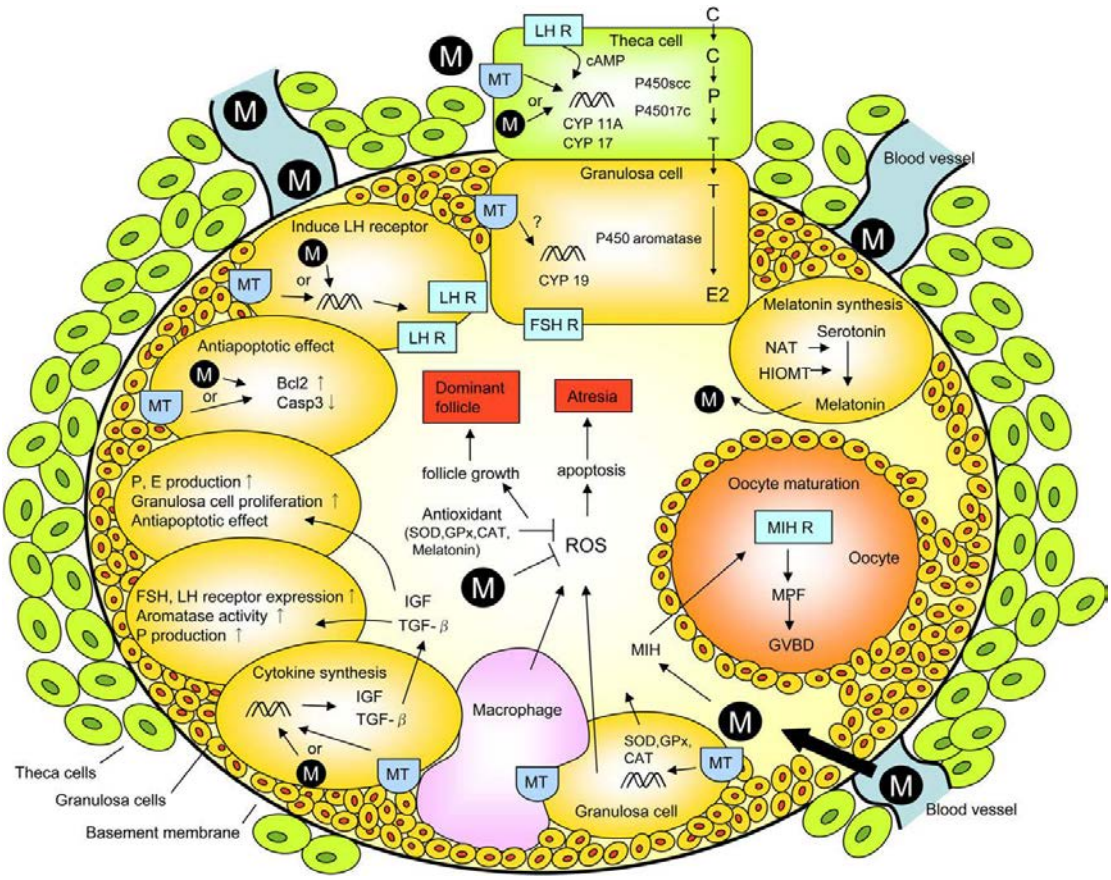

Figure 5: Melatonin effects in the ovarian antral follicle (from Tamura et al.[186]). Melatonin in the follicular fluid (FF) mainly comes from granulosa cell (GC) production and the circulating blood. Melatonin regulates sex steroid production, prevents apoptosis and follicular atresia, and acts as an antioxidant. These actions are mediated by regulation of gene expression, control of enzyme activity, and ROS scavenging. The follicle can be rescued by melatonin and continue to grow to a dominant follicle. P (progesterone); E (estradiol); M (melatonin); MT (melatonin receptors); LHR (LH receptor); FSHR (FSH receptor); C (cholesterol); NAT (N-acetyltransferase); HIOMT (hydroxyindole-O-methyltransferase); MIH (maturation-inducing hormone); MIF (maturation-promoting factor); GVBD (germinal vesicle breakdown); ROS (reactive oxygen species); SOD (superoxide dismutase); GPx (glutathione peroxidase); CAT (catalase); IGF (insulin-like growth factor); TGF-β (transforming growth factor β); CYP (cytochromes P450).

Melatonin has been added to different steps of IVEP in many species with promising results on embryo development (reviewed by Cruz et al.[42]). The addition of melatonin to IVM medium at a concentration of 10^{-12}-10^{-6} M increases blastocyst production in cattle[174,182,187,188], sheep[185], pig[173,183] and mice[189–191]. Some of these studies also show an improvement in blastocyst quality (sheep[185], pig[173], and cow[174]). Moreover, melatonin can enhance embryo development of low-quality oocytes, as shown in pigs (in oocytes with 1-2 layers of cumulus cells and a prolonged IVM)[192] and cows (in oocytes with less than 3 layers of cumulus cells and with irregular cytoplasm)[193]. However, the success of the treatment is concentration-dependent. For instance, Tian et al.[174] found that 10^{-9} M is the best concentration for IVM of cow oocytes, whereas 10^{-3} M is toxic and decreases embryo development.

The mechanisms of action by which melatonin improves oocyte quality during IVM have also been investigated. The most relevant is the antioxidant effect as shown by lower intra-oocyte ROS levels compared to conventional IVM[180,183,194]. By reducing ROS melatonin also prevents the negative consequences of OS in oocytes: alterations in the meiotic spindle[180,195], apoptosis[36,196] and aging[36,197]. Other actions include increasing intra-oocyte GSH levels[187,191,198], preserving oocyte mitochondrial function and ATP production[187,188,199], and regulating the expression of oocyte maturation-related genes[174] and antioxidant-related genes[188]. Melatonin also up-regulates the gene expression in CCs related to extracellular matrix formation, maturation and antioxidants[174,185,200]. Lastly, the role of melatonin receptors on IVM has been revealed with the addition of melatonin receptor inhibitors such as luzindole. Luzindole prevented the melatonin positive effect on embryo development in cow[174] and sheep oocytes[185], and melatonin antiapoptotic effect on pig granulosa cells[184].

2.5. Improvement of oocyte competence by biphasic IVM

As reviewed in chapter 2.2. *oocyte competence*, during folliculogenesis oocytes go through changes at nuclear and cytoplasmic levels that provide the oocyte the ability to undergo embryo development[43]. The oocyte is maintained at meiotic arrest by the follicle environment and spontaneously resumes meiotic maturation when removed from the follicle[44]. Therefore, in standard IVM meiotic and cytoplasmic maturation are unsynchronized, and oocyte competence impaired. Biphasic IVM is a promising system to overcome *in vitro* limitations.

2.5.1. Follicular regulation of oocyte meiosis

Cho et al.[201] first reported that high oocyte cAMP levels maintain meiotic arrest (figure 6). As reviewed by Gilchrist et al.[48], cAMP is synthesised by adenylyl cyclase (AC) from ATP in

response to GPR activation. The oocyte can synthesise cAMP, but intra-oocyte levels mainly come from surrounding follicular cells via GJs. Cyclic AMP activates protein kinase A (PKA) which blocks the maturation-promoting factor (MPF) and maintains the oocyte at prophase I.

The oocyte also contains potent phosphodiesterases (PDEs) that hydrolyse cAMP into 5'AMP. But the follicle keeps PDEs inhibited via c-type natriuretic peptide (CNP)-cGMP pathway. CNP secreted by granulosa cells activates the natriuretic peptide receptor (NPR2) expressed in CCs, which is a guanylyl cyclase receptor. Resulting cGMP reaches the oocyte via GJs where acts as a competitive inhibitor for PDE3A, the main PDE in the oocyte[202,203]. Hence the activation of NPR2 by CNP increases cGMP in both CCs and oocyte[204].

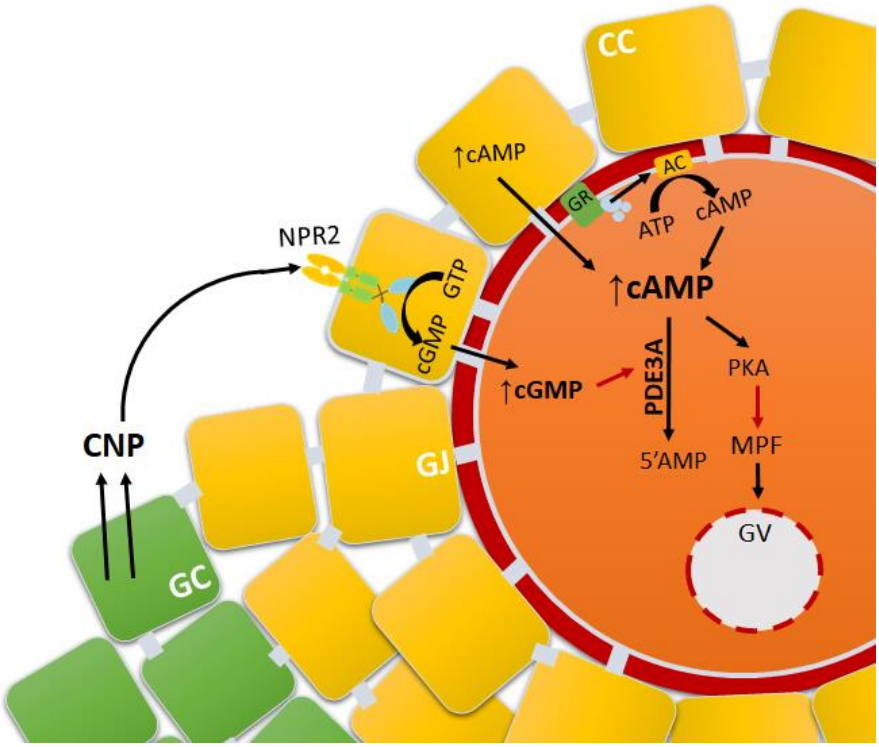

Figure 6: Control of meiotic arrest by the ovarian follicle. Meiotic arrest is maintained by high cAMP. The follicular environment promotes high oocyte cAMP by secreting c-type natriuretic peptide (CNP) that induces cGMP synthesis. Phosphodiesterase 3A (PDE3A) hydrolyses cAMP and is inhibited by cGMP. Black arrows show promotion and red arrows inhibition. GC (granulosa cell); CC (cumulus cell); GJ (gap junction); NPR2 (natriuretic peptide receptor 2); GR (g protein-coupled receptor); AC (adenylyl cyclase); PKA (protein kinase A); MPF (maturation-promoting factor); GV (germinal vesicle).

As shown in figure 7, oocyte meiotic resumption is an orchestrated process initiated by the pre-ovulatory LH surge which promotes oocyte maturation and ovulation (reviewed by Downs[205]). LH induces a transient increase in cAMP levels, followed by a great decline that

activates MPF resuming meiosis. Although the pathways involved are complicated and not fully understood, the rapid cAMP decrease is related to a fall in follicular cGMP which probably ceases the inhibitory effect on PDE3A[202,203]. LH down-regulates CNP expression in granulosa cells[206] and decreases NPR2 activity in cumulus cells[207]. Moreover, the initial cAMP rise induced by LH promotes the EGF network which is involved in CC expansion and closure of GJs[143]. CC-oocyte GJC decreases with oocyte nuclear maturation in an interdependent manner[208]. The loss in GJC contributes to meiotic resumption due to the interruption of cGMP diffusion[202,203]. At the same time the maintenance of high cAMP levels prolongs GJC[140,209].

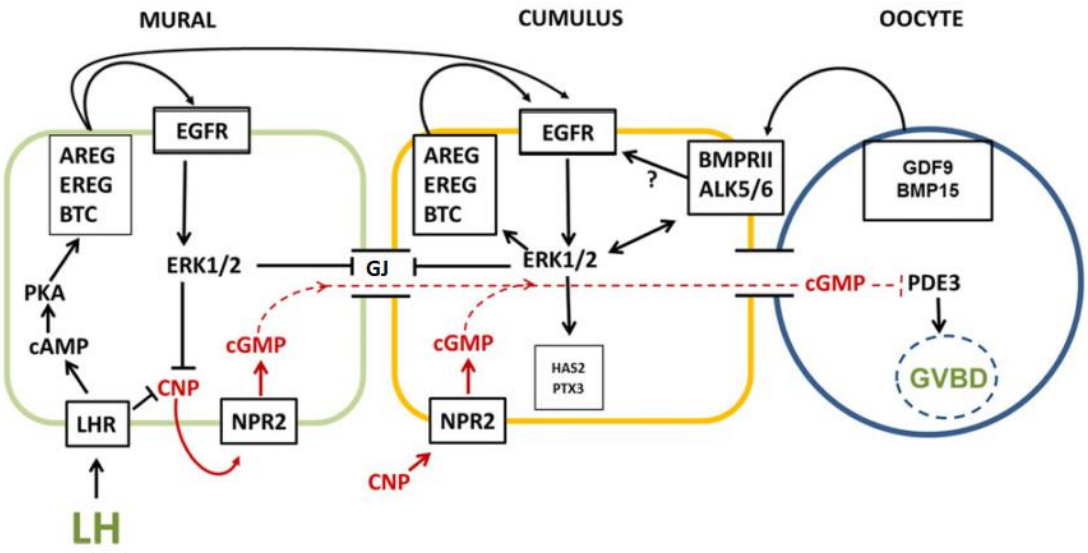

Figure 7: Oocyte meiotic resumption and maturation orchestrated by luteinizing hormone (LH) and epidermal growth factor (EGF) signalling network (modified from Richani & Gilchrist[210]). The LH surge up-regulates EGF network (amphiregulin, AREG; epiregulin, EREG; betacellulin, BTC) which transmits the maturation signal from follicle cells to the oocyte. This induces mural granulosa cell luteinisation, closure of gap junctions (GJs) and cumulus extracellular matrix formation (hyaluronan synthase-2, HAS2; pentraxin 3, PTX3). There is a simultaneous down-regulation of the meiotic inhibitory signal mediated by c-type natriuretic peptide (CNP) and cGMP, inducing oocyte nuclear maturation. Black arrows indicate up-regulation, red arrows down-regulation, and dashed lines decreased cGMP diffusion. LHR (LH receptor); NPR2 (natriuretic peptide receptor 2); EGFR (EGF receptor); PKA (protein kinase A); ERK (extracellular signal-regulated protein kinase); GDF9 (growth differentiation factor 9); BMP15 (bone morphogenetic protein 15); BMPRII (BMP receptor 2); ALK (TGFβ type I receptor kinase); PDE3 (phosphodiesterase 3); GVBD (germinal vesicle breakdown).

2.5.2. Cyclic AMP-mediated IVM

On *in vitro* conditions, oocytes resume meiosis even without LH stimulation. After COC isolation from the follicular environment and its cGMP-PDE meiotic inhibition, there is a fast loss of cAMP[144] and CC-oocyte GJC[211]. Meiosis resumption can even take place during the oocyte collection process, especially if it is performed on saline, PBS or holding medium instead of follicular fluid (reviewed by Gilchrist et al.[48]). FSH used on standard IVM produces a transient increase in cAMP[212], but cAMP hydrolysis occurs fast in this system leading to GVBD in around 1 h in mouse and 6 h in cattle.

New IVM systems focus in controlling cAMP levels throughout maturation to better resemble physiological maturation and improve oocyte embryo development competence (reviewed by Gilchrist et al.[48]). These systems include a pre-IVM culture period with cAMP modulators to prevent spontaneous meiotic resumption (figure 8).

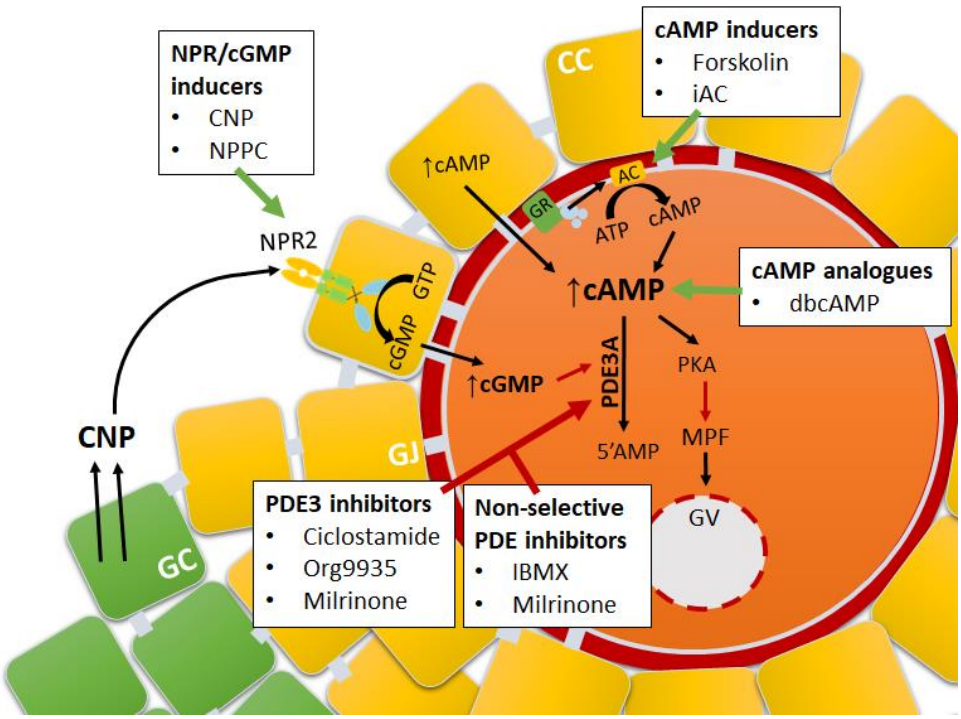

Figure 8: *In vitro* oocyte meiotic inhibition via cAMP-modulators and its mechanisms of actions. Green arrow indicates promotion and red arrow indicates inhibition.

The most promising cAMP-mediated IVM was named simulated physiological oocyte maturation (SPOM)[209,213]. SPOM induces high cAMP levels by combining different types of modulators such as AC activators, PDE inhibitors and cAMP analogues. This approach achieved 86% blastocyst rate in bovine oocyte (compared to 55% in control group), but the results have

not been repeatable in other studies. SPOM includes agents that can be detrimental for oocyte function if not used at the precise period with a correct dose, hence extensive washout between phases is essential.

In recent years biphasic IVM, which follows a more conservative protocol, has been more widely spread although having a moderate success. Biphasic IVM (figure 9) consists in two maturation culture phases: pre-IVM and standard IVM. Pre-IVM includes a PDE inhibitor that maintains moderate levels of cAMP and can last from 4 to 48 h, depending on the animal species and the agents used (reviewed by Gilchrist et al.[48]). Pre-IVM is followed by standard IVM without PDE inhibitors that induces a rapid cAMP decrease and enables oocyte maturation.

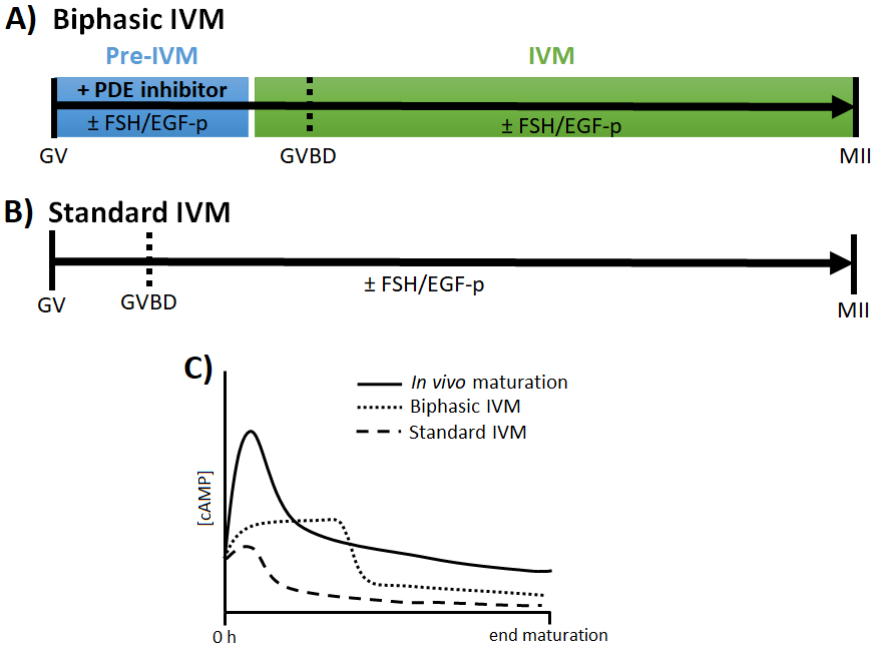

Figure 9: Biphasic *in vitro* maturation protocol (A) compared to standard IVM (B) (modified from Gilchrist et al.[48]). Biphasic IVM includes a PDE inhibitor during a pre-IVM phase followed by washout of the PDE inhibitor and IVM phase. C) Schematic illustration of cAMP concentration in cumulus-oocyte complexes with biphasic IVM compared to standard IVM with FSH and oocytes matured *in vivo*. FSH (follicle-stimulating hormone); EGF-p (epidermal growth factor-like peptides); GV (germinal vesicle); GVBD (germinal vesicle breakdown); MII (metaphase II); PDE (phosphodiesterase).

2.5.3. Effect of maintaining high cAMP levels on oocyte and cumulus cells

As above mentioned, high intra-oocyte cAMP levels maintain meiotic arrest during a pre-IVM phase. This would provide the oocyte with the time to acquire competence prior to IVM. Depending on the system and the animal species GVBD is prevented[214,215] or delayed[209,216]. When nuclear maturation resumes, the time for reaching MII after GVBD is shortened[217],

probably due to the production of meiosis-inducing factors related to the EGF network during pre-IVM (reviewed by Gilchrist[48]). Thus, the combination of pre-IVM and IVM decreases meiotic asynchrony among the oocyte pool[218].

On the other hand, high cAMP levels prolong the CC-oocyte GJC[140,209,216] and improve oocyte metabolism by different mechanisms. High cAMP increases lactate production by COCs which suggests an enhancement of CC glycolysis[141]. The glycolytic pathway metabolizes glucose producing energy and metabolites needed by the oocyte[219]. High cAMP also rises intra-oocyte GSH levels[140,141], hence improving the oocyte antioxidant defences[140]. Prolonged GJC are responsible for this effect, as oocyte GSH depends on the flow from CC via GJs. This was shown by blocking GJC which prevented the increase of GSH[140,141]. Moreover, cAMP-mediated IVM enhances mitochondrial and oxidative metabolism with higher O_2 consumption and ATP:ADP ratio[141,220]. Lastly, a microarray analysis of CC after 6 h of exposure to cAMP-modulators showed an up-regulation of genes involved in cell communication, cell metabolism, cell survival, steroidogenesis and formation of extracellular matrix[221]. Figure 10 summarises possible cAMP mechanisms that lead to better oocyte developmental competence.

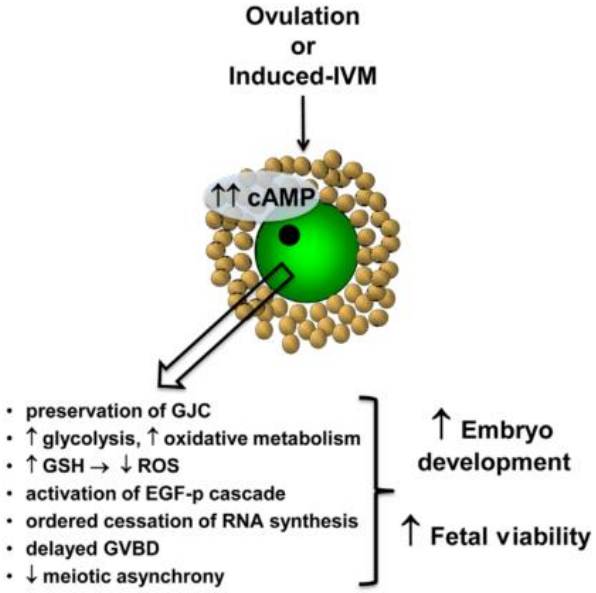

Figure 10: Positive effects of maintaining high cAMP levels on oocyte developmental competence (from Gilchrist et al.[48]). EGF-p (epidermal growth factor-like peptides); GJC (gap junctional communication); GSH (glutathione); GVBD (germinal vesicle breakdown); ROS (reactive oxygen species)

The positive effect on oocyte developmental competence could be further relevant in oocytes with has not completed follicular development (reviewed by Luciano et al.[122]). This was shown by Dieci et al.[222] by culturing COCs of different initial grade of development. Pre-IVM improved

embryo development in growing oocytes but was detrimental in fully grown oocytes. Similarly, pre-IVM can increase the competence of juvenile oocytes as reported in prepubertal calves[223] and juvenile mice[5].

2.5.4. Biphasic IVM with IBMX and C-type natriuretic peptide

For pre-IVM in biphasic IVM systems, various PDE inhibitors are used. Yet, 3-isobutyl-1-methylxanthine (IBMX), a non-specific PDE inhibitor, is probably the most tested one. Either individually (500 μM) or combined with forskolin has been effective at maintaining high cAMP levels and delaying meiotic resumption in ruminant oocytes[140,224–226]. This pre-IVM phase has a positive impact on embryo rate and quality in cattle[140,224,227], and improves embryo quality in sheep although having no effect on embryo yield[225]. Pre-IVM with IBMX also maintains GJC in bovine COCs[140,224], and improves antioxidant defences[140] and mitochondrial activity[138] in bovine oocytes. In addition, IBMX is added to oocyte collection medium to prevent meiotic resumption before pre-IVM, even when other cAMP modulators are used for pre-IVM[48].

More recently, after achieving a better understanding of the physiological pathways that maintain high intra-oocyte cAMP levels in the follicle, CNP has become the focus of research. Being a physiological molecule, it potentially has fewer toxic effects for the oocyte than other PDE inhibitors. In bovine COCs, CNP maintains meiotic arrest by inducing intra-oocyte cGMP via NPR2 localised in oocytes and CCs[220]. In mice, pre-IVM with CNP is able to maintain meiotic arrest for 48 h and achieve a blastocyst yield equivalent to IVF of ovulated oocytes[5]. Blastocyst rate and quality have also been improved in cattle[220,228,229], pig[230] and sheep[231] after IVF, and goat after parthenogenetic activation[232]. It has even been successful at improving oocyte competence in humans with polycystic ovary syndrome[233]. However, effective doses of CNP are higher in ruminants than in mice (100-200 nM vs. 25 nM), and meiotic arrest can only be maintained for 4-8 h, leading to only a mild improvement in blastocyst development[220,229,231,232,234].

Finally, the addition of other agents to the pre-IVM culture medium can improve the system. E2 and GDF9 up-regulate the expression of NPR2 in bovine CCs and oocytes[220], and prolong the GV stage from 24 to 48 h in mice pre-IVM with CNP[215]. On the other hand, several studies have reported the benefits of adding FSH at a low dose during pre-IVM (reviewed by Gilchrist et al.). FSH plays a role in the acquisition of oocyte competence during follicular development because it promotes the expression of EGF receptors[92] and the GJC[93], among other functions. In mice, the combination of CNP, FSH, GDF9 and E2 during the pre-IVM improves oocyte nuclear competence and size, and further enhances embryo development[215].

Chapter 3

Objectives

The main aim of this study is to enhance *in vitro* embryo production in juvenile goats by improving oocyte competence. Four specific objectives were addressed:

1. To improve embryo developmental competence of juvenile-goat oocytes by supplementing the IVM medium with melatonin, as a system for reducing oxidative stress.

2. To study melatonin mechanisms of action during oocyte IVM and the role of melatonin receptors in juvenile goats.

3. To study the effect of two meiotic inhibitors (CNP and IBMX) during a pre-IVM culture on the developmental competence of cattle oocytes, as a model for future studies in juvenile goats.

4. To develop a pre-IVM culture that maintains meiotic arrest and cumulus-oocyte communication in juvenile-goat oocytes.

5. To improve embryo developmental competence of juvenile-goat oocytes with a biphasic IVM system consisting in a pre-IVM culture period followed by standard IVM.

Chapter 4

Beneficial effects of melatonin on in vitro embryo production from juvenile goat oocytes

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https://doi.org/10.1071/RD17170

Chapter 5

Effects of melatonin on oocyte developmental competence and the role of melatonin receptor 1 in juvenile goats

Reprod Domest Anim. 2018 Nov 16. [Epub ahead of print]
https://doi.org/10.1111/rda.13378

Chapter 6

Effect of pre-maturation with C-type Natriuretic Peptide and 3-Isobutyl-1-methylxanthine on cumulus-oocyte communication and oocyte developmental competence in cattle

Effect of pre-maturation with C-type Natriuretic Peptide and 3-Isobutyl-1-methylxanthine on cumulus-oocyte communication and oocyte developmental competence in cattle.

Sandra Soto-Heras[a], Maria-Teresa Paramio[a], Jeremy Thompson[b,c,d,*]

[a]Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona, 08193, Spain

[b]Robinson Research Institute, School of Paedriatics and Reproductive Health, The University of Adelaide, Adelaide, South Australia, 5005, Australia.

[c]Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, 5005, Australia

[d]Australian Research Council Centre of Excellence for Nanoscale BioPhotonics and Institute for Photonics and Advanced Sensing, The University of Adelaide, Adelaide, South Australia, 5005, Australia.

[*]Corresponding author at: The University of Adelaide, School of Medicine, Level 2 Medical School South, Frome Road, Adelaide, South Australia, 5005, Australia. E-mail address: jeremy.thompson@adelaide.edu.au

ABSTRACT

In vitro embryo production depends on healthy oocyte developmental competence, which is acquired *in vivo* during folliculogenesis, involving cytoplasmic and nuclear processes. *In vitro* maturation (IVM) induces spontaneous resumption of meiosis, preventing full competence acquisition. Biphasic maturation systems that incorporate a pre-IVM phase to maintain meiotic arrest enable enhanced cytoplasmic maturation. We designed a pre-IVM combining C-type natriuretic peptide (CNP) with IBMX (a non-specific phosphodiesterase inhibitor) with the aim of improving the developmental competence of bovine oocytes. In a preliminary experiment, COCs were cultured with increasing CNP concentrations and nuclear stage was assessed. Both 100 and 200 nM CNP maintained higher germinal vesicle (GV) rates than control for 6 h (79.3%, 76.4% and 59.2%, respectively). In a second experiment, we found that 100 nM CNP plus 500 µM IBMX retained more oocytes in GV (92.0%) at 6 h compared to either CNP or IBMX alone (74.8% and 86.7%, respectively). We then assessed the influence of the biphasic maturation system consisting of 6-h pre-IVM (CNP plus IBMX) on development, followed by 20-h IVM and compared to control 24-h IVM. Blastocyst rate was increased after pre-IVM (45.1 vs. 34.5%). Pre-IVM also enhanced mitochondrial activity in matured oocytes, but did not affect intra-oocyte glutathione levels. Analysis of the density of transzonal projections showed

that pre-IVM prolonged this potential communication pathway for longer after IVM. In conclusion, CNP and IBMX work synergistically to arrest meiosis in bovine oocytes during a pre-IVM phase, which improves cumulus-oocyte communication and embryo development.

Keywords: Pre-maturation; C-type natriuretic peptide; IBMX, Meiotic arrest; Oocyte competence; Transzonal projections

1. Introduction

In vitro embryo production (IVEP) is an important artificial reproductive technology for cattle breeding programs because when coupled to genetic selection, it increases the rate of genetic gain relative to natural mating and artificial insemination (Granleese et al., 2015). Nevertheless, both production of blastocysts and subsequent pregnancy rate post-transfer are lower than for *in vivo*-produced embryos, limiting widespread adoption (Rizos et al., 2002).

During folliculogenesis, oocytes go through changes at nuclear and cytoplasmic levels essential for acquiring developmental competence (Gilchrist and Thompson, 2007), which is defined as the ability to develop to the blastocyst stage, induce pregnancy and bring healthy offspring to term (Sirard et al., 2006). Oocytes are arrested at the germinal vesicle (GV) stage within the follicle, but they spontaneously resume meiotic maturation when removed and cultured *in vitro* (Edwards, 1965). This creates a disconnection between meiotic and cytoplasmic maturation. To counter this disconnection, meiotic inhibitors have been employed to provide the time to complete cytoplasmic maturation before IVM (Gilchrist et al., 2016). However, variable results are observed following oocyte meiosis arrest *in vitro* with meiotic inhibitors of various classes. The most efficacious of these in improving oocyte quality has been when meiosis is inhibited by an intra-oocyte cAMP-regulator during a pre-maturation (pre-IVM) phase (Gilchrist et al., 2016).

In vivo, meiotic arrest is controlled by high intra-oocyte cAMP levels (Cho et al., 2018). The maintenance of high levels of cAMP also prolongs the gap junction communication (GJC) between cumulus cells (CC) and oocytes (Albuz et al., 2010; Li et al., 2016), which is essential for acquiring oocyte competence (Gilchrist, 2010). Moreover cAMP-mediated IVM has an effect on oocyte metabolism, for instance it stimulates CC glycolysis (Zeng et al., 2014), increases the oocyte glutathione (GSH) levels which improve oocyte antioxidant defence (Li et al., 2016; Zeng et al., 2014), and enhances mitochondrial and oxidative metabolism (Xi et al., 2018; Zeng et al., 2014).

One such cAMP modulator is 3-isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase (PDE) inhibitor. Oocyte cAMP is hydrolysed by PDE3A (Zhang et al., 2010), which is inhibited by IBMX thereby preventing the degradation of cAMP. In bovine oocytes, pre-IVM with IBMX + forskolin (activates adenylyl cyclase activity) delays meiotic resumption and increases blastocyst rate and quality (Albuz et al., 2010; Li et al., 2016). C-type natriuretic peptide (CNP) is secreted by granulosa cells and stimulates the production of cGMP by CC which inhibits the PDE3/4 (Zhang et al., 2010). In mice pre-IVM with CNP and oestradiol maintains meiotic arrest for 48 h, increasing blastocyst development rate to a level comparable to oocytes matured *in vivo* (Romero et al., 2016). In cattle, pre-IVM with CNP can improve blastocyst yield and quality (Franciosi et al., 2014; Xi et al., 2018; Zhang et al., 2016), as in other livestock species (Zhang et al., 2018, 2015; Y. Zhang et al., 2017). However, meiotic arrest can only be held for about 6 h and blastocyst development is only slightly improved (Franciosi et al., 2014; Xi et al., 2018; T. Zhang et al., 2017).

In the present study we hypothesize that combining IBMX and CNP in a pre-IVM phase will prolong meiotic arrest, assist cumulus-oocyte communication and improve oocyte quality.

2. Materials and methods

Unless indicated otherwise, chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.1. COC collection and culture

Bovine ovaries were obtained from adult cows of various ages at an abattoir and transported to the laboratory in warm saline (30-35ºC) within 2 h after recovery. COCs were aspirated from 3-8-mm follicles with an 18-gauge needle and a 10-mL syringe. COCs were maintained in follicular fluid until transferred to IVM or pre-IVM medium. COCs were incubated at 38.5ºC with 6% CO_2 in air in a humidified atmosphere for different time periods. Control culture medium for Pre-IVM consisted of VitroMat (IVF Vet Solutions, Adelaide, Australia) supplemented with 4 mg/mL fatty acid-free bovine serum albumin (BSA; ICPbio Ltd, Auckland, NZ) and 100 nM β-Estradiol. Pre-IVM medium was supplemented with CNP and IBMX depending on the experimental design. IBMX was previously diluted in DMSO (0.1% final DMSO concentration). IVM culture medium was VitroMat supplemented with 4 mg/mL BSA and 100 mIU/mL recombinant human follicle stimulating hormone (FSH; Puregon, Organon).

2.2. Assessment of meiotic arrest

At 6 and 24 h after pre-IVM, oocytes were mechanically denuded by pipetting and fixed in 4% (v/v) paraformaldehyde for 30 min. Fixed oocytes were incubated with 1 µL/mL 4',6-

diamidino-2-phenylindole (DAPI) solution in phosphate buffer saline (PBS) with 4 mg/mL BSA for 15 min at room temperature (RT). Oocytes were washed in PBS with 1 mg/mL BSA and mounted on a slide with glycerol. The nuclear maturation stage was analysed under an epifluorescence microscope (Olympus BX51; excitation: 340-380 nm; emission: 440-480 nm). Nuclear stage was classified as: germinal vesicle (GV) and germinal vesicle breakdown (GVBD) at 6 h; and GV, GVBD, Metaphase I (MI) and Metaphase II (MII) at 24 h.

2.3. Assessment of oocyte glutathione levels and mitochondrial activity

Monochlorobimane (MCB) and MitoTracker™ deep red FM (Molecular Probes; Eugene, OR) were used together to quantitatively assess cytoplasmic maturation, as established by Sutton-McDowall et al. (2015). MCB binds to thiol compounds and has high affinity for GSH (99% of intracellular fluorescence) (Keelan et al., 2001). MitoTracker™ deep red emits more fluorescence depending on the mitochondria membrane potential, assessing mitochondrial activity. Oocytes were denuded by mechanical pipetting and cultured first with 12.5 µM MCB for 15 min, and second with 200 nM MitoTracker™ deep red FM for 15 min, in PBS with 4 mg/mL BSA at 38.5ºC. Oocytes were washed in PBS with 1 mg/mL BSA and transferred in 5-µL drops to a glass-bottom confocal dish. Oocytes were analysed with a Fluoview FV10i confocal microscope (Olympus) using the following filters: MCB 358 nM excitation and 461 nM emission; MitoTracker™ Deep Red FM 644 nM excitation and 665 nm emission. The magnification, laser intensity and image capturing parameters were set and maintained for all replicates. Mean fluorescence intensity in each oocyte was determined with ImageJ software (Version 1.51h; National Institute of Health, Bethesda, MD, USA). Additionally, three patterns of mitochondrial distribution were observed: peripheral (mitochondria beneath the plasma membrane); homogenous (disperse mitochondria throughout the cytoplasm); and semi-peripheral (disperse mitochondria throughout the cytoplasm with less intensity in the center).

2.4. Assessment of transzonal projections

Transzonal projections (TZPs) were assessed by fluorescein isothiocyanate (FITC) conjugated phalloidin, which stains actin filaments (F-actin) using an adapted protocol from Liu et al. (2010). Briefly, COCs were partially denuded, then fixed in cold 4% (v/v) paraformaldehyde for 20 min. At RT, COCs were then permeabilized in 0.25% Triton X-100 in PBS with 4 mg/mL BSA for 30 min and stained with 5 µg/mL phalloidin-FITC solution in PBS with 4 mg/mL BSA for 60 min. Three washes with PBS-BSA were performed between each step. COCs were mounted with fluorescence mounting medium (Agilent, Santa Clara, CA, USA) on coverslips with a reinforcement ring and kept at -20ºC until analysis. TZPs fluorescence signals were examined with a Fluoview FV10i confocal microscope with 495 nm excitation and

513 nm emission. Images were processed with ImageJ software. As described by Romero et al. (2016), TZPs were observed as continuous filaments between the oocyte and cumulus cells. TZP density was determined by measuring the mean pixel intensity within the zona pellucida, delimited by the polygon selection tool (Fig. 1).

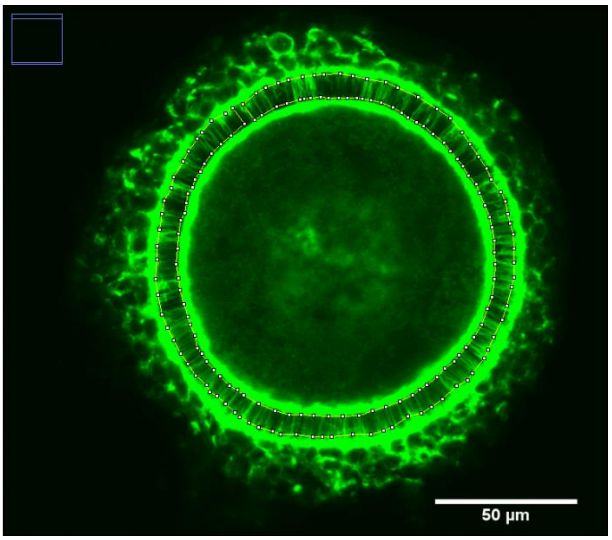

Fig. 1. Assessment of the transzonal projections density in a cumulus-oocyte complex with ImageJ software version 1.51h. The zona pellucida area between the oocyte and cumulus cells was delimited with the polygon selection tool. Mean average pixel intensity of the delimited region was calculated.

2.5. In vitro embryo production

Procedures for *in vitro* fertilization (IVF) and in vitro embryo culture (IVC) were adapted from Hussein et al. (2006). Briefly, after IVM COCs were washed in Wash medium (IVF Vet Solutions) and co-cultured with 1 x 10^6 sperm/mL in 500 µL VitroFert (IVF Vet Solutions) supplemented with 4 mg/mL BSA, 10 IU/mL Heparin (DBL, Hospira, Australia), 12.5 µM hypotaurine, 25 µM penicillamine and 1.25 µM epinephrine at 38.5ºC in 6% CO_2 and a humidified air. Frozen sperm from a single bull of proven fertility was thawed at 30-35ºC and selected with Bovipure density gradient (NidaCon International AB, Mölndal, Sweden) with a 25-min centrifugation at 300 X g at RT. At 24 h post-IVF, presumptive zygotes were washed and denuded by gently pipetting in wash medium. They were cultured in VitroCleave medium (IVF Vet Solutions) supplemented with 4 mg/mL BSA, in 20-µl drops (5 zygotes/drop) overlaid with paraffin oil at 38.5ºC with humidified 7% O_2, 6% CO_2, balance N_2. At 5 days post-IVF, embryos were transferred into 20-µL drops of VitroBlast (IVF Vet Solutions) supplemented with 4 mg/mL BSA and under the same culture conditions. Cleavage and blastocyst rate were recorded at 8 days post-IVF. Blastocysts were directly fixed in ethanol with 25 µg/mL Hoechst

33342 (Molecular Probes, Eugene, OR, USA) and kept at 4 ºC overnight. Stained blastocysts were mounted on a slide with a drop of glycerol and observed under an epifluorescence microscope (Olympus BX51; excitation: 340-380 nm; emission = 440-480 nm) to count the blastocyst cell number.

2.6. Experimental design

Experiment 1: Effects of CNP on oocyte meiotic arrest

To test the effect of CNP on the maintenance of the oocyte meiotic arrest, recovered COCs were cultured for 24 h in a pre-IVM medium supplemented with CNP at different concentrations. The experimental groups were: 0 (Control), 50, 100 and 200 nM CNP. Oocyte meiotic stage was assessed at 6 and 24 h. Between 34-39 oocytes were evaluated per treatment and time point over four replicates.

Experiment 2: Effects of CNP combined with IBMX on oocyte meiotic arrest

To evaluate if the combination of CNP and IBMX further delayed oocyte meiotic arrest, aspirated COCs were cultured for 24 h in a pre-IVM medium supplemented with 100 nM CNP, 500 µM IBMX, or a combination of both. Four experimental groups were tested: Control, CNP, IBMX and CNP + IBMX. Oocyte meiotic stage was assessed at 6 and 24 h. Between 36-41 oocytes were evaluated per treatment and time point over four replicates.

Experiment 3: Effects of Pre-IVM with CNP and IBMX on oocyte embryo developmental competence

In order to evaluate if a pre-IVM with a combination of CNP and IBMX yielded more developmental competent oocytes, COCs were in vitro matured, fertilized and embryos cultured. Pre-IVM medium contained either 100 nM CNP, 500 µM IBMX or a combination of both. Between pre-IVM and IVM, COCs were washed 5 times in IVM medium to remove any residual CNP or IBMX. The pre-IVM system (6 h of pre-IVM followed by 20 h of IVM) was compared to a conventional IVM of 24 h. The four experimental groups comprised of Control (24 h IVM), CNP pre-IVM, IBMX pre-IVM, and CNP + IBMX pre-IVM. Blastocyst yield was recorded at 8 days post-IVF and blastocysts were stained with Hoechst 33343. Between 183 and 192 COCs were cultured per treatment over five replicates and 60-68 blastocysts were stained per treatment group.

185 *Experiment 4: Effect of Pre-IVM with CNP plus IBMX on Glutathione level and*
186 *mitochondrial activity*

187 To determine if the pre-IVM treatments altered the oocyte antioxidant defense and energy
188 metabolism, COCs were denuded and stained with MCB and MitoTracker™ Deep Red FM.
189 COCs from two treatment groups were assessed: Pre-IVM (6-h pre-IVM with 100 nM CNP +
190 500 µM IBMX, followed by 20 h IVM) and Control (20h IVM). A total of 45 oocytes per
191 treatment were evaluated in three replicates.

192 *Experiment 5: Effect of Pre-IVM with CNP plus IBMX on the cumulus-oocyte connections*

193 To determine if the pre-IVM maintained TZPs between cumulus cells and oocytes after IVM,
194 COCs were stained with phalloidin-FITC at different time points after pre-IVM and IVM and the
195 TZPs integrity was observed with confocal microscopy. A total of 5 groups of COCs were
196 analysed: 0h control (immature COCs after aspiration), 6 h Pre-IVM, 6 h IVM, 20 h IVM, 6h Pre-
197 IVM + 20 h IVM. 45 COCs were evaluated per group in four replicates.

198 *2.7. Statistical analysis*

199 Data were analyzed by two-way ANOVA followed by Tukey's multiple-comparison post-hoc
200 test. Treatment was specified as the fixed factor and replicate as the random variable. Prior to
201 ANOVA, data which were not normally distributed (blastocyst rate and mitochondrial
202 distribution) were square root arcsine transformed, and normality and homogeneity of
203 variance were reassessed (and confirmed). The statistical analyses were performed with
204 SAS/STAT® software v 9.4 (SAS institute Inc., Cary, NC, USA). Results were considered
205 statistically significant when $P < 0.05$.

206 **3. Results**

207 *3.1. CNP maintains meiotic arrest for up to 6 h and the combination with IBMX increases*
208 *the efficiency of arrest (experiment 1 and 2)*

209 In experiment 1 we examined the effect of 6 and 24 h of pre-IVM treatment with different
210 CNP concentrations (50, 100 and 200 nM) on oocyte meiotic progression (Fig. 2). Both 100 and
211 200 nM of CNP were able to maintain the oocyte in GV stage at 6 h compared to control group
212 without CNP ($P < 0.05$), but no differences were observed at 24 h. In experiment 2 we
213 combined CNP (100 nM) with IBMX (500 µM) to assess if IBMX could enhance the effect of
214 CNP on the oocyte nuclear stage (Fig. 3). The combination of IBMX and CNP significantly
215 maintained meiotic arrest up to 6 h with a higher rate than CNP ($P < 0.01$), but no differences
216 were observed at 24 h.

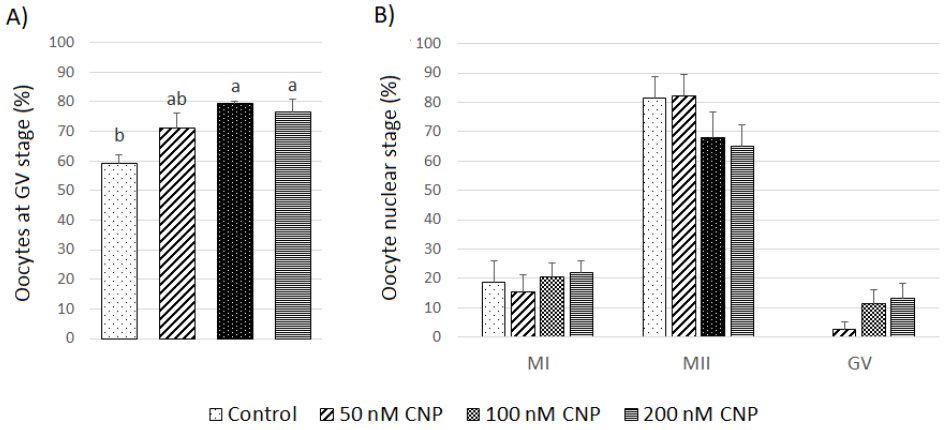

Fig. 2. Effect of CNP on the progression of nuclear maturation in bovine oocytes matured *in vitro* for 6 h (A) and 24 h (B) with 0 (Control), 50, 100 or 200 nM CNP. Oocyte nuclear maturation was assessed with DAPI and classified as: Germinal vesicle (GV), Metaphase (MI) and Metaphase II (MII). Each bar represents mean + s.e.m. Four replicates were performed with at least 33 oocytes assessed per treatment and time point. Different superscript letters (a-c) in each column represent statistically significant differences ($P < 0.05$).

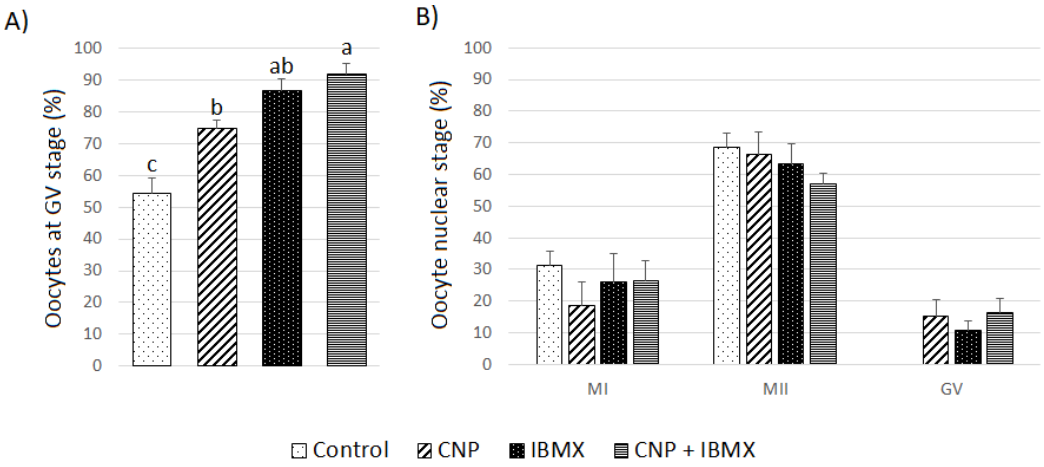

Fig. 3. Effect of CNP and IBMX on the progression of nuclear maturation in bovine oocytes *in vitro* cultured for 6 h (A) and 24 h (B) with 0 (Control), 100 nM CNP, 500 µM IBMX or 100 nM CNP + 500 µM IBMX. Oocyte nuclear maturation was assessed with DAPI and classified as: Germinal Vesicle (GV), Metaphase (MI) and Metaphase II (MII). Each bar represents mean + s.e.m. Four replicates were performed with at least 33 oocytes assessed per treatment and time point. Different superscript letters (a-c) in each column represent statistically significant differences ($P < 0.05$).

3.2. Pre-IVM with CNP and IBMX during 6 h followed by a conventional IVM improves embryo development (experiment 3)

We assessed the embryo development at 8 days post-fertilization after 6 h of pre-IVM followed by a 20-h IVM, compared to control 24-h IVM (Fig. 4). Pre-IVM with CNP plus IBMX

significantly increased the blastocyst rate compared to control group ($P < 0.05$). Pre-IVM with IBMX increased the cleavage rate compared to control group ($P < 0.05$) but had no effect on blastocyst rate. Pre-IVM with CNP did not have an effect on cleavage and embryo development rates. No differences were observed in blastocyst total cell number in any experimental group.

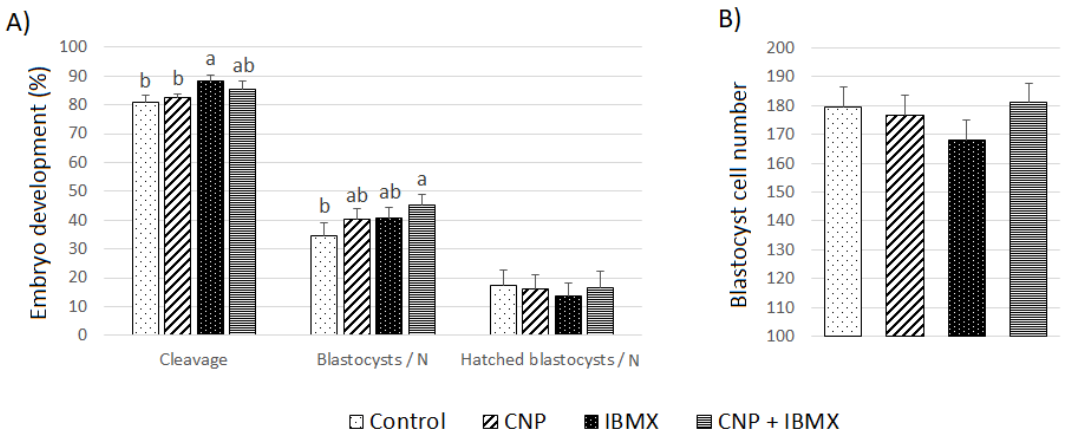

Fig. 4. Effect of 6 h pre-IVM with CNP and IBMX followed by standard IVM on bovine embryo yield (A) and total cell number (B) at 8 days post-fertilization. COCs were cultured in a Pre-IVM medium for 6 h supplemented with 100 nM CNP, 500 µM IBMX or 100 nM CNP + 500 µM IBMX, followed by 20 h IVM. A group of COCs were IVM for 24 h without previous pre-IVM (Control). Five replicates were performed with at least 183 oocytes cultured and 60 blastocysts assessed for cell number per treatment. N = nº of immature oocytes. Each bar represents mean + s.e.m. Different superscript letters (a-c) in each column represent statistically significant differences ($P < 0.05$).

3.4. Pre-IVM with CNP and IBMX improves mitochondrial activity and does not affect GSH levels of matured oocytes (experiment 4)

Oocyte GSH levels (MCB staining) and mitochondrial activity (MitoTracker™ deep red FM staining) were assessed after IVM (Fig. 5). Pre-IVM with CNP plus IBMX during 6 h followed by 20 h of IVM showed significantly higher mitochondrial activity compared to 20-h control IVM ($P < 0.001$), but did not have an effect on the GSH content. No differences were observed in the mitochondrial distribution patterns: control group showed 9.4% peripheral, 59.3% semi-peripheral and 31.3% homogenous distribution; pre-IVM group showed 5.9% peripheral, 47.7% semi-peripheral and 46.4% homogenous distribution.

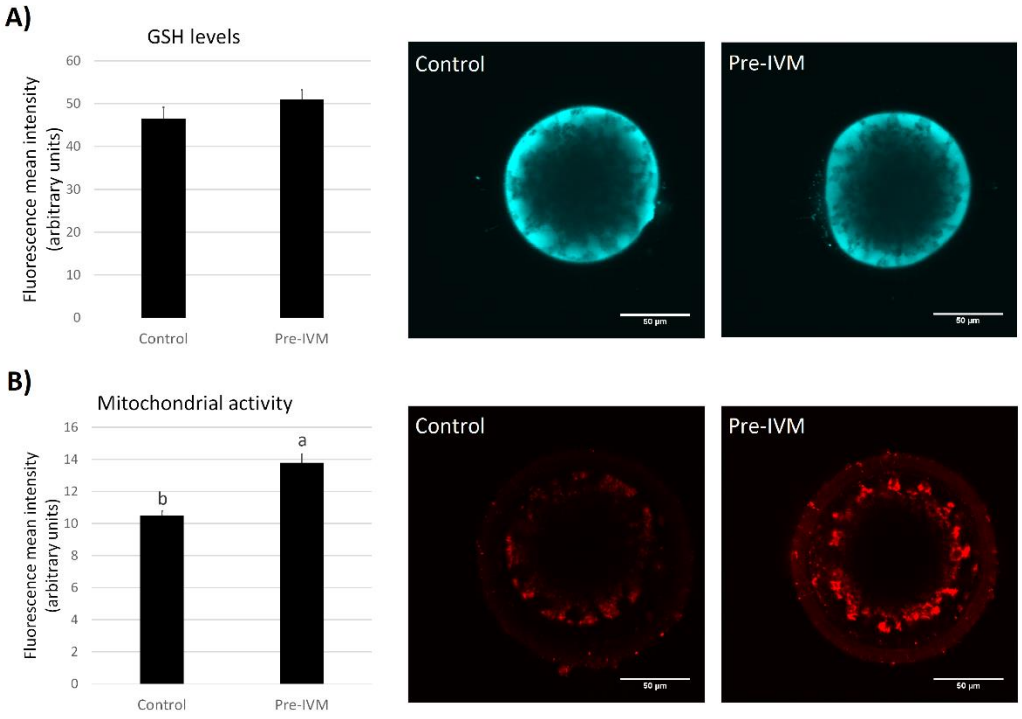

Fig. 5. Effect of 6 h pre-IVM with CNP plus IBMX on GSH levels (A) and mitochondrial activity (B) of bovine oocytes. COCs were either cultured for 6 h in a pre-IVM with 100 nM CNP plus 500 µM IBMX followed by 20 h IVM (Pre-IVM group), or culture for 20 h (Control). 45 oocytes per group were stained with MCB and MitoTracker™ deep red FM in 3 replicates and the oocyte average pixel intensity was quantified with Image J. Each bar represents mean + s.e.m. Different superscript letters (a-b) in each column represent statistically significant differences ($P < 0.0001$).

3.5. Pre-IVM with CNP and IBMX maintains transzonal projections of matured oocytes (experiment 5)

TZP density of COCs was evaluated with Phalloidin-FITC staining after follicular recovery (Control 0h), 6 h of IVM, 6 h of pre-IVM, 20 h of IVM, and 6 h of pre-IVM + 20h of IVM (Fig. 6). There was a significant increase in the density of TZPs after 6 h pre-IVM and 6 h IVM, compared to Control 0h group ($P < 0.05$). There was also a decrease after 20 h of IVM and 6 h of pre-IVM + 20 h of IVM, compared to 6 h of pre-IVM and 6 h of IVM ($P < 0.05$). The pre-IVM significantly maintained the density of TZPs after 20 h of IVM compared to control 20-h IVM ($P < 0.05$).

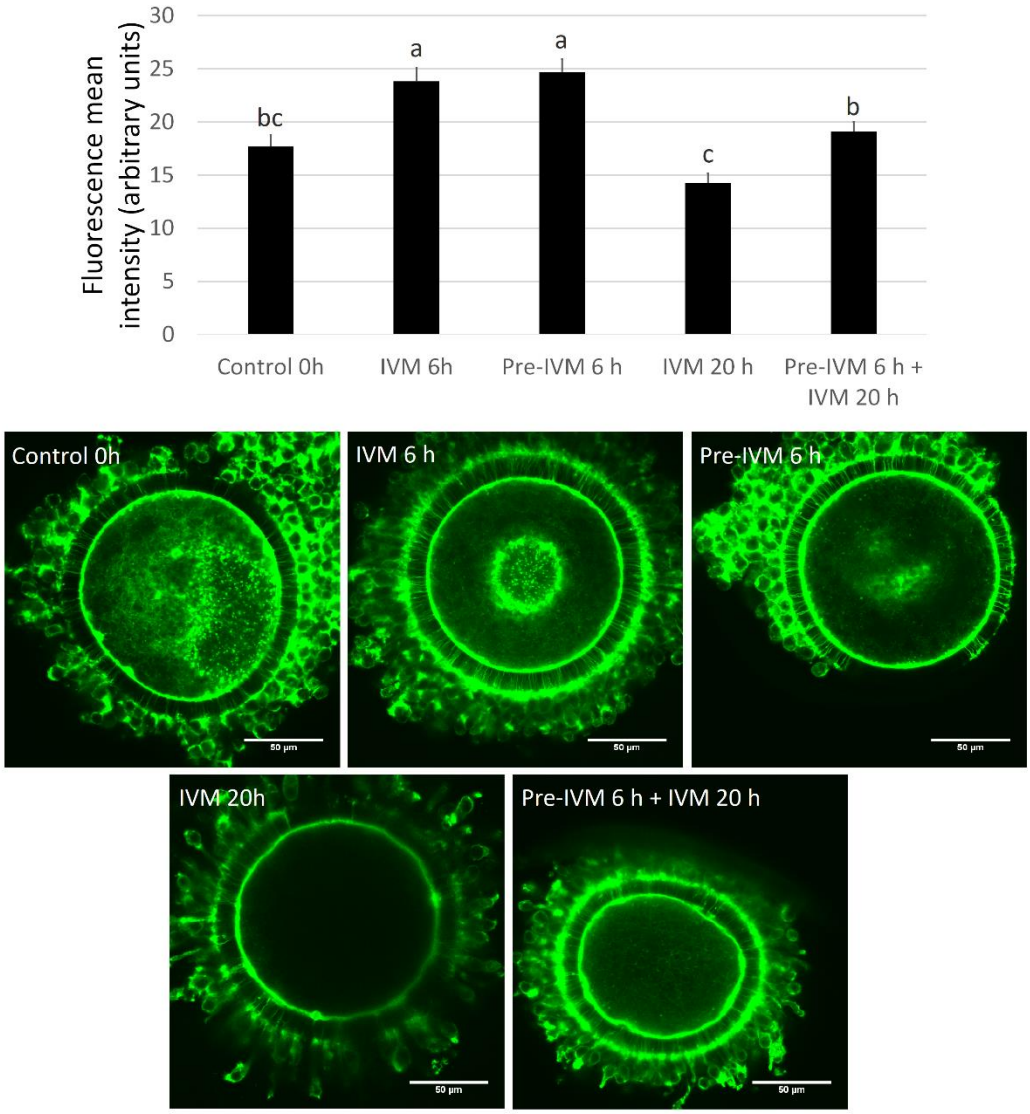

Fig. 6. Effect of 6 h pre-IVM with CNP plus IBMX on transzonal projections of bovine COCs. COCs were stained with Phalloidin-FITC after recovery (Control 0 h), 6 h of conventional IVM, 6 h of pre-IVM with 100 nM CNP + 500 µM IBMX, 20 h of IVM, and 6 h of pre-IVM followed by 20 h IVM. Average pixel intensity in the region between the oocyte and cumulus cells was quantified with ImageJ. 45 COCs per group were assessed in 4 replicates. Each bar represents mean + s.e.m. Different superscript letters (a-c) in each column represent statistically significant differences ($P < 0.05$).

4. Discussion

In the present study we investigated the effect of CNP with and without IBMX on the oocyte meiotic maturation. We hypothesized that a combination of both cAMP modulators in a pre-IVM phase would maintain germinal vesicle stage for a longer time than when used individually. We aimed to improve bovine oocyte developmental competence by applying this biphasic IVM protocol to IVEP.

A combination of CNP and IBMX was able to maintain meiotic arrest for 6 h in more than 90% of the oocytes, a higher rate than when either of the meiotic inhibitors were tested individually. Previous studies with bovine COCs have already shown that CNP (Xi et al., 2018; T. Zhang et al., 2017) and its precursor (NPPC) (Franciosi et al., 2014) can maintain GV stage for 6-8 h, and IBMX together with forskolin (an adenylate cyclase activator) for 9 h (Albuz et al., 2010). Oocyte meiotic arrest is maintained by high intra-oocyte cAMP levels (Cho et al., 2018). While IBMX is a broad spectrum PDE inhibitor which prevents cAMP hydrolysis , CNP stimulates the synthesis of cGMP which antagonizes PDE activity (Gilchrist et al., 2016). Our results suggest a synergy between both meiotic inhibitors. However, we were not able to prolong these effects for 24 h, whereas in other species, CNP could arrest meiosis for at least 24 h in mouse (Romero et al., 2016) and human (Sánchez et al., 2017) COCs. These differences between species could be explained by the higher PDE8 activity (60%) in bovine CC compared to a predominant PDE4 activity in mouse CC; IBMX does not inhibit PDE8 (Sasseville et al., 2009). Moreover, PDE8 has 100-fold higher affinity for cAMP than PDE4 (Bender, 2006), hence the higher cGMP levels induced by CNP could be more efficient at inhibiting PDE4 than PDE8.

Based on the above results, a biphasic IVM was tested in which a 6-h pre-IVM was followed by a 20-h IVM. The shorter IVM period, compared to the conventional 24 h, was chosen to prevent oocyte aging due to the increase of the total time of culture, and to design a more practical protocol that would adapt to laboratory working hours and could be translate to breeding programs. The pre-IVM with CNP and IBMX improved oocyte developmental competence observed as higher blastocyst rate, although it did not have an effect on blastocyst cell number. These results are in agreement with previous studies in which the meiotic arrest induced by pre-IVM with CNP or PDE inhibitors improved blastocyst development in cattle (Albuz et al., 2010; Li et al., 2016; Sugimura et al., 2018; Xi et al., 2018; T. Zhang et al., 2017). However, in the present study CNP individually did not improve blastocyst rate compared to control IVM. The disparities between studies could be related to different maturation periods. For instance, Xi et al. (2018) found that the pre-IVM with CNP only enhanced blastocyst rate when it was followed by a longer IVM (26 h).

Cumulus-oocyte communication is essential for oocyte maturation (Russell et al., 2016). Some studies have shown that cAMP modulators such as CNP and IBMX prolong cumulus-oocyte GJC, which otherwise rapidly decreases during meiotic maturation (Albuz et al., 2010; Franciosi et al., 2014; Li et al., 2016; Luciano et al., 2011). Open GJs allow the bidirectional transfer of important maturation-related molecules between cumulus cells and the oocyte, which has a positive effect on oocyte GSH levels (Li et al., 2016), oocyte chromatin remodelling

and transcription (Franciosi et al., 2014; Luciano et al., 2011), and oocyte metabolism (Zeng et al., 2014). In the present study we did not observe any change to oocyte GSH levels after IVM. Yet mitochondrial activity was enhanced, which has been related to higher embryo developmental competence (Ge et al., 2012). Other biphasic IVM protocols in bovine oocytes have shown either a higher mitochondrial activity (Huang et al., 2016) or an increase in mtDNA copy number (Xi et al., 2018; T. Zhang et al., 2017). As reviewed by Van Blerkom (2011), mitochondrial ATP synthesis is essential for oocyte maturation and early embryo development. Nevertheless, discussion of differences in mitochondrial activity results should be conducted cautiously, because mitochondria are also responsible for ROS production and triggering apoptosis (reviewed by Dumollard et al., 2007). Simultaneous quantification of intra-oocyte GSH, ROS levels and mitochondrial activity may provide a more complete picture (McDowall et al., 2015). Nevertheless, in the present study mitochondrial activity was considered a marker of improved oocyte quality, as it was associated with improved embryo development in the pre-IVM group.

Communication between cumulus and oocyte is partly mediated by TZPs; actin filaments that connect CC cells to the oolemma by traversing the zona pellucida (Macaulay et al., 2014). Prior to maturation, they connect with GJs (Hyttel et al., 1997). Macaulay et al. (2014) reported that in bovine COCs, TZPs were already withdrawing from the oolemma at 9 h of IVM and were completely separated by 22 h. However, in the present study we observed an increase after 6 h of culture in either pre-IVM or IVM medium and some still remained, although at a lower density, after 20 h of conventional IVM. It has been reported that pre-IVM with CNP can maintain TZPs in mice (Romero et al., 2016) and human COCs (Sánchez et al., 2017). In our experiment COCs which have undergone a 6-h pre-IVM presented higher density of TZPs after 20 h IVM compared to COCs after 20 h of IVM alone. This could have relevance to oocyte competence, as TZPs allow the transfer of mRNA and metabolic molecules essential for oocyte maturation (Macaulay et al., 2016, 2014).

5. Conclusion

In conclusion, we have observed that a combination of CNP and IBMX can efficiently maintain meiotic arrest for 6 h in bovine oocytes. We have designed a biphasic IVM protocol consisting in 6 h of pre-IVM with CNP plus IBMX followed by 20 h of IVM. This two-step maturation system improved the cumulus-oocyte communication by TZPs which led to enhanced oocyte developmental competence. The present study adds evidence to the benefits of biphasic IVM on IVEP in livestock species, compared to conventional IVM system. Biphasic IVM research in the cow also has relevance for human assisted reproductive technology,

because improving human IVM efficiency would enable the reduction in use of hormonal ovarian stimulation and its side effects.

Conflicts of interest

J. G. Thompson is the Founder of a company, ART Lab Solutions Pty Ltd, which manufactures bovine IVF media. All other authors declare they have no conflicts of interest whatsoever.

Acknowledgements

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Chapter 7

Biphasic in vitro maturation with C-type natriuretic peptide and estradiol enhances embryo developmental competence of juvenile-goat oocytes

Biphasic in vitro maturation with C-type natriuretic peptide and estradiol enhances embryo developmental competence of juvenile-goat oocytes

Sandra Soto-Heras[a], Irene Menéndez-Blanco[a], Maria-Gracia Catalá[a], Dolors Izquierdo[a], Jeremy Thompson[bcd], Maria-Teresa Paramio[a]*

[a]Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

[b]Robinson Research Institute, School of Paedriatics and Reproductive Health, The University of Adelaide, Adelaide, South Australia, 5005, Australia.

[c]Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, 5005, Australia

[d]Australian Research Council Centre of Excellence for Nanoscale BioPhotonics and Institute for Photonics and Advanced Sensing, The University of Adelaide, Adelaide, South Australia, 5005, Australia.

*Corresponding author: Edifici V, Campus UAB, 08193 Bellaterra (Cerdanyola del Vallès) Barcelona, Spain. Tel. number: +34 93 581 1456. E-mail address: teresa.paramio@uab.cat

Abstract

In vitro embryo production success in juvenile animals is compromised due to inherently lower oocyte quality. Conventional *in vitro* maturation (IVM) can impair oocyte competence by inducing spontaneous meiotic resumption. A series of experiments were performed to determine if maintaining meiotic arrest during a pre-IVM phase improves juvenile-goat oocyte competence. In experiment 1, COCs were cultured with C-type natriuretic peptide (CNP; 0, 50, 100, 200 nM) for 6 and 8 h. Nuclear stage and chromatin configuration were assessed, but no differences were observed. In experiment 2, same CNP concentrations were tested plus 10 nM estradiol (known promoter of CNP receptor; NPR2). CNP (200 nM) plus estradiol maintained germinal vesicle (GV) rate for 6 h (74.7% vs. 28.3% in control, $P < 0.05$) with predominant condense-clumped chromatin configuration. In experiment 3, relative mRNA quantification showed *NPR2* expression was down-regulated after 6 h of pre-IVM culture. In experiment 4, analysis of transzonal projections indicated that pre-IVM maintained cumulus-oocyte communication after oocyte recovery from the follicle. For experiments 5 and 6, biphasic IVM (6 h pre-IVM with CNP plus estradiol, followed by 24 h IVM) and control IVM (24 h) were compared. Biphasic IVM enhanced oocyte antioxidant defenses (higher GSH and lower ROS levels), up-regulated *DNA-methyltransferase 1* and *pentraxin 3* expression, and produced higher blastocyst rate than control IVM (30.2% vs. 17.2%, $P < 0.05$). In conclusion, we have

developed a biphasic IVM system including a pre-IVM phase with CNP plus estradiol that maintains oocyte meiotic arrest for 6 h and enhances the developmental competence of juvenile-goat oocytes.

Keywords: oocyte competence, biphasic IVM, CNP, estradiol, meiosis, cumulus-oocyte communication

Introduction

Juvenile *in vitro* embryo transfer (JIVET) has great potential for improving breeding programs as it can increase the genetic gain rate by reducing the generation interval (Morton 2008), and ovaries from juvenile females provide higher number of oocytes than adults (Koeman *et al.* 2003). However oocytes come from small follicles (< 3 mm) with a heterogeneous grade of development and quality leading to lower blastocyst rates compared to adults (reviewed by Paramio & Izquierdo 2014).

Oocyte *in vitro* maturation (IVM) is a limiting step for *in vitro* embryo production (IVEP). Conventional IVM can impair oocyte competence, which is the ability to sustain embryo development and lead a pregnancy to term (Sirard *et al.* 2006). Competence acquisition depends on changes at nuclear and cytoplasmic levels that occur during folliculogenesis prior to final oocyte maturation (reviewed by Gilchrist & Thompson 2007). But oocytes spontaneously resume meiosis *in vitro* after being retrieved from the follicles (Edwards 1965) which prevent the oocyte to fulfill this process.

High intra-oocyte cyclic AMP levels sustain meiotic arrest (Cho *et al.* 2018) by preventing the activation of the maturation-promoting factor (Gilchrist *et al.* 2016). The follicular environment maintains high cAMP via C-type natriuretic peptide (CNP) and its receptor (NPR2). CNP increases cyclic GMP levels in cumulus cells and oocytes (Zhang *et al.* 2010; Xi *et al.* 2018) which inhibits phosphodiesterase 3A (PDE3A), the main cAMP hydrolysing enzyme (Norris *et al.* 2009; Vaccari *et al.* 2009). After COC liberation from the follicle, PDE3A is released from cGMP inhibition and there is a rapid cAMP decrease (Luciano *et al.* 2004).

Novel cAMP-mediated IVM systems can better simulate physiological oocyte capacitation and maturation (reviewed by Gilchrist *et al.* 2016). One such system is biphasic IVM that consists in a pre-IVM phase using a PDE inhibitor, which maintains meiotic arrest by preventing cAMP degradation, followed by standard IVM. Recently, CNP has been tested for pre-IVM in different animal species and has succeeded at maintaining meiotic arrest and improving embryo development (Franciosi *et al.* 2014; Zhang *et al.* 2015a, b, 2017a, 2018; Xi *et al.* 2018).

Interestingly in juvenile mice, IVM-oocytes developed to blastocyst stage at comparable rate to *in vitro* fertilization (IVF) of ovulated oocytes (Romero *et al.* 2016), showing that pre-IVM with CNP can overcome IVEP limitations even in oocytes from juvenile females. As reviewed by Luciano *et al.* (2018) cAMP-mediated IVM could be especially beneficial for oocytes with lower inherent developmental competence.

The success of cAMP-modulated IVM has been related to the maintenance of gap junction communication (GJC) between cumulus cells (CC) and oocytes (Albuz *et al.* 2010; Li *et al.* 2016). Moreover, maintaining high cAMP levels increases intra-oocyte glutathione (GSH) levels (Zeng *et al.* 2014; Li *et al.* 2016). This could further benefit juvenile oocytes which are more sensitive to reactive oxygen species (ROS) due to lower GSH synthesis (Jiao *et al.* 2013). Mitochondrial number and activity are also enhanced (Zeng *et al.* 2014; Xi *et al.* 2018), which are related to oocyte competence (Huang *et al.* 2016; Lamas-Toranzo *et al.* 2018a). Lastly, cAMP-modulated IVM up-regulates genes involved in cell communication and metabolism, steroidogenesis and formation of extracellular matrix, in cumulus cells (Khan *et al.* 2015). Biphasic IVM could also promote other pathways related to competence acquisition hence it would be interesting to study other genes such as *DNA methyltransferase 1* (Uysal & Ozturk 2017), *growth-differentiation factor 9* and *bone morphogenetic protein 15* (Gilchrist *et al.* 2008), *follicle stimulating hormone receptor* (Ferreira *et al.* 2009), *pentraxin 3* and *TNF alpha induced protein 6* (Brown *et al.* 2013).

Considering above mentioned results, we hypothesized that biphasic IVM could improve IVEP in juvenile-goat oocytes. The aim of this study was to design a pre-IVM using CNP that sustains meiotic arrest and CC-oocyte communication, providing the oocyte with additional embryo developmental competence.

Material and methods

Unless indicated all chemicals were purchased from Sigma-Aldrich® Chemical Co (St. Louis, USA).

Oocyte recovery

Ovaries from juvenile goats (1 to 2 months old) were obtained at a local slaughterhouse and maintained at 35-37ºC in phosphate buffered saline (PBS). Cumulus oocyte complexes (COCs) were recovered by slicing of the ovary surface in TCM-199 with HEPES and supplemented with 2.2 mg/mL $NaHCO_3$, 50 mg/mL gentamycin and 11.1 mg/mL heparin. In pre-IVM experimental groups the slicing medium was also supplemented with 500 µM 3-Isobutyl-1-methylxanthine (IBMX; a non-specific PDE inhibitor) to avoid meiotic resumption during oocyte recovery, but

not in IVM control groups. Oocytes with at least two layers of compact cumulus cells and homogeneous cytoplasm were selected.

Oocyte *in vitro* maturation

Oocytes were cultured in pre-IVM, pre-IVM plus IVM (biphasic IVM) or IVM depending on the experiment.

Pre-IVM

COCs were cultured in 100-µL drops of pre-IVM medium covered with mineral oil for 6 or 8 h at 38.5ºC in humidified air with 5% CO_2. Prior to culture, COCs were washed five times in the same medium to eliminate residual IBMX from the slicing medium. Basic pre-IVM medium was TCM-199 with 4 mg/mL bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1 mM glutamine, 100 µM cysteamine and 5 µg/mL gentamycin. The basic medium was supplemented with CNP (0, 50, 100, 200 nM) and 10 nM 17β-estradiol (E2), depending on the experimental group.

IVM

COCs were washed five times and cultured in IVM medium (adapted from Catalá *et al.* 2011): TCM-199 supplemented with 5 µg/mL LH, 5 µg/mL FSH, 1 µg/mL E2, 10 ng/mL epidermal growth factor (EGF), 0.2 mM sodium pyruvate, 1 mM glutamine, 10% (v/v) fetal bovine serum (FBS) and 5 µg/mL gentamycin. COCs were cultured in 100-µL drops covered with mineral oil for 24 h at 38.5ºC in humidified air with 5% CO_2.

Assessment of oocyte nuclear stage

For evaluating nuclear maturation oocytes were stained with orcein adapting Prentice-Biensch *et al.* (2012) protocol. Briefly, oocytes were denuded and fixed in ethanol:acetic (3:1) overnight at 4ºC. Oocytes were mounted in a slide, covered with a wax supported coverslip and stained with 1% orcein (w/v) in 45% acetic acid solution (v/v). Nuclear stage was assessed with a phase-contrast microscope (Olympus BX50) and classified as (figure 1): germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII). GVs were also classified according to chromatin configuration and nucleolus size, as described by Sui *et al.* (2005) (figure 1). For posterior analysis classification was simplified to: GV1, GV net-like (GV2n + GV3n), GV clumped (GV2c + GV3c + GV4).

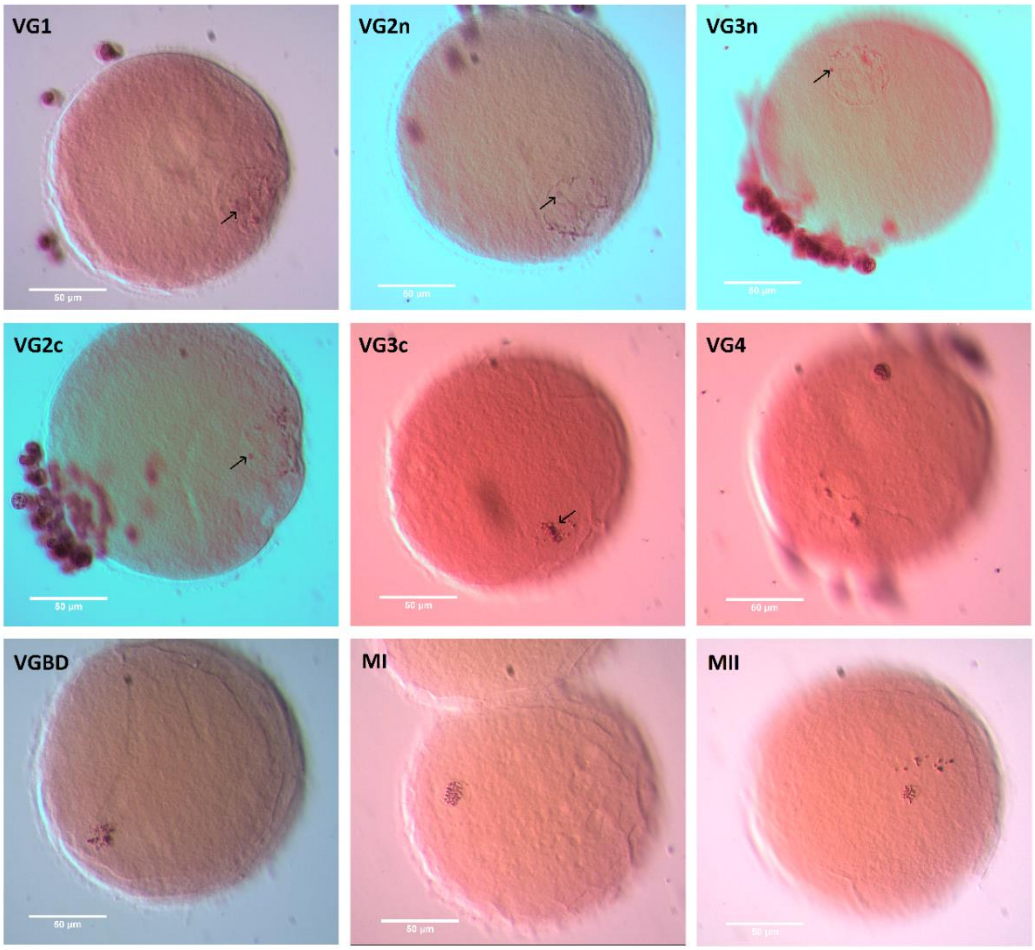

Figure 1. Nuclear stage and GV classification of juvenile-goat oocytes stained with orcein and evaluated by phase-contrast microscopy. GVBD: germinal vesicle breakdown, broken nuclear membrane and chromatin condensing at prophase I. MI: metaphase I, diploid chromosomes aligned at the meiotic spindle. MII: metaphase II, haploid chromosomes aligned at the meiotic spindle and an extruded polar body. GV: germinal vesicle, intact nuclear membrane. GV1: large nucleolus and diffuse filamentous chromatin. GV2n: medium-size nucleolus and condense net-like chromatin. GV2c: medium-size nucleolus and condense clumped chromatin. GV3n: small nucleolus and condense net-like chromatin. GV3c: small nucleolus and condense clumped chromatin. GV4: no nucleolus and condense clumped chromatin. Arrows point the nucleolus.

Assessment of transzonal projections

Transzonal projections (TZPs) were evaluated by staining with fluorescein isothiocyanate labeled phalloidin (phalloidin-FITC), which binds to actin filaments, adapting Liu *et al.* (2010) protocol. COCs were partially denuded and fixed in 4% paraformaldehyde (PF; w/v) for 20 min at 38ºC. COCs were permeabilized with 0.25% triton X-100 in PBS (v/v) for 30 min and incubated with 5 µg/mL phalloidin-FITC in 0.4% BSA-PBS (w/v) for 60 min at room temperature (RT). COCs were then counterstained with 1 µg/mL Hoechst 33258 (Invitrogen, Eugene, OR,

USA) for 10 min. COCs were mounted in a poly-L-lysine-treated coverslip with a drop of Vectashield® mounting medium (Vector laboratories, Burlingame, CA, USA) and a reinforcement ring, sealed and kept at -20ºC until analysis with confocal laser microscopy (Spectral Leica TCSSP5, Mannheim, Germany). Images were taken with 63 x magnification under mineral oil (laser excitation: 488 for TZPs; 405 nm for chromatin) and analysed with ImageJ software (Version 1.51h; National Institute of Health, Bethesda MD, USA). As described by Romero *et al.* (2016), TZPs were seen as continuous filaments going from cumulus cells to the oocyte, and TZP density was quantified by measuring average pixel intensity in the zona area delimited by polygon selection tool.

Assessment of reactive oxygen species (ROS) and glutathione (GSH) levels

Oocyte ROS level was measured by staining with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes Inc., Eugene, OR, USA). Hydrogen peroxides oxidize H_2DCF-DA to its fluorescent form (2',7'-dichlorofluorescein, DCF), hence emitted fluorescence is directly related to ROS concentration in the cell. As previously described (Park *et al.* 2014), oocytes were denuded and incubated for min with 10 µM H_2DCF-DA in 0.4% BSA-PBS at 38.5ºC.

Oocyte GSH content was measured with monochlorobimane (MCB) probe with reacts with reduced thiols (-SH) emitting fluorescence (99% of light is related to GSH binding). Protocol was adapted from Keelan *et al.* (2001). Oocytes were denuded and incubated for 15 min with 12.5 µM MCB in 0.4% BSA-PBS at 38.5ºC.

After incubation with either H_2DCF-DA or MCB, oocytes were washed three times in 0.1% BSA-PBS, immediately transferred with a 10-µL drop to a slide and observed under Olympus BX50 epi-fluorescent microscope with 10 x magnification (excitation: 460 nm for ROS; 370 nm for GSH). Exposure time and gain were maintained for all images. Average fluorescence intensity per oocyte was measured with Image J software and normalized with the background average intensity.

RNA relative quantification

RNA relative quantification was performed for *DNMT1*, *GDF9*, *BMP15*, *NPR2*, *PTX3*, *TNFAIP6* and *FSHR* (primers listed in table 1). Groups of 10 COCs were washed in PBS with 0.3% polyvinylpyrrolidone (PVP, w/v), snap-frozen in buffer RLT (Qiagen RNeasy® mini kit; Qiagen, Ambion Inc., Austin TX, USA) and kept at -80ºC until analysis. RNA was extracted with Qiagen RNeasy® mini kit and eluded in 30 µL RNase-free water. RNA concentration was assessed with Qubit™ RNA HS assay kit (Thermo Fisher Scientific, Waltham MA, USA) and RNA integrity (RIN) with Agilent RNA 6000 pico chip on Agilent 2100 bioanalyzer (Agilent technologies, Waldbronn,

Germany). RNA concentration ranged from 7.11 to 29.7 ng/µL and RIN from 7.3 to 8.6. DNase treatment was performed prior to reverse transcription (RT) with Turbo DNA-free™ kit (Applied biosystems, Foster City CA, USA). RT was performed with High-Capacity cDNA Reverse Transcription Kit (Applied biosystems) in 30 µL reaction volume with 9 µL RNA, and a Bio rad T100 thermal cycler (Bio Rad Laboratories, Hercules CA, USA) set to 25ºC 10 min, 37ºC 120 min and 95ºC 5 min. For real-time quantitative PCR (RT-qPCR), SYBR® Select Master Mix (Applied biosystems) was used as fluorophore with 15 µL reaction volume. RT-qPCR was performed in Quant Studio™ 12K Flex Real-rime PCR system (Applied biosystems) and the plate was set up by a robotic distributor (Eppendorf epmotion 5075, Eppendorf, Hamburg, Germany). Thermo-cycling conditions consisted in: initial holding (50ºC 2 min) and denaturation (95ºC 10 min) steps, amplification stage (95ºC 15 s and 60ºC 1 min, repeated 40 cycles), and final melting curve (95ºC 15 s, 60ºC 1 min, 95ºC 15 s). Three replicates for each sample and primer were performed. Prior to final analysis, a standard curve was done for each gene to determine the PCR efficiency (80-110%). RNA was quantified with the $2^{-\Delta\Delta C}{}_{T}$ method described by Livak & Schmittgen (2001), which calculates the increase in cycle threshold (Ct) relative to reference genes (*RPL19* and *RPS9*) and reference group. The RT app on Thermo Fisher cloud was used for this calculation.

Table 1. Primer detailed information for each gene analyzed

Gene	Sequence (5'-3')	GenBank accession no.	Fragment size (bp)
RPL19	*Forward:* AGATTGACCGCCACATGTATCAC *Reverse:* TCCATGAGAATCCGCTTGTTTT	NC_030826.1	79
RPS9	*Forward:* ACAAACGTGAGGTCTGGAGGG *Reverse:* GGGTCTTTCTCATCCAGCGTC	NC_030825.1	88
DNMT1	*Forward:* GGTGAAAAGGCTCTTCTTGGC *Reverse:* AATAGTGGTGCGTACTCTGGGC	NC_022299.1	83
GDF9	*Forward:* TCTACAACACTGTTCGGCTCTTCA *Reverse:* CACAACAGTAACACGATCCAGGTT	NC_022299.1	122
BMP15	*Forward:* TCGGGTACTATACTATGGTCTCAATTC *Reverse:* GCCTCAATCAGAAGGATGCTAATGG	NW_017189516.1	141
NPR2	*Forward:* TCTGTACGCCGAAGTCCTGAA *Reverse:* CGTCCTTGCATCTTCTCGACA	NC_030815.1	87
PTX3	*Forward:* TGGACAACGAAATAGACAATGGAC *Reverse:* TCGGAGTTCTCACGACTGCA	NC_030808.1	76
TNFAIP6	*Forward:* GGAATCCGTCTCAATAGAAGTGAAA *Reverse:* TGTAAACACACCACCACACTCCTT	NC_030809.1	81
FSHR	*Forward:* GTTTTGAAAGTATGATTGTATGGCTGAG *Reverse:* GAGTTGGGTTCCATTGAATGC	NC_030818.1	80

Reference genes: *RPL19* (ribosomal protein L19), *RPS9* (ribosomal protein S9); Quantified genes: *DNMT1* (DNA methyltransferase 1), *GDF9* (growth-differentiation factor 9), *BMP15* (bone morphogenetic protein 15), *NPR2* (natriuretic peptide receptor 2), *PTX3* (pentraxin 3), *TNFAIP6* (TNF alpha induced protein 6), *FSHR* (follicle stimulating hormone receptor).

Quantification of mtDNA copy number

Mitochondrial DNA was quantified with qPCR as described by Lamas-Toranzo *et al.* (2018b), using primers GTTAAACGGCCGCGGTATTC (forward) and TCACCCCAACCAAAACTGCT (reverse) that amplify a 262 bp specific product from goat mitochondrial DNA (GenBank accession no: NC_005044.2). Oocytes were completely denuded and zona pellucida was removed with 0.5% protease from *Streptomyces griseus* in 0.3% PVP-PBS (w/v). Oocytes were individually placed in 0.2-ml tubes, snap frozen and stored at -80ºC until analysis. Oocytes were digested with 8 µl PicoPure™ DNA extraction kit (Applied Biosystems) by incubating at 65ºC for 1 h followed by inactivation at 95ºC for 10 min. A standard curve was done by cloning the specific goat mitochondrial product in the vector pMD20 (Takara, Kusatsu, Japan). Quantitative PCR was performed with Gotaq® qPCR Master Mix (Promega, Madison WI, USA) on a MIC quantitative thermo-cycler (Biomolecular Systems, Upper Coomera, Australia). Thermo-cycling conditions consisted in: initial denaturation step (95ºC 5 min), amplification step (94ºC 15 s, 56ºC 30 s, 72ºC 20 s, repeated 40 cycles), and a final melting curve. DNA was quantified following the comparative quantification cycle method as described by Bermejo-Álvarez *et al.* (2008). Briefly, Ct value was determined in the region of the amplification curve where increasing one cycle was equivalent to doubling the amplified PCR product. The ΔCt was normalized by subtracting from each Ct value the highest average Ct of the experimental groups, i.e. the average Ct of the group with the lowest mtDNA number. Fold changes were determined using the $2^{-\Delta\Delta C_T}$ formula.

In vitro embryo production

After IVM, COCs were co-cultured with 4 x 10^6 sperm/mL in 100-µL drops of BO-IVF medium (IVF Bioscience, Falmouth, United Kingdom) covered with mineral oil at 38.5ºC in humidified air with 5% CO_2. Frozen sperm from 2 bucks of proven fertility was thawed at 36ºC for 1 min and selected with BoviPure™ density gradient (Nidacon EVB S.L., Barcelona, Spain) by centrifuging for 20 min at 250 X g. After 20 h of IVF, presumptive zygotes were completely denuded and cultured in 10-µL drops of BO-IVC medium (IVF Bioscience) covered with Nidoil (Nidacon, Mölndal, Sweden) at 38.5ºC in humidified air with 5% CO_2 and 5% O_2. Cleavage was recorded at 48 h post-fertilization (hpf) and blastocyst rate at 8 days post-fertilization (dpf).

Assessment of blastocyst quality

Blastocyst quality was assessed by differential staining as described by Thouas *et al.* (2001). Blastocysts were incubated in TCM-199 with 1% Triton X-100 (v/v) and 100 µg/mL propidium iodide for 25 s, and transferred to pure ethanol with 25 µg/mL Hoechst 33258, where were

kept at 4ºC overnight. Blastocysts were mounted with a drop of glycerol and observed under Olympus BX50 epifluorescence microscope (370 nm excitation). Differential cell count was performed with Image J software: inner cell mass (ICM, blue) and trophectoderm (TE, red).

Experimental design

Experiment 1. Effect of pre-IVM with CNP on meiotic arrest

COCs were cultured in pre-IVM medium supplemented with 0 (control), 50, 100 or 200 nM CNP. Nuclear stage was evaluated with orcein staining after 6 and 8 h of culture. A total of 47-48 oocytes were assessed per treatment and time point (four replicates).

Experiment 2. Effect of pre-IVM with CNP and estradiol on meiotic arrest

COCs were cultured in pre-IVM medium supplemented with CNP and E2: 0 (control), E2 (10 nM), 50 nM CNP + E2, 100 nM CNP + E2, or 200 nM CNP + E2. Nuclear stage was evaluated with orcein staining after 6 and 8 h of culture. GV chromatin configuration was also recorded. A total of 46-50 oocytes were assessed per treatment and time point (four replicates).

Experiment 3. Effect of pre-IVM on the expression of natriuretic peptide receptor (NPR2)

COCs were cultured for 6 h in pre-IVM medium supplemented with: 200 nM CNP, or 200 nM CNP + 10 nM E2. An additional group was tested: uncultured COCs which were frozen after recovery from the ovary (control 0 h). Relative expression of *NPR2* was analyzed by RT-qPCR. A total of five samples (10 COCs/sample) were analyzed per group.

Experiment 4. Effect of pre-IVM with CNP and estradiol on cumulus-oocyte communication

CC-oocyte communication was assessed by staining TZPs with phalloidin-FITC. Biphasic IVM was compared to control IVM. For biphasic IVM, COCs were cultured for 6 h in pre-IVM medium with 200 nM CNP plus 10 nM E2, followed by 24 h IVM. For control IVM, oocytes were cultured for 24 h in IVM medium. COCs were fixed and stained at different time points: after oocyte recovery (Control 0 h), 6 h pre-IVM, 6 h IVM, 6 h pre-IVM + 24 h IVM, 24 h IVM. A total of 32-46 COCs were assessed per condition (five replicates).

Experiment 5. Effect of biphasic IVM (6 h pre-IVM plus 24 h IVM) on oocyte quality

Various parameters related to oocyte quality were assessed at the end of IVM. Two experimental groups were tested: biphasic IVM (6 h pre-IVM with 200 nM CNP plus 10 nM E2, followed by 24 h standard IVM) and control IVM (24 h). The following parameters were assessed: nuclear stage (46-47 oocytes per group, four replicates), GSH levels (30 oocytes,

three replicates), ROS levels (30 oocytes, three replicates), mtDNA copy number (30 oocyte, three replicates), and relative mRNA quantification of *DNMT1*, *GDF9*, *BMP15, PTX3*, *TNFAIP6* and *FSHR* (five samples, 10 COCs/sample). For mtDNA copy number and mRNA quantification and additional group was evaluated: uncultured COCs after follicle recovery (control 0 h).

Experiment 6. Effect of biphasic IVM (6 h pre-IVM plus 24 h IVM) on embryo development

In order to determine if biphasic IVM improves oocyte developmental competence, COCs were fertilized and embryo cultured after IVM. Two experimental groups were tested: biphasic IVM (6 h pre-IVM with 200 nM CNP plus 10 nM E2, followed by 24 h IVM) and control IVM (24 h). Cleavage and blastocyst rates were recorded at 48 hpf and 8 dpf, respectively. A total of 148-151 oocytes were cultured per group (four replicates). Expanded and hatched blastocysts were differentially stained for quality assessment (16-24 blastocysts per group, three replicates).

Statistical analysis

Nuclear stage, embryo production, blastocyst cell number, ROS levels, GSH levels and TZPs density were analyzed with two-way ANOVA followed by Tukey's multiple-comparison test. Treatment was set as the fixed factor and replicate as the random variable. Data from nuclear stage and embryo development did not present a normal distribution and were square root arcsine transformed prior to analysis. SAS/STAT® software version 9.4 (SAS institute Inc., Cary, NC, USA) was used for these statistical analyses. The mtDNA copy number was analyzed by one-way ANOVA using the SigmaStat software (Jandel Scientific, San Rafael, CA). The relative mRNA quantification was analyzed with the RT app on Thermo Fisher cloud using a model of integrated correlation. Results were considered statistically significant when $P < 0.05$.

Results

Pre-IVM with CNP maintains meiotic arrest in the presence of estradiol, with predominant condensed clumped chromatin configuration (experiments 1, 2 and 3)

In experiment 1 (figure 2A) the effect of various CNP concentrations (0, 50, 100 and 200 nM) on oocyte meiotic arrest was evaluated. No differences were found among treatments. In experiment 2 (figure 2B) same CNP concentrations were tested combined with 10 nM E2. Pre-IVM with 200 nM CNP + E2 maintained oocyte meiotic arrest for 6 h: GV rate was higher than control, E2 and 50 nM + E2 groups (75%, 28%, 33% and 48%, respectively; $P < 0.05$). After 8 h, there were no differences among treatments and GV rate in 200 nM + E2 group decreased ($P < 0.05$). In experiment 3 (figure 2C) *NPR2* expression in COCs was quantified after pre-IVM with or without E2, compared to uncultured COCs (control 0 h). There was a decrease after 6 h of

pre-IVM with CNP ($P < 0.0001$) and CNP + E2 ($P < 0.001$). There were no significant differences between treatment groups although a tendency to higher expression in CNP + E2 group ($P = 0.113$). Moreover, in experiment 2 chromatin configuration analysis after 6 h of pre-IVM (figure 3) showed higher GV rate with condensed clumped chromatin (GV2c + GV3c + GV4) in 200 nM CNP + E2 group compared to control, E2 and 50 nM CNP + E2 groups ($P < 0.05$).

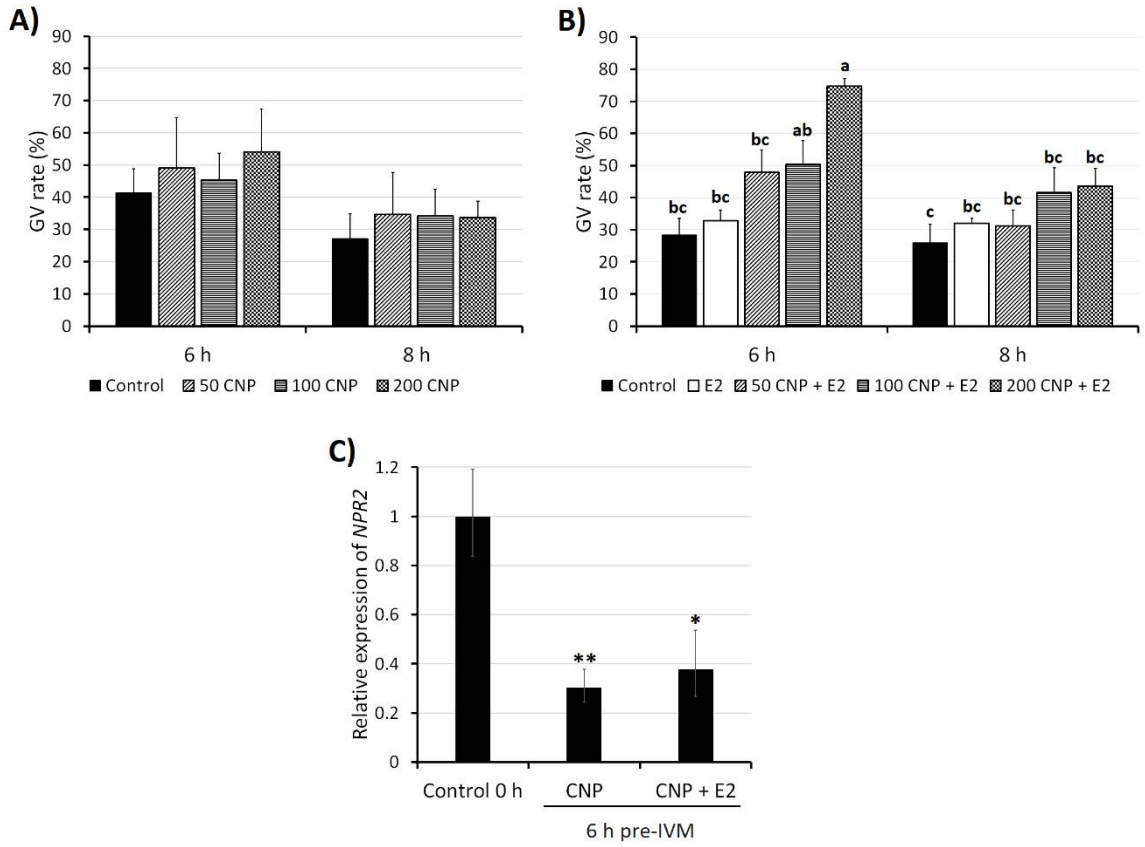

Figure 2. Effect of pre-IVM with CNP and estradiol on the maintenance of meiotic arrest in juvenile-goat oocytes. (A) Germinal vesicle (GV) rate of oocytes cultured for 6 h and 8 h with 0 (Control), 50, 100 or 200 nM CNP. A total of 47-48 oocytes were stained per condition (4 replicates). (B) GV rate of oocytes cultured for 6 h and 8 h with 0 (Control), 10 nM estradiol (E2), 50 nM CNP + E2, 100 nM CNP + E2 or 200 nM CNP + E2. A total of 46-50 oocytes were assessed per condition (4 replicates). Each bar represents mean + s.e.m. Different superscript letters (a-c) in each column indicate statistically significant differences ($P < 0.05$). (C) Relative gene expression of natriuretic peptide receptor (*NPR2*) in COCs after 6 h of pre-IVM with CNP and E2. Five samples were tested per group (10 COCs per sample). Each bar represents relative quantification (RQ), and error bars show RQ max and RQ min. Superscript symbols indicate statistical differences relative to control 0 h: (*) $P < 0.001$; (**) $P < 0.0001$.

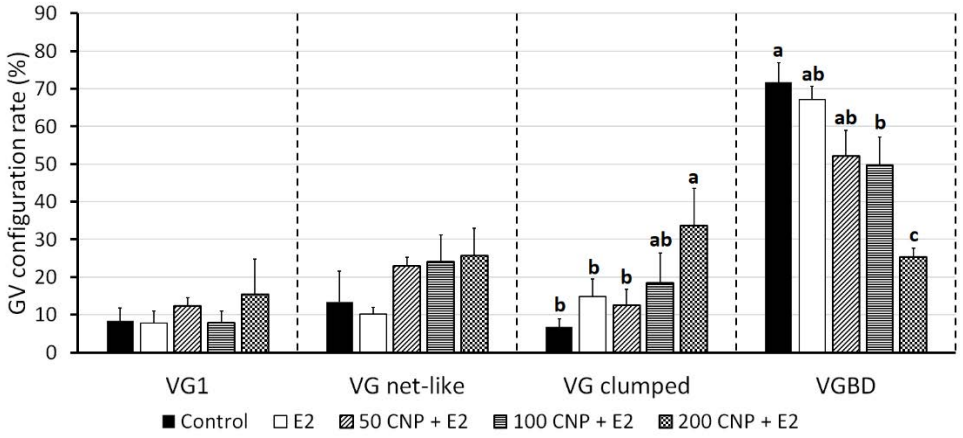

Figure 3. Effect of pre-IVM with CNP and estradiol for 6 h on germinal vesicle chromatin configuration of juvenile-goat oocytes. Treatment groups were: Control, 10 nM estradiol (E2), 50 nM CNP + E2, 100 nM CNP + E2, and 200 nM CNP + E2. Germinal vesicles (GV) were classified as GV1: diffuse filamentous chromatin; GV net-like: GV2n + GV3n, condensed net-like chromatin; GV clumped: GV2c + GV3c + VG4, condensed clumped chromatin; GVBD: broken nuclear membrane. A total of 46-50 oocytes per treatment were assessed (4 replicates). Each bar represents mean + s.e.m. Different superscript letters (a-c) in each column indicate statistically significant differences ($P < 0.05$)

Pre-IVM with CNP and estradiol maintains cumulus-oocyte communication (experiment 4)

In experiment 4 (figure 4) the effect of pre-IVM with CNP and E2 on CC-oocyte communication was evaluated, compared to conventional IVM. Pre-IVM maintained TZP density at the same level than uncultured COCs after follicle recovery (control 0 h), whereas there was a decrease after 6 h of IVM ($P < 0.05$). At the end of IVM there was a great decrease in TZP density relative to control 0 h, 6 h of pre-IVM and 6 h of IVM ($P < 0.001$).

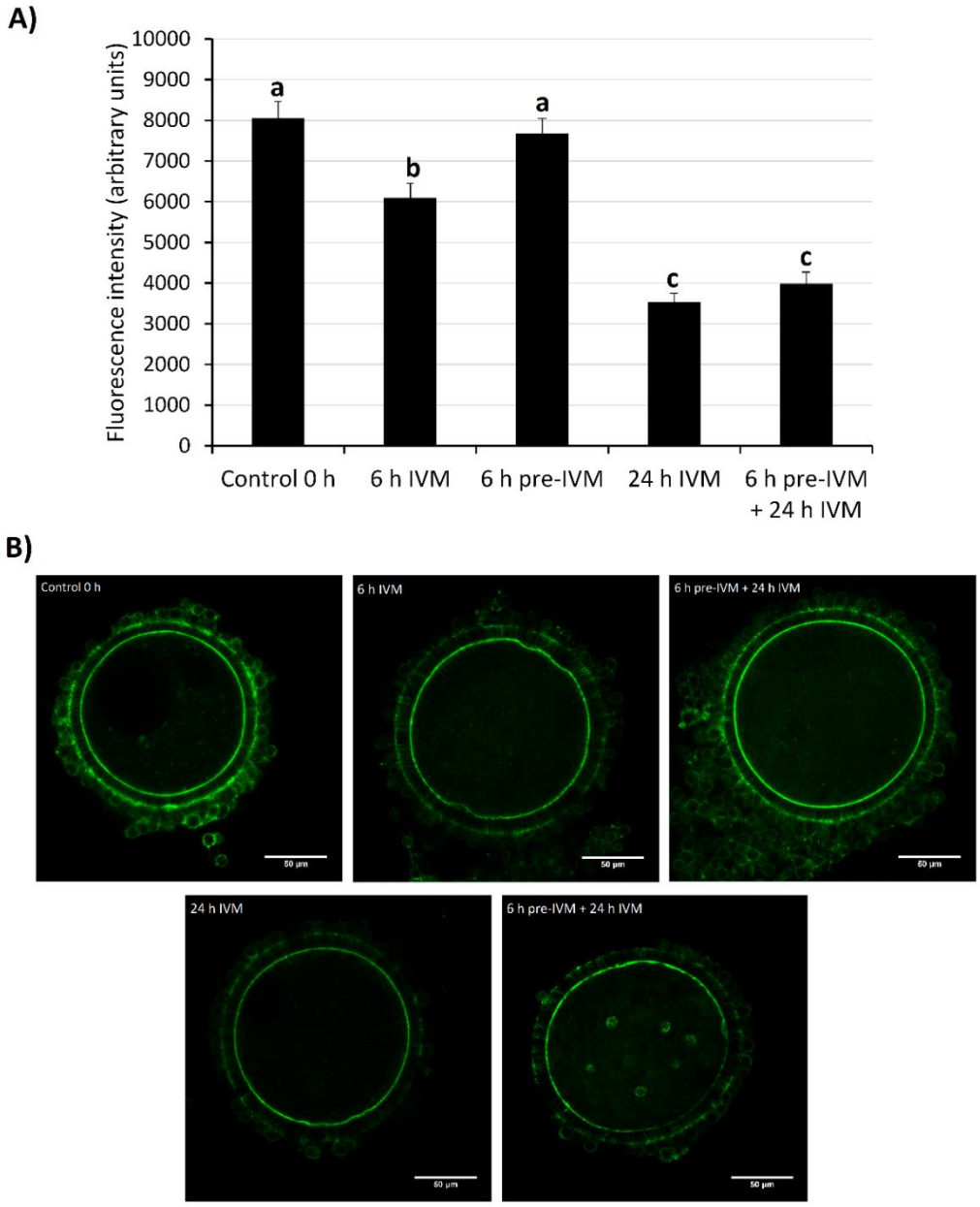

Figure 4. Effect of pre-IVM with CNP and estradiol on transzonal projections (TZPs) density of juvenile goats. COCs were stained with Phalloidin-FITC after recovery (control 0 h), 6 h of IVM, 6 h of pre-IVM with CNP and E2, 24 h of IVM, and 6 h of pre-IVM followed by 24 h IVM. (A) Average fluorescence intensity in the zona area. At least 32 COCs were assessed per group (4 replicates). Each bar represents mean + s.e.m. Different superscript letters (a-c) in each column indicate statistically significant differences ($P < 0.05$). (B) Representative confocal images. Positive actin filaments are observed as continuous filaments going from the cumulus cells to the oocyte through the zona region.

Biphasic IVM enhances the oocyte antioxidant defenses and up-regulates the expression of maturation-related genes, but has no effect on nuclear maturation and mitochondria DNA copy number (experiment 5)

In experiment 5 we assessed various parameters related to oocyte competence at the end of IVM, comparing biphasic IVM (6 h pre-IVM with CNP plus E2, followed by 24 h IVM) with control IVM (24 h). Assessment of nuclear stage (figure 5) showed that MII rate was around 80% for both treatments. Regarding mitochondria (figure 6), neither biphasic IVM nor control IVM modified mtDNA copy number after oocyte recovery (control 0 h). As shown in figure 7, biphasic IVM enhanced intra-oocyte GSH levels compared to control IVM ($P < 0.001$), which was related to a decrease in intra-oocyte ROS levels ($P < 0.001$). Lastly, COCs expression of target genes was evaluated compared to uncultured COCs (control 0 h; figure 8). Both biphasic and control IVM up-regulated *GDF9* ($P < 0.05$), but only biphasic IVM up-regulated *DNMT1* ($P < 0.01$). Both *PTX3* and *TNFAIP6* were up-regulated after biphasic IVM and control IVM ($P < 0.0001$). Yet, *TNFAIP6* expression after was higher after biphasic IVM than control IVM ($P < 0.05$). *FSHR* was down-regulated either after control IVM and biphasic IVM ($P < 0.0001$).

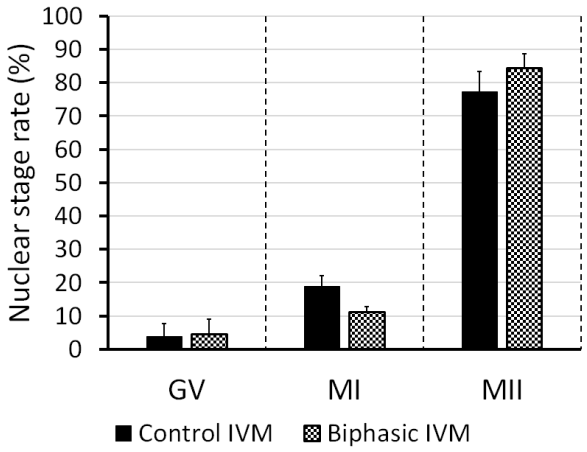

Figure 5. Nuclear maturation rate of juvenile-goat oocytes after biphasic IVM (6 h pre-IVM with CNP and estradiol, followed by 24 h IVM) and control IVM (24 h). Nuclear stage was classified as GV: germinal vesicle; MI: metaphase I; MII: metaphase II. A total of 40 oocytes were assessed per group (4 replicates). Each bar represents mean + s.e.m.

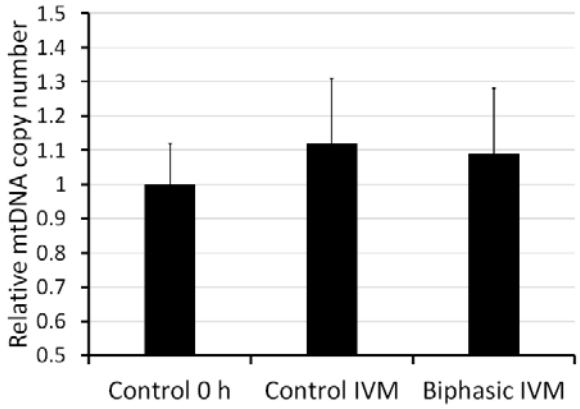

Figure 6. Mitochondrial DNA copy number in juvenile-goat oocytes after recovery from the follicle (control 0 h), biphasic IVM (6 h pre-IVM with CNP plus estradiol, followed by 24 h IVM) and control IVM (24 h). A total of 30 oocytes per group were assessed (3 replicates). Each bar represents mean + s.e.m.

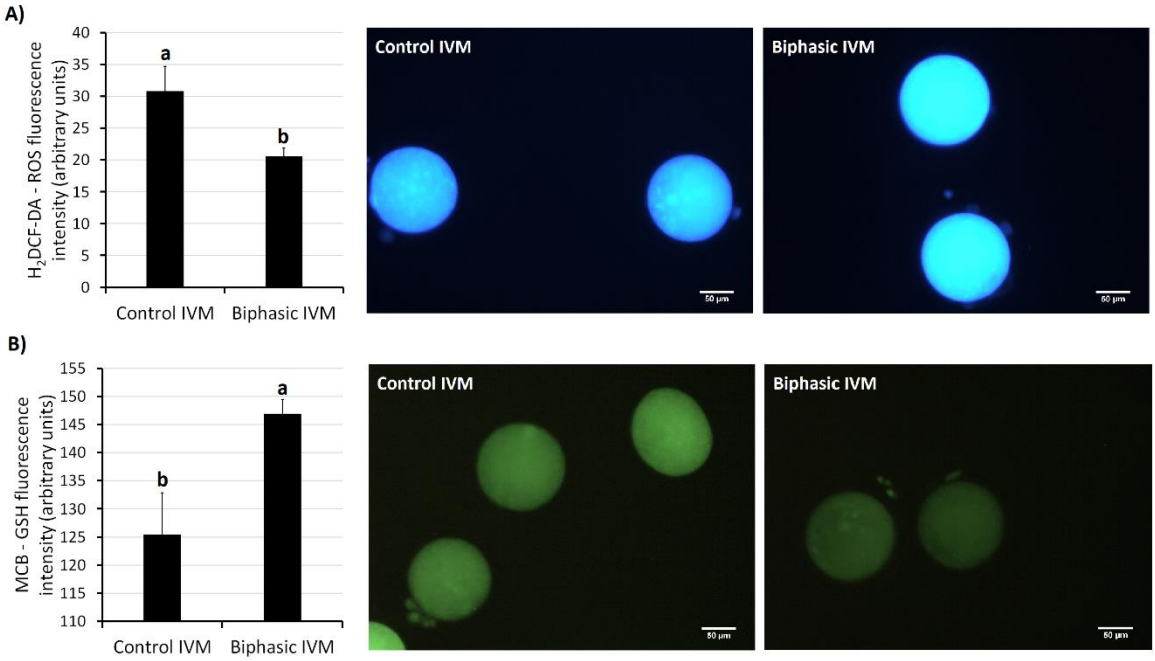

Figure 7. Effect of biphasic IVM on GSH and ROS levels of juvenile-goat oocytes. Oocytes were stained with MCB (GSH) and H_2DCF-DA (ROS) after biphasic IVM (6 h pre-IVM with CNP and E2, followed by 24 h IVM) and control IVM (24 h). (A) MCB-GSH average fluorescence intensity per oocyte and representative images. (B) H_2DCF-DA-ROS average fluorescence intensity per oocyte and representative images. A total of 30 oocytes were assessed per group (3 replicates). Each bar represents mean + s.e.m. Different superscript letters (a, b) in each column indicate statistically significant differences ($P < 0.01$).

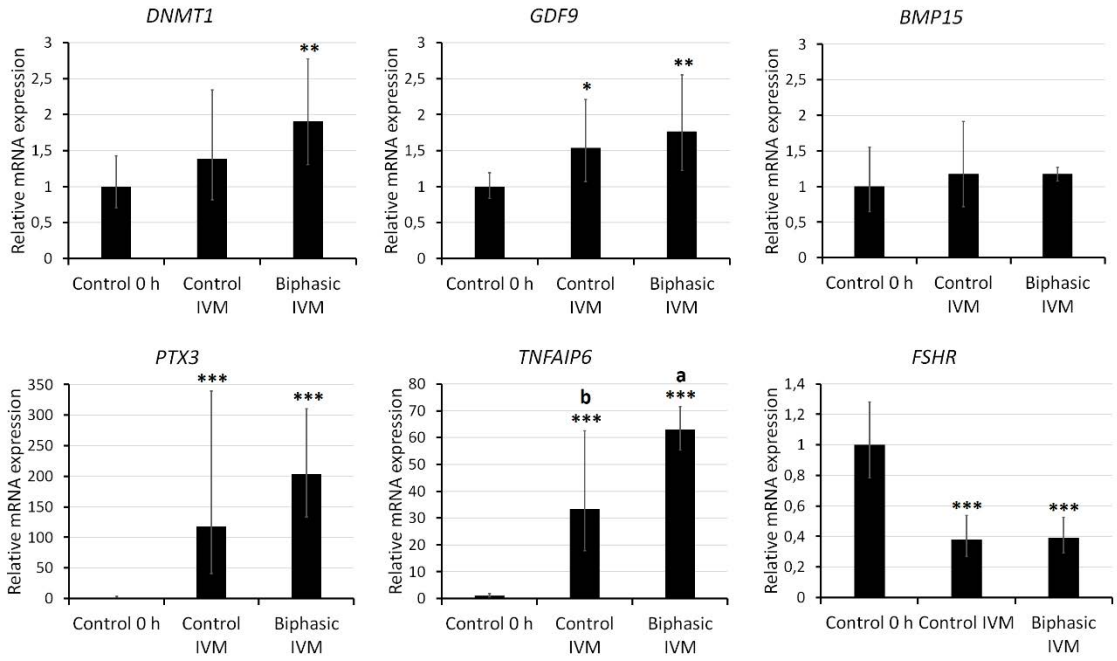

Figure 8. Relative gene expression of *BMP15*, *DNMT1*, *GDF9*, *FSHR*, *PTX3* and *TNFAIP6* in juvenile-goat COCs after biphasic IVM (6 h pre-IVM with CNP and E2, followed by 24 h IVM), control IVM (24 h), and oocyte recovery (control 0 h). Five samples were tested per group (10 COCs per sample). Each bar represents relative quantification (RQ), and error bars show RQ max and RQ min. Superscript symbols indicate statistical differences relative to control 0 h: (*) $P < 0.05$; (**) $P < 0.001$; (***) $P < 0.0001$. Different superscript letters (a - b) indicate statistical differences between treatment groups ($P < 0.05$).

Biphasic IVM enhances embryo development (experiment 6)

In experiment 6 (table 2) oocytes were *in vitro* fertilized and embryo cultured for 8 days after biphasic IVM (6 h pre-IVM followed by 24 h IVM) and control IVM (24 h). Biphasic IVM induced higher blastocyst rate compared to control IVM (30.2% vs. 17.2%; $P < 0.05$). However, there were no differences in blastocyst cell number (table 3).

Table 2. Cleavage (17 hpf) and blastocyst development (8 dpf) of juvenile-goat oocytes *in vitro* matured with biphasic IVM (6 h pre-IVM with CNP plus E2, followed by 24 h IVM) and control IVM (24 h).

Treatment	**N**	**Cleaved/N (%)**	**Blastocysts/N (%)**	**Blastocysts/CV (%)**	**Hatched-blastocysts/N (%)**
Control IVM	148	70.2±7.9	17.2±4.5^{b}	24.1±6.4^{b}	7.5±1.9
Biphasic IVM	151	71.6±10.8	30.2±8.0^{a}	39.6±6.8^{a}	9.8±4.1

N: cultures oocytes; CV: cleaved oocytes. Data are presented as mean % ± s.e.m. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

Table 3. Effect of biphasic IVM (6 h pre-IVM with CNP plus E2, followed by 24 h IVM) on the cell number of *in vitro* produced blastocysts from juvenile-goat oocytes.

Treatment	N	ICM	TE	ICM/TE	Total cell number
Control IVM	16	19.9±2.2	107.4±14.6	0.25±0.05	127.3±13.9
Biphasic IVM	24	23.1±2.3	125.8±12.0	0.23±0.04	148.9±12.0

N: blastocysts; ICM: inner cell mass; TE: trophectoderm. Data are presented as mean ± s.e.m.

Discussion

In the present study we aimed to improve IVEP in juvenile goats by implementing a biphasic IVM system which consists in a pre-IVM phase with CNP followed by standard IVM. First, we developed a pre-IVM phase with CNP and estradiol that sustained meiotic arrest and cumulus-oocyte communication for 6 h. Second, we evaluated the effect of biphasic IVM on oocyte embryo developmental competence.

Pre-IVM with 200 nM CNP delayed GVBD when combined with estradiol. Previous studies have already shown that CNP can sustain meiotic arrest for 6 h in cattle (Franciosi *et al.* 2014; Zhang *et al.* 2017a; Xi *et al.* 2018), sheep (Zhang *et al.* 2018) and adult goat (Zhang *et al.* 2015b), and for at least 24 h in mouse (Romero *et al.* 2016). In our study estradiol was essential for enabling CNP effect. Similarly, the addition of estradiol prolonged CNP meiotic arrest from 4 to 6 h in adult goats (Zhang *et al.* 2015b) and from 24 to 48 h in juvenile mice (Romero *et al.* 2016). This is related to the estradiol effect of promoting CNP receptor (NPR2), as observed in bovine cumulus cells and oocytes (Xi *et al.* 2018). In our study, *NPR2* expression considerably decreased after 6 h of pre-IVM despite the presence of estradiol, although there was a tendency to a lower decline. Analyzing *NPR2* at other time-points during pre-IVM could have revealed a slower decrease with estradiol. Oocyte secreted factors (OSFs) such as GDF9 and BMP15 can also up-regulate *NPR2* (Xi *et al.* 2018), hence could further prolong meiotic arrest during pre-IVM in juvenile-goat oocytes.

On the other hand, pre-IVM with CNP and estradiol increased the rate of GVs with condensed clumped chromatin, according to Sui *et al.* (2005) classification. During follicular development chromatin changes progressively from disperse to condense configurations related to acquisition of meiotic and developmental competence (Lodde *et al.* 2007; Luciano *et al.* 2011). Pre-IVM with CNP also induced condense chromatin configurations in mice (surrounded nucleolus; Romero *et al.* 2016) and cow (GV2; Franciosi *et al.* 2014).

For determining if the pre-IVM period could also sustain cumulus-oocyte communication, TZPs were assessed. TZPs are actin filaments that go from cumulus cells to the ooplasm traversing

the zona pellucida (Macaulay *et al.* 2014) and forming GJs at the union with the oocyte (reviewed by Russell *et al.* 2016). TZPs control information exchange during follicular development and start disrupting after the onset of oocyte maturation (reviewed by Albertini *et al.* 2001). In our study, there was a progressive decrease in TZP density during IVM. But pre-IVM maintained TZP density for 6 h after follicular recovery, in accordance to previous results in mice (Romero *et al.* 2016) and humans (Sánchez *et al.* 2017). Pre-IVM with IBMX plus forskolin also prevents the loss of GJC (Zeng *et al.* 2014; Li *et al.* 2016). Prolonging CC-oocyte communication for 6 h could have a positive impact on the acquisition of oocyte competence. For instance, TZPs enable the transfer of mRNA and metabolites essential for oocyte maturation (Macaulay *et al.* 2014, 2016).

Considering above results, for following experiments we compared two IVM systems: biphasic IVM (6 h pre-IVM with CNP plus estradiol, followed by 24 h IVM) and control IVM (24 h). And we evaluated different parameters that indicate oocyte competence: nuclear maturation, mtDNA copy number, GSH and ROS levels, and COC gene expression. The assessment of nuclear maturation showed a high rate of oocytes reaching MII after both biphasic and control IVM (around 80 %). Whereas in adult goats biphasic IVM with CNP increased MII rate (Zhang *et al.* 2015b). Funahashi *et al.* (1997) also stated that temporary maintaining meiotic arrest synchronizes the time of reaching MII stage after the onset of IVM among the oocyte pool.

Oocyte mtDNA copy number was not increased after biphasic IVM compared to uncultured oocytes (control 0 h). The mtDNA copy number is correlated to the number of mitochondria and is a marker of oocyte competence (reviewed by Fragouli & Wells 2015). The mitochondria number increases during folliculogenesis (Cotterill *et al.* 2013), but is stable during oocyte maturation and early embryo development (Van Blerkom 2009). Pre-IVM with CNP, which simulates the latest phase of follicular development, increased mtDNA copy number in cow (Zhang *et al.* 2017a; Xi *et al.* 2018) and mitochondrial activity in sheep (Zhang *et al.* 2018).

Biphasic IVM increased intra-oocyte GSH levels and decreased ROS compared to control IVM. ROS induce oxidative stress which impairs maturation and embryo development (reviewed by Tamura *et al.* 2008), whereas GSH is the oocyte main non-enzymatic antioxidant (reviewed by Guérin *et al.* 2001) and positively affects the male pronucleus formation after IVF (Perreault *et al.* 1988). In mouse and cow oocytes, the raise in GSH levels after pre-IVM with IBMX plus forskolin is associated to the maintenance of GJC that enables GSH transfer from CCs (Zeng *et al.* 2014; Li *et al.* 2016). Our results suggest a similar mechanism in juvenile-goat oocytes which is promising for improving JIVET. The low oocyte competence in juvenile females is related to a

454 higher exposure to ROS due to impaired GSH synthesis (Jiao *et al.* 2013). Thus in our laboratory
455 we observed that juvenile-goat oocytes have lower GSH levels than adults, and increasing GSH
456 by adding cysteamine to IVM leads to higher embryo rates (Rodríguez-González *et al.* 2003).

457 Lastly, biphasic IVM up-regulated *DNMT1* in juvenile-goat COCs, the main methyltransferase in
458 bovine oocytes (Heinzmann *et al.* 2011). As reviewed by Uysal & Ozturk (2017), DNA
459 methylation by DNMTs increases during follicular development and oocyte maturation and is
460 essential for early embryo development. An up-regulation of extracellular matrix-related genes
461 (*TNFAIP6* and *PTX3*) after IVM was also observed, which was higher after biphasic IVM. These
462 proteins are promoted by the EGF during oocyte maturation, but are down-regulated *in vitro*
463 consequently impairing oocyte competence (reviewed by Brown *et al.* 2013). Sugimura *et al.*
464 (2018) reported that pre-IVM with IBMX and dbcAMP enhances the expression of *TNFAIP6* and
465 other extracellular matrix genes in bovine CCs. However, there was a drastic decrease in *FSHR*
466 expression after IVM regardless of the culture system, in accordance to Sugimura et al. study.
467 FSH is essential for acquiring oocyte competence during follicular development prior to
468 maturation (El-Hayek *et al.* 2014) and its receptor (FSHR) expression is considered a predictor
469 of oocyte quality (reviewed by Ferreira *et al.* 2009).

470 Overall above results suggested that biphasic IVM improves juvenile-goat oocyte competence.
471 This was further confirmed by assessing embryo development: oocytes cultured with biphasic
472 IVM produced more blastocysts than control IVM. An improvement in embryo development
473 has been previously reported in cow (Franciosi *et al.* 2014; Zhang *et al.* 2017a; Xi *et al.* 2018),
474 sheep (Zhang *et al.* 2018), pig (Zhang *et al.* 2017b) and mouse (Romero *et al.* 2016) after IVF,
475 and goat after parthenogenetic activation (Zhang *et al.* 2015b). However, embryo rate has only
476 been slightly improved in ruminants. Whereas, juvenile-mice oocytes developed to similar
477 blastocyst rate than ovulated oocytes fertilized *in vitro* (Romero *et al.* 2016). The inability to
478 prolong meiotic arrest for more than 6 h in ruminants, compared to 48 h in mice, is probably
479 accountant for the different success. In addition, other medium components that can improve
480 oocyte quality during pre-IVM should be considered. In mice the drastic improvement is only
481 achieved when FSH and GDF9 are included in pre-IVM with CNP (Romero *et al.* 2016).

482 In conclusion, we developed a pre-IVM with CNP and estradiol that inhibits meiotic resumption
483 for 6 h in juvenile-goat oocytes while maintaining cumulus-oocyte communication. This pre-
484 IVM followed by standard IVM in a biphasic IVM system improves oocyte protection against
485 oxidative stress, up-regulates gene expression related to DNA methylation and extracellular
486 matrix formation, and enhances blastocyst development. This study shows that biphasic IVM

provides additional developmental competence to juvenile-goat oocytes and is a promising procedure for improving JIVET. Future experiments should focus in prolonging the temporary meiotic arrest and refining the pre-IVM medium with FSH and OSFs that could further enhance oocyte competence.

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Chapter 8

General discussion

Oocyte IVM is a limiting step for IVEP. *In vitro* matured oocytes lead to lower embryo development and pregnancy rates than oocytes recovered after *in vivo* maturation and then fertilized in vitro[3–5]. Embryo rates are especially poor in JIVET[25] because oocytes mainly come from small follicles (< 3 mm) with related lower developmental competence (reviewed by Paramio & Izquierdo[1]). In addition, juvenile oocytes have higher ROS levels compared to adults due to lower GSH synthesis[52]. The aim of the present study was to improve embryo developmental competence of juvenile-goat oocytes by two IVM strategies: A) reducing oocyte exposure to oxidation by supplementing the IVM medium with melatonin; B) applying a pre-IVM culture phase with meiotic inhibitors prior to IVM (biphasic IVM).

The positive effect of melatonin on IVEP has already been shown in several species (reviewed by Cruz et al.[42]). OS caused by ROS is responsible for a reduced oocyte developmental competence *in vitro* (reviewed by Guérin et al.[35]). Thus, antioxidants are routinely added to IVM medium. Melatonin can be more powerful than other antioxidants because it prevents oxidation by different mechanisms such as scavenging ROS[191,194] and promoting the expression of enzymatic antioxidants[188]. Melatonin can also improve embryo development of low-quality oocytes[192,193]. But prior to this study it has not been tested in JIVET. To determine the effect of melatonin on IVM of juvenile-goat oocytes we conducted two studies.

In the first study (Chapter 4), we evaluated the effect of melatonin on oocyte embryo developmental competence. First, we measured melatonin in follicular fluid. Melatonin concentration increased with follicular size: from 0.57 x 10^{-9} M in small follicles (< 3 mm) to 1.07 x 10^{-9} M in large follicles (> 5 mm). Similar results have been observed in humans[186], which suggests that melatonin is related to follicular development and oocyte competence. Second, we tested a series of increasing melatonin concentrations (0, 10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M) on IVM. The 10^{-7} M concentration showed a higher, but statistically insignificant, blastocyst rate compared to control group. We hypothesized that cysteamine, which was also present in the basic IVM medium, may hide the ability of melatonin to act as antioxidant. Hence, we carried out the following two-treatment factorial experiment. We compared the effects of melatonin (10^{-7} M), cysteamine, cysteamine plus melatonin and no antioxidants whatsoever. IVM with either cysteamine or melatonin increased blastocyst rate compared to IVM without antioxidants (21.3%, 28.9% and 11.7%, respectively). Melatonin also improved blastocyst quality compared to cysteamine. Moreover, we measured intra-oocyte ROS levels after IVM. Melatonin decreased ROS compared to IVM without antioxidants, as previously reported[180,188], but cysteamine also reduced ROS at similar levels. This all suggested that melatonin had other

effects on juvenile-goat oocytes apart from reducing ROS that lead to better embryo development compared to cysteamine.

We designed a second study (Chapter 5) to determine melatonin mechanisms of actions and the role of melatonin receptors. First, using immunocytochemistry we localized MT1 in oocytes and CCs before and after IVM. MT1 has also been detected in cows[174,182] and sheep[185] COCs. Second, we assessed different oocyte quality parameters after IVM with melatonin (10^{-7} M) and melatonin plus luzindole (a MT1/2 antagonist) to identify receptor-mediated actions. IVM with melatonin increased mitochondrial activity and ATP levels while decreasing ROS, consistent with results in cows[188]. This indicates that melatonin can prevent the rise in ROS induced by high mitochondrial activity, as previously reported[235]. On the other hand, we further confirmed a positive impact on blastocyst development: although no significant differences on blastocyst rate were observed (35.6% vs. 30.6% in control), melatonin increased the ICM number of hatched blastocysts, which is correlated to blastocyst quality[120]. However, IVM with melatonin plus luzindole showed no significant differences in mitochondrial activity, ROS levels and blastocyst quality compared to melatonin and control groups. Whereas luzindole prevented the positive effect of melatonin on mitochondria distribution in cow oocytes[188], and embryo development in cow[188] and sheep[185]. We concluded that MT1 may partly mediate melatonin actions in juvenile-goat oocytes, but it is not determinant for melatonin positive effect.

Considering results in both studies, we observe that melatonin had a variable effect on embryo development. Previous studies also show great variability in the embryo production success. In cattle[174,182,188], pigs[173,183] and mice[180,189,190], IVM with 10^{-12} to 10^{-6} M melatonin improved embryo development. Whereas in other studies in cattle[236,237] and sheep[238] melatonin was ineffective at similar concentrations. As shown in our first study, the combination of various antioxidants can hide melatonin effect. As for the second study, for the first time we used commercial IVF and embryo culture mediums which enhanced overall IVEP success: embryo rate of control group was 11.7% in the first study and 30.6% in the second one. Hence melatonin benefits depend on general culture conditions. Furthermore, we have only focused on IVM, but the addition of melatonin in other IVEP steps can potentiate melatonin positive effects as shown in mice[189], cow[239] and pig[240]. Nevertheless, our two studies indicate that melatonin improves IVEP in juvenile goats (either blastocyst production or quality) being more powerful than cysteamine. We can relate these results to a clear antioxidant role, but also to other effects on oocyte competence like the enhancement of mitochondrial function.

The second strategy consisted in improving oocyte competence with a biphasic IVM system. Conventional IVM can prevent full acquisition of oocyte competence by inducing spontaneous meiotic resumption regardless of oocyte grade of development (reviewed by Gilchrist et al.[48]). The identification of the mechanisms that regulate oocyte meiotic arrest by maintaining high intra-oocyte cAMP levels[201] has enabled the development of new IVM systems. Biphasic IVM consists in a pre-IVM phase with cAMP modulators that maintain oocyte meiotic arrest, followed by standard IVM. Biphasic IVM has proved to benefit oocytes with a low inherent competence, such as those from small antral follicles[211,233] and from juvenile animals (calves[223] and mice[5]). Therefore, we hypothesized that biphasic IVM could improve the developmental competence of juvenile-goat oocytes. We conducted two studies in which we used two known cAMP modulators: IBMX, which prevents cAMP hydrolysis by inhibiting various PDEs; and CNP, which promotes cGMP synthesis that inhibits PDE3A[48]. The first study was performed at the University of Adelaide (Australia) under the supervision of Jeremy Thompson, who is an expert in cAMP-modulated IVM. We tested pre-IVM in bovine oocytes in order to learn biphasic-IVM procedures in a simpler model and be able to design a similar system in juvenile goats. The second study was performed in our laboratory applying biphasic IVM to juvenile-goat IVEP.

In the first study with bovine oocytes (Chapter 6), we evaluated the effect of pre-IVM with CNP and IBMX on oocyte meiotic maturation and developmental competence. Previous studies have reported that these molecules can sustain meiotic arrest: CNP in cattle[220,229,234], sheep[231] and adult goat[232] for 6 h, and in mice for 24 h[5]; IBMX with forskolin in cattle for 9 h[209]. In our study the combination of both agents (100 nM CNP plus 500 µM IBMX) sustained a higher GV rate for 6 h (more than 90%) suggesting a synergic meiotic inhibition. This 6-h pre-IVM followed by 20 h IVM (biphasic IVM) increased blastocyst rate (45.1% vs. 34.5% in control IVM). Similarly, biphasic IVM with CNP[220,234] and IBMX plus forskolin[140] improves blastocyst development in bovine oocytes. We also investigated the effect of biphasic IVM on CC-oocyte communication and oocyte metabolism after IVM. CC-oocyte communication mediated by TZPs and GJs is essential for oocyte maturation (reviewed by Russel et al.[139]). TZPs enable the transfer of mRNA and other metabolites[96,97]. After the onset of maturation GJs are rapidly closed and TZPs progressively disrupted. In our study, COCs presented higher TZPs density after biphasic IVM than control IVM. Pre-IVM with CNP also sustains TZPs in mice[5] and human COCs[233]. In addition cAMP-modulated IVM prolongs GJC[140,209,211,229], which has a positive impact on oocyte GSH levels[140], chromatin remodeling and transcription[211,229] and metabolism[141]. Lastly, we observed higher mitochondrial activity after biphasic IVM, in accordance to other pre-IVM studies with bovine oocytes[138].

In the second study with juvenile-goat oocytes (Chapter 7), we developed a similar experiment but only CNP was tested as a meiotic inhibitor for pre-IVM, avoiding the use of IBMX (a non-physiological agent). IBMX was, however, added to the slicing medium because in a preliminary experiment meiotic resumption was detected during oocyte recovery prior to pre-IVM. Unlike in the cow study, where follicular intrinsic meiotic inhibitors were present during this process since COCs were recovered by aspiration. First, we achieved a meiotic arrest with 200 nM CNP only when it was combined with 10 nM E2 and for 6 h (GV rate was 74.7% vs. 28.3% in control). Estradiol promotes the CNP receptor (NPR2) in cumulus cells and oocytes[220] and enables a longer meiotic arrest than pre-IVM with only CNP (from 4 to 6 h in adult-goat oocytes[232]; and from 24 to 48 h in mice[215]). In our study estradiol effect was not clear: *NPR2* expression was decreased after 6 h of pre-IVM, although the addition of E2 showed a tendency to slow the rate of decline. Pre-IVM with CNP plus E2 had also a potential positive effect on oocyte competence. Oocytes presented higher GV rate of condensed clumped chromatin configuration, similar to previous results in other species[215,229]. Remodeling from disperse to condense chromatin takes place during follicular development and is related to oocyte competence acquisition[211,241]. On the other hand, pre-IVM culture sustained CC-oocyte communication showing a high TZP density comparable to COCs after follicular recovery.

Second, to determine if pre-IVM could provide additional developmental competence to oocytes we compared biphasic IVM (6 h pre-IVM with CNP plus E2, followed by 24 h standard IVM) with control IVM (24 h). Biphasic IVM improved oocyte antioxidant defenses as observed by higher intra-oocyte GSH levels and a lower ROS. Other biphasic IVM systems increases GSH in bovine oocytes by sustaining GJC which enables the transfer from CCs[140,141]. The similar mechanism revealed in this study is promising for JIVET. As mentioned before, juvenile oocytes have lower GSH levels than adults[40,52]. Moreover, biphasic IVM up-regulated the expression of *DNMT1,* which is essential for oogenesis and early embryo development (reviewed by Uysal and Ozturk[242]), and extracellular matrix-related genes (*TNFAIP6* and *PTX3*), which are down-regulated *in vitro* leading to poor oocyte competence (reviewed by Brown et al.[243]). Pre-IVM with IBMX and dbcAMP also up-regulates *TNFAIP6* and other extracellular matrix genes in bovine CCs[227]. Lastly, biphasic IVM increased blastocyst rate (30.2% vs. 17.2% in control), in accordance with previous results in sheep[231] and adult goat with parthenogenetic activation[232].

We have developed a pre-IVM with CNP and estradiol in juvenile goats that sustains oocyte meiotic arrest and CC-oocyte communication for 6 h, and improves oocyte developmental competence. It is worth noticing that the blastocyst rate improvement was higher in the juvenile-goat study (almost two-fold increase compared to control IVM) than in the cow study.

Hence biphasic IVM is a promising procedure for improving IVEP with low-quality oocytes. Yet its full potential has probably not been exploited. Juvenile-mice oocytes cultured in a biphasic IVM with CNP developed to similar blastocyst rate than oocytes matured *in vivo* and fertilized *in vitro*[215], showing that biphasic IVM can overcome *in vitro* limitations. Whereas in ruminants there is only a slight improvement on embryo development[220,230,234,244]. SPOM system, which induces high cAMP levels by combining various cAMP modulators, achieved an 86 % blastocyst rate with bovine oocytes[209,213]. But the success of this protocol depends on the accurate combination of different agents and culturing periods that can otherwise be detrimental for oocyte quality. Thus, a more conservative protocol like pre-IVM with CNP is preferable.

We suggest two approaches that could improve pre-IVM. First, maintaining meiotic arrest for more than 6 h could enable full competence acquisition before IVM. CNP arrests meiosis for at least 24 h in mice[5], but only for 6 h in ruminants. A possible explanation is the predominant PDE: bovine CCs have higher PDE8 activity compared to higher PDE4 in mice[245]. PDE8 has 100-fold higher affinity for cAMP than PDE4 (reviewed by Bender and Beavo[246]). Hence the high cGMP induced by CNP may not be as efficient at inhibiting PDE8 in ruminants as PDE4 in mice. Other cAMP modulators such as specific PDE8 inhibitors could further maintain meiotic arrest in ruminants. Moreover, CNP effect is limited by the drastic decrease in *NPR2* expression after 6 h of pre-IVM, which can be prevented by different agents. OSFs (GDF9 and BMP15) up-regulate *NPR2* in bovine CC[220], and GDF9 plus E2 prolonged CNP meiotic arrest from 24 to 48 h in mice[5]. Second, pre-IVM medium could be enriched in order to better simulate the physiological process of competence acquisition. Both OSFs and FSH are essential for promoting CC-oocyte communication and oocyte development before ovulation (reviewed by Sugimura et al.[91]). In mice, the drastic improvement in blastocyst rate is only achieved by adding FSH and GDF9 to pre-IVM with CNP[5].

In conclusion, we have improved developmental competence of juvenile-goat oocytes by two approaches. First, we reduced oxidation during IVM by adding melatonin, and second, we improved oocyte competence by applying a pre-IVM with CNP prior to IVM. These are promising methods to improve JIVET in other species. Both procedures have been previously tested in adult ruminants but not in juvenile, which are more sensitive to oxidation and have inherently lower competence. In previous studies of our group, blastocyst rates of juvenile-goat oocytes ranged from 6 to 20 %. Although in the present study we have overcome these results, there is still great variability between experiments and replicates which limits the application of JIVET.

Chapter 9

Conclusions

1. Melatonin is present in follicular fluid of juvenile goats with increasing concentration correlated to follicular size, and melatonin receptor 1 is localized in juvenile-goat oocytes and cumulus cells before and after IVM.

2. The IVM with melatonin supplementation improves the developmental competence of juvenile-goat oocytes leading to higher blastocyst rate and quality.

3. Melatonin positive effects on oocyte IVM in juvenile goats are related to two mechanisms of action: reduction of intra-oocyte ROS levels and improvement of mitochondrial activity.

4. IBMX and CNP can efficiently sustain oocyte meiotic arrest for 6 h during a pre-IVM culture period and improve oocyte developmental competence in cattle.

5. The pre-IVM culture with CNP and estradiol sustains oocyte meiotic arrest and cumulus-oocyte communication for 6 h in juvenile goats.

6. Biphasic IVM system, consisting of 6 h pre-IVM followed by 24 h IVM, improves oocyte antioxidant defenses, up-regulates the expression of genes related to DNA methylation and extracellular matrix formation, and enhances the embryo development of juvenile-goat oocytes.

Chapter 10

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