STUDIES ON *IN VITRO* MATURATION OF DOG OOCYTES TO IMPROVE MATURATION RATE AND DEVELOPMENTAL POTENTIALS

By

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Abstract

In vitro maturation of dog oocytes has always been the main obstacle preventing reproductive biologist from producing canine *in vitro* cultured embryos. The unsuccessful oocyte maturation in canine species originates from their unique physiological and biological specifications. Ovulation of dominant follicles in bitch (6-12 in each oestrous cycle) occurs at prophase I stage of oocyte nucleus and meiotic resumption develops during 3-5 days of oviductal transition. During this PhD thesis, studies were designed in order to speculate characteristics of canine oocyte maturation *in vitro* in terms of maturation media components, gas composition of the incubator and hormonal requirements. Level of oxidative stress during 72h (culture period) of *in vitro* maturation showed that 5%O_2, 5% CO_2 and 90% N_2 composition improves meiotic resumption and reduces degeneration rate significantly compared to 5% CO_2 in air. Utilization of caffeine as a non specific phosphodiesterase inhibitor at 10mM for 12h at the beginning of the 72h culture (12+60) also improved MII maturation rate (16.9% ± 2.4; $P < 0.05$). Among several hormonal treatments recombinant porcine Growth Hormone (PGH) at 100ng/ml and Melatonin (MTN) at 100nM concentrations had outstanding improvement over meiotic resumption (28.9% ±10.0 and 56.2% ±8.6 respectively; $P < 0.05$). Attempts were made to study developmental potentials of optimally matured oocytes by parthenogenetic activation (PA) and *in vitro* fertilization (IVF) using chilled semen. Partial digestion of the zona pellucida prior to IVF improved the cleavage rate at 48h 6.4% ± 0.3 and resulted in production of a single 8 cell embryo. Moreover; canine follicular cells were culture in order to characterize their primary culture morphology and steroidogenic responsiveness to physiological and pharmaceutical substances. Immunolocalization of aromatase (CYP19) positive cell clumps, presumptive oestrogen producing colonies, was identified. This primary culture also maintained its steroidogenic machinery up to 96h (measured by radioimmunoassay) with a significant increase in production of estradiol and progesterone after 72h compare to the start of the culture.

Author's declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Bedfordshire.

It has not been submitted before for any degree or examination in any other University.

Name of candidate: Signature:

Date:

I dedicate this work to my beloved mother and brother who has never stopped believing in me.

In the memory of Dr. Keykavous Salavati (1948-2010)

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..... Believing in nothing and opening up to the poetry of reality takes lots of courage and determination. Human kind is not known for its bravery and most certainly not for its determination. Truth is the only reality which sets us free, far from superstition and confusion. Science is the only rational tool to unveil the truth about who we are. The beauty lied within the scientific poetry of reality is all the hope I have left, to be a better and wiser human being every single day I live. Not having the certain answer ever stopped me from asking more questions and I'm planning to live by it for the rest of my life.

Truly Yours

Mazdak Salavati

Publications arisen from this thesis

Published papers:

1. **Salavati M,** Ghafari F, Zhang T & Fouladi-Nashta AA 2012 *Effects of oxygen concentration on in vitro maturation of canine oocytes in a chemically defined serum-free medium. Reproduction* **144** 547-556

2. **Salavati M,** Ghafari F, Zhang T & Fouladi-Nashta AA 2013 *Influence of caffeine pre-treatment on biphasic in vitro maturation of dog oocytes. Theriogenology* **80** 784-792

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1. Effects of caffeine on *in vitro* maturation of dog oocytes: Arrest or Promote. Poster presentation. *Society of Reproduction and Fertility Annual Conference July 2011 Brighton, UK*

2. Effects of caffeine on *in vitro* maturation of canine COCs. Poster presentation. University of Bedfordshire *Annual Conference June 2011 Luton, UK*

3. Contrasting effects of porcine growth hormone and foetal bovine serum on *in vitro* maturation of dog oocytes. Poster presentation. *Society of Reproduction and Fertility Annual Conference July 2012 Edinburgh, UK*

4. Effects of oxygen concentration on *in vitro* maturation of canine oocytes. Oral presentation (ePoster). *International Congress of Animal Reproduction August 2012 Vancouver, BC, Canada*

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6. Improving effects of melatonin on *in vitro* maturation of canine oocytes. Poster presentation. *Society of Reproduction and Fertility Annual Conference July 2013 Cambridge, UK.*

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List of Abbreviations

Abbreviation	Standing for
17 βHSD	17 β Hydroxysteroid dehydrogenase
25OHC	25 Hydroxy cholesterol
3HSD	3 Hydroxysteroid dehydrogenase
6DMAP	6 Dimethyl amino purine
AD	Androstenedione
AI	Anaphase I
ARC	Arcuate nucleus
AREG	Amphiregulin
ASMT	Acetyl O-methyltransferase
AVPv	Anteroventral periventricular preoptic area
BAD	BCL2 associated death promoter
BAK	BCL2 homologous antagonist killer
BAX	BCL2 associated X protein
BCB	Brilliant cresyl blue
BCL2	B-cell lymphoma 2 apoptosis regulatory protein family
BCLw	BCL2 family member
BLAST	Basic linear alignment search tool
bp	base pair
BSA	Bovine serum albumin
BTC	Beta cellulin
Ca^{2+}	Calcium ion
CAT	Catalase
cDNA	complementary DNA
CL	Corpus luteom
CO_2	Carbon dioxide
COC(s)	Cumulus oocyte complex (es)
CYP17	Cytochrome p oxidase 17
CYP19	Cytochrome p oxidase 19 (Aromatase)
dbcAMP	Dibutyryl cyclic Adenosine mono phosphate
DCF	2', 7' – dichlorofluorescein
DCHF	2', 7' – dicholorodihydrofluoroescein
DCHFDA	2',7' – dicholorodihydrofluoroescein diacetate
DDW	Deionized distilled water
Degen	Degenerated
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
E2	17 β-Estradiol (estrogen)
EBS	Estrus bitch serum
ECL	Enhanced chemiluminescence

Abbreviation	Standing for
ECS	Estrus canine serum
EGF	Epithelial growth factor
EGFR	EGF receptor
EREG	Epiregulin
ERK	Extracellular regulated kinase
ESS	Estrus sheep serum
FBS	Fetal bovine serum
FC	Follicular cells
FGF-10	Fibroblast growth factor - 10
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
FSHR	FSH receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCS	Glutathione Complex Synthase
GH	Growth hormone
GnRH	Gonadotropin releasing hormone
GPR54	G coupled protein receptor 54
GPRi	G coupled protein receptor inhibitory component
GPRs	G coupled protein receptor stimulatory component
GPX	Glutathione peroxidase
GSH	Glutathione
GSR	Glutathione reductase
GV	Germinal vesicle (prophase I)
GVBD	Germinal vesicle break down
H_2O_2	Hydrogen peroxide
hCG	Human chorionic gonadotropin
HKG	Housekeeping gene
HPG	Hypophysial pituitary gonadal
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IAP	Inhibitor of apoptosis proteins
IBMX	1-methyl-3-isobutylxanthine
ICSI	Intracytoplasmic sperm injection
IDV	Integrated density value
IGF-1	Insulin like growth factor - 1
IGFBP	IGF binding protein
IgG	Immunoglobulin G
ION	Ionomycin
IVC	In vitro culture of embryo
IVEP	In vitro embryo production
IVF	In vitro fertilization
IVM	In vitro maturation
kDa	kilo Dalton

Abbreviation	Standing for
LH	Luteinizing hormone
LHR	LH receptor
LN_2	Liquid nitrogen
MAPK	Mitogen activated protein kinase (ERK)
ME	Median eminence
MI	Metaphase I
MII	Metaphase II
miRNA	micro RNA (micro interfering RNA)
MPF	Maturation promoting factor (CDK1/P^{34}CDC2 + Cyclin B)
MPN	Male pronuclear formation
MR	Meiotic resumption
MSP	Monospermic penetration
MTN	Melatonin
MTNRA1	Melatonin receptor A1
MTNRB1	Melatonin receptor B1
MW	molecular weight
N_2	Nitrogen
NaCl	Sodium chloride
O_2	Oxygen
P4	Progesterone
P450SCC	P450 Side chain cleavage (cytochrome oxidase)
PA	Parthenogenetic activation
PAGE	Poly Acrylamide gel electrophoresis
PAPPA	Pregnancy associated plasma protein A
PB	Polar body
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PGH	Porcine growth hormone
PI3	Inositol tri-phosphate
PKA	Protein kinase A (cAMP dependent)
PKB	Protein kinase B (PI3K/AKT)
PKC	Protein kinase C
POA	Pre optical area
PVP	Polyvinylpyrrolidone
PZD	Partial Zona digestion
RIA	Radioimmunoassay
ROS	Reactive oxygen species
rRNA	regulatory RNA
RT-PCR	Realtime PCR
SC	Stromal cells
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate

Abbreviation	Standing for
SH	Standing heat
SOD	Superoxide dismutase
SOF	Synthetic oviductal fluid
StAR	Steroid transfer acute regulatory protein
SUZI	Subzonal insemination
TAE	Tris-acetate EDTA buffer
TCM199	Tissue culture medium 199
TG	Target gene
TGF	Tumor growth factor
TI	Telophase I
TT	Testosterone
WB	Western blotting
ZP	Zona pellucida
βME	β Mercapto ethanol (2ME)

List of Figures and Tables

1. Literature Review

1.1. Introduction

Dogs have been the most popular companion animals in the history of mankind and have always been part of the social and personal activities of modern human life. Despite all efforts, a successful system for *in vitro* maturation (IVM) and fertilization (IVF) have not been established for canid family (Songsasen & Wildt 2007, Rodrigues & Rodrigues 2010).

Regarding phylogenic classifications beside caniform families of bears, pandas, skunks, minks and sea lions there is a Canid family consisting of 4 wild dog species, the Asian dhole, 20 other fox species and the most popular *Canis* genera (Concannon *et al.* 2009). Canis species are mainly categorize as 3 subspecies of wolves, Jackals and coyotes of which *Canis lupus familiaris* will be recognized as the descendent of grey wolf alongside its red and Ethiopian brothers (Concannon *et al.* 2009). *Canis lupus familiaris* or the domestic dog has always been the most attended representative of canid family in the reproductive biology and other modeling research projects. However unique features of this specie have made it one of the most complicated animal models to fit in assisted reproductive techniques. Establishment of assisted reproductive technologies for canid family unlike other domestic animals such as cattle, sheep and etc. has been a conundrum for the past 50 years (Songsasen & Wildt 2007, Rodrigues & Rodrigues 2010). The *in vitro* culture of canine oocytes due to their delayed nuclear maturation and prolonged period of oviductal meiotic resumption is of great complexity (Rodrigues & Rodrigues 2010). This study was designed to elucidate technical aspects of an optimized *in vitro* maturation (IVM) system for canine oocytes. The main challenges integrated in to this system originate from hindered nuclear maturation of oocytes from prophase I at the point of ovulation up to metaphase II. Moreover the high lipid content of these cells in combination with their fragile immature status at point of collection staggers the yield of IVM systems. The unsynchronized hormonal exposure, severe predisposition to

degenerate *in vitro* (specifically due to oxidative stress) and impaired developmental potentials were addressed during this thesis. Studies presented in the next experimental chapters are an ensemble of attempts to set up an efficient IVM system for canine oocytes.

1.2. Reproductive cycle

Although canine ancestors had tendencies toward seasonal breeding and wolves/foxes still follow the same photoperiodic reproductive pattern, domestic dog has been categorized as non-seasonal breeder (Concannon *et al.* 2009, Concannon 2011). Hard evidence elucidating the seasonality of domestic dog is still missing and limited studies concerning this issue have controversial results (Concannon 2011). While dogs are not seasonal breeders, estrous cycle is mostly observed once annually (interestrous intervals of 7 months). They will normally reach the puberty between 6-13 months after birth and tend to mate every 8 months or year around; thus, three pregnancies in every two years are expected (Golden 2009, Concannon 2011). The oestrous cycles in dogs consist of four distinctive stages (Fig. 1.1):

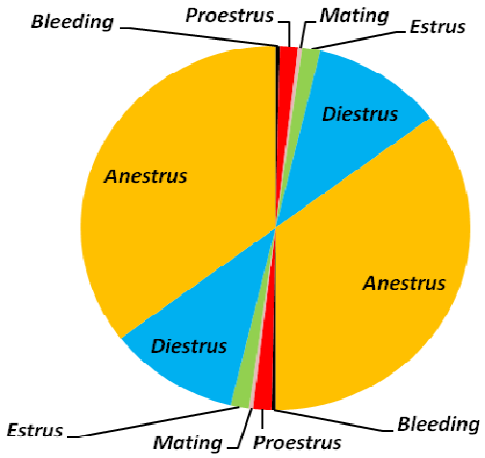
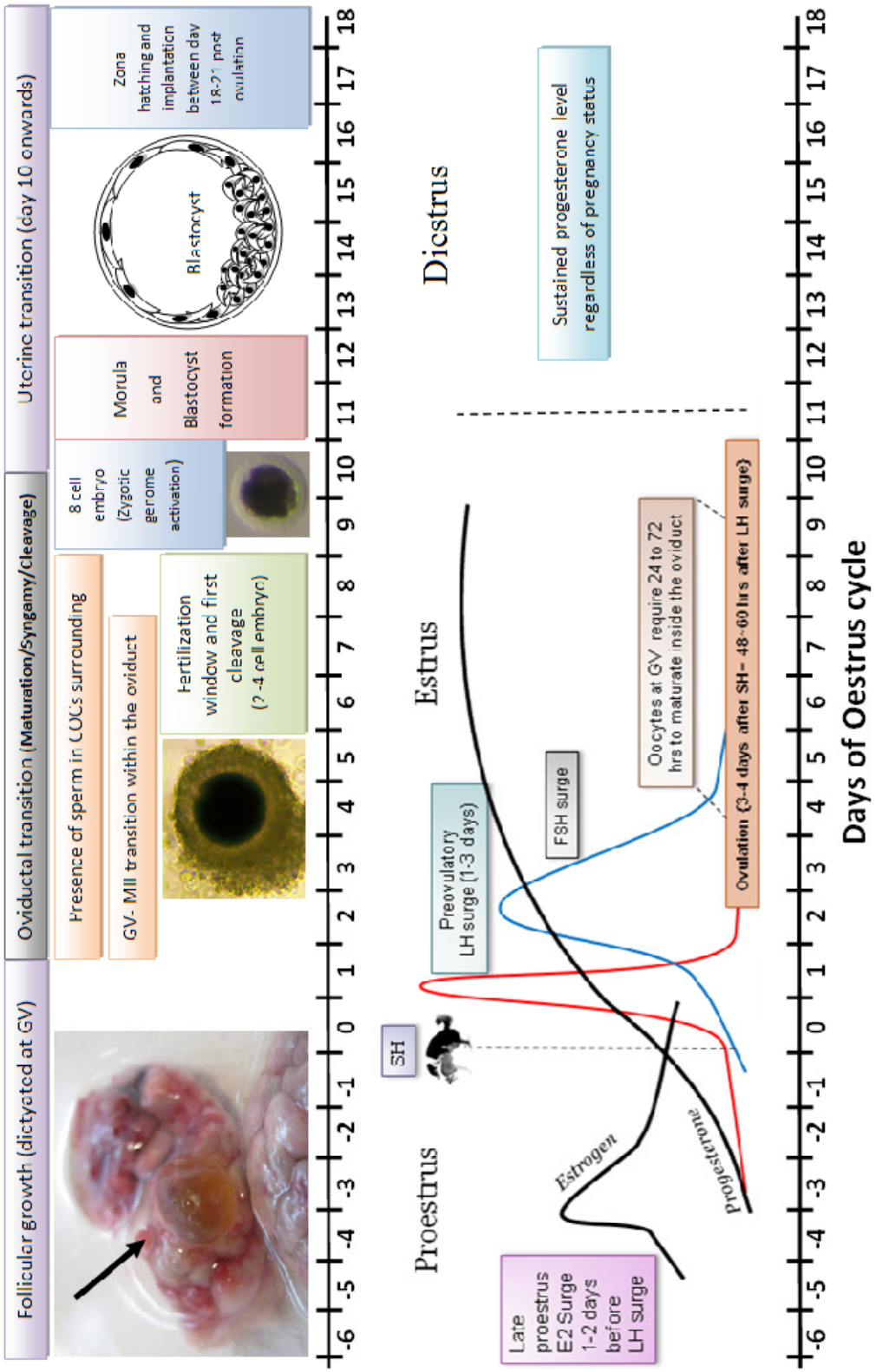


Figure 1.1 - Canine Oestrous Cycle. Graph illustrates the oestrous cycle in canine species which follows a circulating pattern of Proestrus, Estrus, Metestrus/Diestrus and Anestrus every 7-10 months.

1.2.1. Proestrus phase: this stage of the estrous cycle is characterized by the peculiar bitch behavior which attracts males but won't stand to be mounted or participate in the copulation. During this period due to follicular growth and development in the ovaries, animal physiology and behavior is under governance of estrogens originated from growing subordinate and preovulatory follicles (Fig 1.2). Swollen vulvar labia and vaginal wall, appearance of blood in the mucoid grayish vulvar discharge and increased sexual receptivity (libido) are the most important indicators of the proestrus in the bitch. It's been hypothesized that swelling and the inflammation in the reproductive organs is so painful for the animal which prevents copulation during proestrus. This stage of reproductive cycle lasts around 9 days on average (Lane & Lofstedt 1996, Senger 2003). The progesterone (P4) production temporal profile is unique and precedential in canine species (Concannon 2011). The pre-ovulatory luteinisation of canine follicles and above 2.5 ng/ml plasma P4 level in the presence of cumulus oocyte complexes (COCs) within the follicular environment is the exceptional specifications of canine ovaries (Concannon 2011).

1.2.2. Estrus phase: Declining concentrations of plasma estrogen (E2) during the last two days of proestrus accompanied by the rising level of P4 due to early lutenization of granulosa cells surrounding the preovulatory follicle, defines bitch transition from proestrus to estrus phase. At this stage female dog will start to seek males and stands for intercourse to take place (beginning of estrus). Sexual receptivity of female animal during the estrus or heat is also referred to as "Standing Heat" (SH) (Fig.1.2). Ovulation of the oocytes will occur 2-3 days after the SH but before that a drastic peak in circulatory luteinizing hormone (LH) is detectable in the first day of estrus. (Fig.1.2) This preovulatory LH peak which holds around 36hrs is followed by a preovulatory follicle stimulating hormone surge (rarely concomitantly) with wider range of 100-120hrs (de Gier *et al.* 2006a). Estrus phase duration may vary from as short as 5 days up to 10 day depending on the breed, body score and reproductive fertility profile of bitch. On average it last 9 days before entrance to Metestrus (Concannon *et al.* 2009).



Adapted from Canine and Feline Endocrinology and Reproduction Edward C. Feldman, 2004, P537 Fig17-14

Figure 1.2 - Hormonal profile and schematic illustrations of ovulatory events around standing heat in bitch. The graph illustrated the hormonal changes during proestrus, estrus and diestrus/metestrus phase in canine seric profile and reproductive cycle. Standing heat (SH) is on day 0 which is the last day of Proestrus and the beginning of Oestrus. Luteinizing Hormone (LH). Follicle Stimulating Hormone (FSH), 17β estradiol (E2) and Progesterone (P4) plasma levels are shown throughout. Embryonic development of canine oocyte in vivo starts with cleavage at 96-128h after ovulation, followed by zygotic maternal transition between days 7-9. Embryonic genome activation occurs in 8cell stage and morula enters the uterus around day 10. Blastocyst formation and further development carries on till day 18-20 when embryo hatches and starts implantation process within the uterine endometrial.

1.2.3. Metestrus phase: Metestrus and diestrus in canine reproductive terminology are used as synonyms. Unlike polyestrous animals, carnivores have a prolonged metestrus/diestrus for 64±1 days. During this stage of reproductive cycle life span of the corpus luteom (CL) which forms after the ovulation of antral follicle is interestingly long regardless of the pregnancy status. Progesterone that is the main product of CL during metestrus will remain above 1-2ng/ml for average of 70 days in plasma (Concannon 2011). The difference between pregnant and non-pregnant bitches is pronounced clearly through rising estrogen and prolactin concentrations in pregnancy during the last 40 days of metestrus rather than the progesterone level or the duration of metestrus itself (Concannon *et al.* 2009). In the pregnant animal estrogen will reach its highest concentration one day before parturition and prolactin will be secreted even after parturition in a pulsatile manner (Concannon *et al.* 2009). In case of pregnancy after 3 months of lactation, otherwise after the metestrus without any acute luteolytic mechanism animal will enter the anestrous phase (Concannon 2011).

1.2.4. Anestrus phase: The non-seasonal anestrous in dog immediately after metestrus or the lactation period can last up to 2-10 months (Okkens & Kooistra 2006). Reaching the progesterone below 1ng/ml concentration is the most

reliable indicator for the start of anestrous. During this stage animal will undergo the reproductive quiescence and the physiology of bitch will get prepared for the next ovarian rebound. Slight increase in pulse frequency and overall concentration of FSH by the late anestrous is remarkable (Okkens & Kooistra 2006). The main objective of this phase is to increase the sensitivity of pituitary to hypothalamical gonadotropin releasing hormone (GnRH) and facilitate the responsiveness of ovarian cortex toward LH and FSH. Within the signaling pathway of GnRH secreting neurons in hypothalamus and the regulatory feed paths of these neurons, KiSS1 gene has the key role in production of a family of neurotransmitter peptides already known as Kisspeptins. KiSS1 (Metastatin) is a metastatic suppressor gene first discovered in 1996 (Lee *et al.* 1996) and then was described as the coding sequence of a family of neuropeptides called Kisspeptins (Kotani *et al.* 2001) which they are all functioning through an orphan G coupled protein receptor known as GPR-54 (Oakley *et al.* 2009). There is a possibility that this gene family be the key to reproductive rebound in dog non seasonal anestrus. After mRNA translation of KiSS1, a 145 amino acid precursor polypeptide (KP 145) rises which will be cleaved to a 54 aa peptide (KP 54) and also could be truncated to shorter peptides such as 14,13,10 aa (KP14-KP13-KP10) (Roseweir & Millar 2009). The important role of Kisspeptins is to govern GnRH secretion from GnRH neurons in Hypothalamus (Pre optical Area–POA) and Median Eminence (ME). KiSS1 neurons in anteroventral periventricular preoptic area (AVPv) and Arcuate Nucleus (ARC) are under influence of sex steroids (e.g. Estrogen, Progesterone, Testosterone) , leptin, melatonin secreted from pineal gland, glucocorticoids and other environmental factors (Roseweir & Millar 2009). In terms of reproductive endocrinology Kisspeptins are the introductive backbone to the Hypophysial Pituitary Gonadal axis (HPG) and in the ovariohisterectomized animals they stay at highest levels of production in hypothalamus after the operation; in addition, in the seasonal breeders during the non-reproductive seasons kisspeptins will also be at the lowest levels (baseline) (Oakley *et al.* 2009, Roseweir & Millar 2009).

1.3. Folliculogenesis and Oogenesis

The other unique specification of canine cumulus oocyte complex (COC) is the nuclear stage of oocyte in terms of meiotic resumption at the time of ovulation. In contrast to the majority of mammals, canine oocytes are ovulated at the prophase of the 1st meiotic division (germinal vesicle, GV) and complete the maturation process to metaphase II stage (MII) 48-72h after ovulation in the oviduct (Hewitt & England 1999, De los Reyes *et al.* 2011). Primordial follicles of ovarian cortex contain the non-growing primary oocytes dictyated at the diplotene stage of first meiotic division (Scaramuzzi *et al.* 2011). During the commitment of the follicle to non-gonadotropin related folliculogenesis and even after conversion of primary follicles to gonadotropin responsive secondary and tertiary, oocyte will maintain its diplotene stage. Alongside the primordial and preantral gonadotropin independent oogenesis which is governed by growth factors (insulin like growth factor [IGF-1], Fibroblast growth factor [FGF-10] and Epidermal growth factor [EGF]) and kit ligands (Scaramuzzi *et al.* 2011), the canine nuclear maturation is timely separated from gonadotropic induction (Concannon *et al.* 2009) by a wide effective window.

In most of mammals the pre-ovulatory LH surge leads in to disruption of oocyte arrest and resumption of meiotic changes up to extrusion of first polar body and formation of metaphase II (Luvoni *et al.* 2005, Suzukamo *et al.* 2009). However in dogs the ovulated oocytes still need to progress their nuclear maturation throughout the oviductal transition period (Reynaud *et al.* 2006, Concannon 2011). These nuclear changes are as following:

1. Prophase I (Germinal Vesicle = GV)

1. A) Leptotene (visible strand of chromatin)

1. B) Zygotene (Synapsis inside tetrads)

1. C) Pachytene (Crossover at chiasmata exchange sites)

1. **D) Diplotene** (Slight uncoiling accompanied by DNA transcription)

1. **E) Diakinesis** (Germinal Vesicle Break Down = **GVBD** + chromatin condensation)

2. Metaphase I = MI when bipolar meiotic spindles [kinetochore microtubules] from two centrioles attach to their respective partners and homologous chromosomes will be arranged equatorially at metaphysial plate that bisects the spindles.

3. Anaphase I = AI In which kinetochore microtubules shorten and pull the bivalents apart from each other to the opposite end of spindle.

4. Telophase I = TI The 1st polar body formation and arrival of chromosome sets at opposite poles.

5. Metaphase II = MII Characterized by the margination of the tightly packed first polar body and the rearrangement of the chromosomes back to chromatin conformation.

When the oocyte reaches the MII stage, it can remain fertile in bitch's oviduct up to several days (Reynaud *et al.* 2006). In the case that the fertility window for matured oocytes in other species, normally hovers around several hours before ageing takes over (Reynaud *et al.* 2005, Reynaud *et al.* 2006). The completion of second meiosis division will take place after the penetration of the sperm and the hardening of zona pellucida (ZP).

1.4. Anatomical and Histological peculiarities

Dog reproductive system has outstanding features which are not common among other domestic animals. Dog ovaries are caudally located to kidneys suspended in the abdomen. In a 25 pound dog they weight approximately 0.3g with a dimension of 1.5 cm (L) x 0.7 cm (W) x 0.5 cm (D) on average (Evans 1993). Canine ovaries are formed of modula (blood and lymphatic vessels, nerves, smooth muscle and connective tissue) and cortex (germ cells [primordial

follicles]). Like other mammals follicles develop from primordial status to primary and then secondary follicle through proliferation of granulosa cells and oogenesis (Evans 1993, Concannon 2011). Recruitment, selection and dominance of follicles happens like other mammals and between 7 to 10 oocytes are ovulated in each estrus cycle (Evans 1993). Ovaries during anestrous are oval and there is no appreciable follicular structure on their surface (Noakes *et al.* 2001). At the end of proestrus preovulatory follicles 0.7-1.1cm in diameter are visible on the cortex with unusually thick follicular wall. The preovulatory lutenization of follicle transforms the follicular wall very similar to corpus luteom (CL) at this stage (Noakes *et al.* 2001). The CL structure is not severely protruding as in bovine and ovine ovaries and they are mainly embedded into the cortex (Evans 1993).

Ovarian bursa which originated from *mesosalpinx* (part of the broad ligament of uterus that enfolds the oviducts) covers bitch ovaries in 360 degrees manner. In other animals this bursa normally covers half, 2/3 or maximum 3/4 of the ovarian surface (Senger 2003). Another extraordinary feature is multi-oocytic follicles in the cortex of the dog ovaries in contrary with the each follicle, one oocyte routine. There are reports of 5-14% prevalence of follicles containing 13-17 oocytes which actually ovulate that population in a single event (Luvoni *et al.* 2005, Payan-Carreira & Pires 2008, Reynaud *et al.* 2009). Although only the biggest oocyte regarding the size has the competency required for further development, this ability is still preserved in this poly ovulatory animal (Reynaud *et al.* 2006).

1.4.1. Lipid yolk

Speaking of oocytes it's noteworthy that canine female gametes contain large amounts of lipid yolk which represents the importance of energy supply during the prolonged period of oviductal travel and the maternal zygotic transition period. (Fig. 1.3)

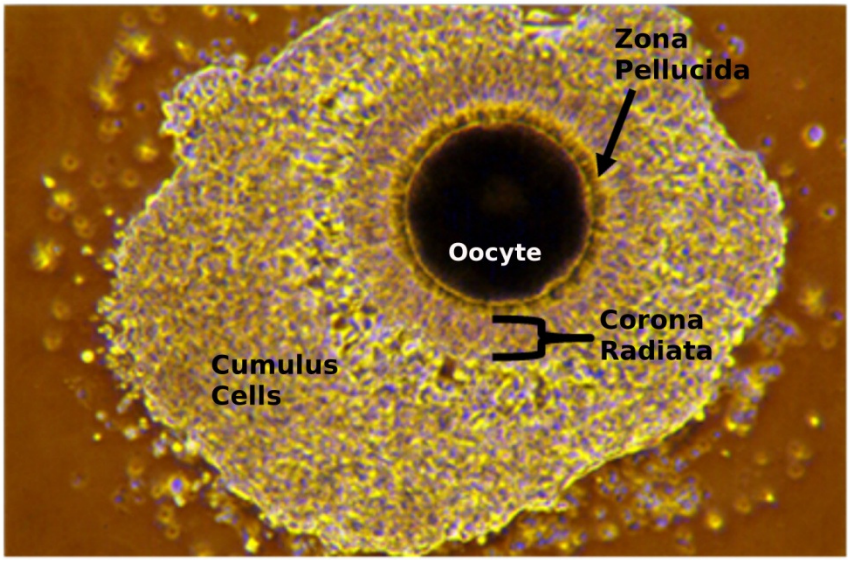


Figure 1.3 – Canine COC cultured in vitro. Picture of canine COC was taken using a digital camera at the beginning of the culture (20X). The oocyte has been squashed between a coverslip and a slide in order to visualize different layers and compartments of the COC.

Abundant lipid droplets in canine oocytes which occupy 80-90% of the visible ooplasm surface (Guraya 1965, Tesoriero 1982, Songsasen *et al.* 2009) have been a persistent issue in chromatin content visibility of these oocytes (Reynaud *et al.* 2005).

1.4.2. Zona pellucida

In the majority of mammals the main organism in charge of zona pellucida (ZP) production is either oocyte or it will orchestrate cumulus cells to provide sufficient material to shoulder the production pressure. On the contrary in canid family ZP-A proteins are secreted from oocyte and ZP-B, ZP-C are originated from corona radiata cells (Blackmore *et al.* 2004). These proteins transcription is completely sequential during folliculogenesis from two different sources. (A > B /C)(De los Reyes *et al.* 2009a) Due to entrapment of two or three most inner layers of cumulus cells inside the ZP structure of oocytes it is difficult to denude them using routine and normal denuding protocols. ZP surface changes during maturation are crucial to sperm penetration and further monospermic syngamy protective mechanisms. Regarding the smoothness and the pore size of the

protein matrix there is difference between dogs and other domestic animals. During *in vitro* maturation in bovine and porcine oocyte the pore size of ZP protein matrix will decrease gradually in 24-48 hrs. However in dog this pore size rises from 0 h up to 72hrs of maturation and then will reduce drastically by 96 hrs. It's been elucidated that sperm penetration is also less successful after 96hrs rather than 72hrs of culture(De los Reyes *et al.* 2009a).

1.5. Assisted reproductive technologies (ART) in dogs

First attempts to produce *in vitro* matured oocytes has been done in 70's when Mahi and Yangimachi (Mahi & Yanagimachi 1976) tried to carry out canine oocyte IVM with ovaries collected at random stages of reproductive cycle. Moreover they succeeded to achieve 25 % maturation to M I/II stages after 48-72 hours of culture. Subsequently they conducted IVF and reported 70 % of sperm penetration with 20% male pronuclear (MPN) formation. Several years later Yamada *et al.* in two close attempts in 1992 (Yamada *et al.* 1992) and 1993 (Yamada *et al.* 1993) improved canine oocyte maturation to M II stage up to 32% and even succeeded to report the ability of developing 8 cell embryos from *in vitro* fertilized oocyte during those studies. After that Nickson *et al.* in 1993 (Nickson *et al.* 1993) cultured oocytes in Tissue culture media 199 (TCM 199) enriched with estrus canine serum which succeeded to have 39% first polar body extrusion by 24 hours. Then oocytes were inseminated with fresh semen and obtained 37.5% of pronucleus formation but no further development was reported. Combining steroid hormones with maturation mediums Hewitt *et al.* in 1997 (Hewitt & England 1997) conducted experiments in different reproductive periods and multiple hormonal treatments. Their results showed that high levels of steroid hormones have no significant effect on oocytes quality and their highest M II maturation rate never exceeded 20%. In 1998 Bolamba *et al.* utilized Dulbecco's Modified Eagle Medium (DMEM) enriched with 20% BSA and in combination with FSH, Human Chorionic Gonadotropin (hCG) and Estradiol in 24, 48, 72 h of culture and reported the meiotic resumption percentage of 5% , 11.5 % and 9.9% respectively (Bolamba *et al.* 1998). Later Hewitt and England in 1999

(Hewitt & England 1999) used Synthetic Oviductal Fluid (SOF) which was recommended for the first time by Tervit *et al.* 1972 for IVM in ovine oocytes(Tervit *et al.* 1972). Their culture media was supplemented with high concentrations of BSA and improvement of maturation was obtained in the co-culture of oocytes with oviductal monolayer cells (MI/AI/MII = 6%). Otoi *et al.* (Otoi *et al.* 1999) investigated the effect of adding canine serum of different reproductive stages to the IVM culture medium and showed improving impact of Estrus Canine Serum (ECS) on meiotic resumption. They concluded that 10% of ECS in culture medium is the optimal concentration for improving the maturation rate. These authors also showed that oocytes diameter is a crucial factor for its developmental competence and the required range is at least 110-120 µm of diameter (Otoi *et al.* 2000a, Otoi *et al.* 2000b). Otoi *et al.* also studied IVM protocol for a 72 h period in 2001 and reported 16.2 % of M II matured oocytes (Otoi *et al.* 2001). In the same year Luvoni *et al.* (Luvoni *et al.* 2001) compared oocytes collected in late proestrus and anestrus stages and reported 11 % M II maturation rate in late proestrus. Songsasen *et al.* in 2002 (Songsasen *et al.* 2002) using Poly vinyl alcohol (PVA), achieved the highest maturation rate of 33.3% at M II within 48 hours of incubation. Bolamba *et al.* in another attempt in 2002 (Bolamba *et al.* 2002) reported 42% of meiotic resumption, 19.2% of oocytes in M I/II and 16.7 % of M II. Otoi *et al.* repeated their 72 hours remedy again in 2002 (Otoi *et al.* 2002) with some modifications in oocyte culture density in medium drops and announced that the optimal density depends on the reproductive cycle of the oocytes. Another study in 2002 was carried out by Bogliolo *et al.* (Bogliolo *et al.* 2002) in which coculture of infundibular and ampullar monolayer cells with oocytes was studied. They reported that the highest meiotic resumption was in IVM combined with ampullar coculture; moreover, 48 hours was sufficient for nuclear maturation to complete. Willingham-Rocky *et al.* in 2003 (Willingham-Rocky *et al.* 2003) succeeded to achieve 23 % of M II oocytes with 59 % of meiotic resumption in 68 hours culture with a medium enriched by dbcAMP and progesterone. In 2003 Luvoni *et al.*

were able to improve their results to 12.5% M I/II and 59.1 % meiotic resumption by lowering the culture time to 30 hours and IVM in isolated (ligated) oviduct (Luvoni *et al.* 2003). Kim *et al.* in 2004 profoundly investigated the effects of β Mercapto-Ethanol (βME) and Epithelial Growth factor (EGF) and reported the supportive roles of these two supplements in improving the maturation rate of canine oocytes. Later Rodrigues *et al.* (Rodrigues Bde *et al.* 2004) tried to utilize SOF with 4 mg/ml BSA as the IVM medium and succeeded to improve the maturation rate up to 10-14 % in M I/II stage; moreover, they studied the *in vitro* insemination and developmental potentials of canine *in vitro* produced embryos in comparison with the reproductive cycle of oocyte donors and proved that there is no significant correlation between them. Kim *et al.* in 2005 (Kim *et al.* 2005) showed that estradiol or progesterone individually could increase maturation rate. However combination of these two hormones didn't improve the results. On the other hand in 2009 Vannucchi *et al.* (Vannucchi *et al.* 2009) during a similar study proved that estrogen solely has no influence on oocyte maturation unless it's used in combination with progesterone. They have postulated that presence of progesterone in the IVM media will lead into higher GVBD even up to 96hrs of culture. De los Reyes *et al.* 2005 evaluated the influence of human chorionic Gonadotropin (hCG) on canine oocyte maturation. They reported the highest maturation to M II stage in their first 48 hours. In 2009 Alhaider *et al.* reported only up to 5% MII rate when equine and human chorionic gonadotropins were used in the presence of fibroblast and epithelial growth factors (FGF-EGF). They concluded that hCG inhibits the positive effects of growth factors on oocyte maturation (Alhaider & Watson 2009). In 2007 Lee *et al.* (Lee *et al.* 2007a) confirmed that FSH treatment will improve cumulus cells expansion and increase the percentage of MI-MII oocytes. In 2005 Reynaud *et al.* (Reynaud *et al.* 2005) proved that the fertilization and cleavage development of canine embryos *in vivo* follows the same rules as other mammals and IVM is still the biggest obstacle in the long journey of establishment of assisted reproductive technology in canid family (Rodrigues & Rodrigues 2010, Chastant-Maillard *et al.*

2011). Throughout years of several studies and multifaceted experiments there are still so many aspects of canine IVM/IVF which requires profound enlightening.

1.5.1. Oxidative stress

High degeneration (>50%) and very low MII maturation rates (16.2 ± 4.2%) (Luvoni *et al.* 2005, Rodrigues & Rodrigues 2010) are the main features of current IVM in the dog. Dog oocytes contain large amount of lipid which occupies 80-90% of the visible ooplasm surface (Guraya 1965, Tesoriero 1982, Songsasen *et al.* 2009). This energy supply serves the important purpose of maintaining the oocyte and presumptive zygotes (Guraya 1965, Luvoni *et al.* 2005, Lopes *et al.* 2010). However, presence of these abundant lipid droplets in ooplasm has an impeccable influence on predisposition of COCs to oxidative stress introduced by reactive oxygen species (ROS) (Wakefield *et al.* 2008, Whitaker & Knight 2008, Tao *et al.* 2010). Oxygen concentration during the IVM culture period can contribute to the extent and velocity of this oxidative stress of which oocyte nuclear and cytoplasmic maturation and development pattern may perturb (Kim *et al.* 2007, Whitaker & Knight 2008).

Glutathione (GSH) is the main non-enzymatic cellular defense mechanisms against ROS and other free radicals (Guerin *et al.* 2001, Menezo *et al.* 2010). GSH becomes a substrate of glutathione peroxidase (GPX) in alliance with Catalase (CAT) which degrades hydrogen peroxide (H_2O_2) to water and oxygen (Whitaker & Knight 2008). H_2O_2 itself is the resulting product in neutralization of ROS by superoxide dismutase (Cytosolic SOD=Cu-Zn SOD1, Mitochondrial SOD= Mn-SOD2) (Guerin *et al.* 2001). In order to recover GSH oocytes utilize another enzyme, Glutathione reductase (GSR), to reduce the GS-SG (oxidized form of GSH) back to GSH (Guerin *et al.* 2001). Because of lower levels of GSH within *in vitro* matured canine oocytes (<8 pMol/oocyte) in comparison with *in vivo* (19.2 pMol/oocyte) (Kim *et al.* 2007), high levels of H_2O_2 and ROS within COCs during IVM can severely impair maturation and increase degeneration rates (Whitaker

& Knight 2008). These studies showed that the H_2O_2 level in oocytes is a good indication of oxidative stress.

High oxygen level has detrimental effects on cumulus cell survival (Silva *et al.* 2009), which influences expansion during oocyte maturation *in vitro*. In the majority of *in vitro* maturation techniques in domestic animals cumulus cell expansion and nuclear maturation are concomitant phenomena during the culture period. In other words expansion of cumulus cells could be a good indicator of the right conditions for nuclear maturation of oocytes within the IVM process (Chen *et al.* 1990, Qian *et al.* 2003). In canine oocytes the relationship between cumulus expansion and nuclear maturation is quite controversial (Otoi *et al.* 2007, Chebrout *et al.* 2009, Reynaud *et al.* 2009), and there have been counterintuitive interpretations for cumulus expansion in canine IVM (Reynaud *et al.* 2005, Reynaud *et al.* 2006, Lee *et al.* 2007a, Chastant-Maillard *et al.* 2010). *In vivo*, expansion of cumulus cells may not concomitant with meiotic resumption since the maturation occurs after ovulation while the expansion initiation time is not clear (Reynaud *et al.* 2009). *In vitro*, extensive mucification of cumulus cells occurred only in the presence of canine serum in the maturation media (Lopes *et al.* 2011). The credibility of cumulus expansion as a reliable indicator of oocyte maturation is to be further elucidated.

1.5.2. PDE inhibitors and maturation

Collection of ovaries during anoestrus (most common phase in which ovariohisterectomy is conducted in) is expected to provide immature oocytes arrested at the GV stage and from secondary or preantral follicles which were not recruited by preovulatory surge of gonadotropic hormones (Vannucchi *et al.* 2009). During the meiotic arrest the stationary status of oocyte nucleus is preserved through maintaining cAMP level within the cell (Luciano *et al.* 2004). The preovulatory luteinising hormone (LH) surge induces a temporary increase in cAMP level within the granulosa cells and utilizes it as a second messenger to affect downstream molecules such as epithelial growth factors (EGFs)

(Mehlmann 2005, Zhang *et al.* 2009) leading to a decrease in cAMP level and resumption of meiosis in the oocyte. The LH mediated changes in cAMP level has been hypothesized to act via three major pathways (Mehlmann 2005): 1.G-protein coupled receptors (GPRs) and their inhibitory components (G_i) of which GPR3 activation promotes adenylate cyclase (AD) phosphorylation and cAMP production in the cumulus cells. 2. Direct activation of phosphodiesterases 3A (PDE-3A) within the oocyte resulting in degradation of cAMP. 3. Elevation of intracellular calcium (Ca^{2+}) level that inactivates AD and lowers cAMP level (Mehlmann 2005). Downstream key player molecular of all these pathways is cAMP and it seems that gaining control over its concentration changes could be critical in promoting oocyte nuclear maturation.

In canine oocytes the time-course of meiotic resumption is also not clear. The molecular events leading to nuclear changes of the oocytes during meiotic progression are spanned to time period between preovulatory stage up to 2-5 days after ovulation and during oviductal transition (Concannon 2011). Therefore an *in vitro* culture ought to be biphasic in order to mimic the *in vivo* events of gonadotropic induction and oviductal transition. During this critical follicular-oviductal transition of the oocytes, the relative changes in the concentration of cAMP are precedential (Luciano *et al.* 2004, Saint-Dizier *et al.* 2004). The maintained cAMP concentration within the oocytes during meiotic arrest support the activity of cAMP dependent protein kinase A (PKA) which activates WEE1/Myt1 pathway in order to keep maturation promoting factor (MPF) phosphorylated and inactive (Mehlmann 2005, Smitz *et al.* 2011). On the contrary, increased levels of cAMP within the cumulus cells as a second messenger of gonadotropic hormones activates EGF downstream pathway leading to phosphorylation of phosphokinase C & AII (PKC/PKAII) , PI3K/AKT (PKB) and consequently activates MAPKs which promote MPF activity and meiotic resumption (Tripathi *et al.* 2010). The changes in the gap junctions between the oocyte and the cumulus cells are also orchestrated via MAPK activation (Luciano *et al.* 2004). Hence, manipulating the cAMP pathways in

favour of higher similarity to *in vivo* conditions may improve maturation efficiency. Downstream of all molecular pathways leading to activation of MPF and resumption of meiosis is the abrupt decrease of cAMP concentration within the oocyte (Wehrend & Meinecke 2001, Zhang *et al.* 2007, Zhang *et al.* 2009). However, cAMP levels in the cytoplasm of cumulus cells and oocyte have different destinies. Although high levels of cAMP inside the oocyte will maintain the meiotic arrest, in cumulus cells accumulation of cAMP is crucial for activation of MAPK. Besides, the type of phosphodiesterase (PDE) in charge of degradation of cAMP varies between oocytes and cumulus cells (PDE-3 and PDE-4 respectively). Therefore inhibition of PDE-4 and accumulation of cAMP inside cumulus cells improves the cytoplasmic maturation process by activation of MAPK. On the contrary the inhibition of PDE-3 within the oocyte maintains the meiotic arrest (Bornslaeger *et al.* 1984, Bornslaeger & Schultz 1985, Liang *et al.* 2007).

Inhibition of PDE has been utilized to manipulate cAMP level and influence the intracellular pathways employing cAMP as a second messenger (Bornslaeger & Schultz 1985, Tsafriri *et al.* 1996, Lee & Campbell 2006, Wang *et al.* 2008). Methylxanthines, a family of PDE inhibitors, such as caffeine, IBMX (1-methyl-3-isobutylxanthine) and theophylline can serve the purpose of sustaining cAMP level (Minelli & Bellezza 2011). Caffeine has been used to improve male gametes fertilization and female gametes developmental potential as an established culture supplement within a specific range of concentration (Abeydeera *et al.* 1994, Kren *et al.* 2004, Lee & Campbell 2006, Choi *et al.* 2010). Caffeine acts as a non-selective competitive PDE inhibitor within the COC (Minelli & Bellezza 2011). Due to its structural similarity with adenosine molecules, it maintains the cAMP level by occupying PDE enzymes such as PDE3-A in the oocytes and PDE4 and PDE5 in the cumulus cells (Smitz *et al.* 2011). Members of methylxanthine family have been shown to completely prevent germinal vesicle breakdown (GVBD) in rodents and entirely/partially inhibit GVBD in larger animals (Sirard & First 1988, Lonergan *et al.* 2003, Smitz *et al.* 2011). In other terms, sufficient concentration

of caffeine in the maturation media can slow down/arrest the nuclear changes and preserve the intercellular gap junction in the designated time period. However the effects of caffeine have not been examined in dog oocytes yet.

1.5.3. Hormonal supplementations

The selection of basic maturation medium for the prolonged culture of canine oocyte is of great importance, since the components of the maturation medium have vital impact on the COCs nourishment, viability, degeneration and developmental profile (Luvoni *et al.* 2005). Moreover the maturation medium should be able to support hormonal requirements of oocyte during IVM. It's a quintessential fact about canine IVM that an ensemble of 9-day-proestrus hormonal priming (estrogen signal and other unknown factors) and 2-5days post ovulatory oviductal secretions cannot be summarized in 3-4 days of *in vitro* culture(De Lesegno *et al.* 2008). However the maturation media in other species such as bovine (Marei *et al.* 2009), ovine (Natarajan *et al.* 2010), porcine(Tao *et al.* 2010) and feline (Luvoni *et al.* 2006b) consists of combinations of gonadotropins, steroids and growth hormones. As progesterone is one of constant steroid hormones during late folliculogenesis in dogs (Fahiminiya *et al.* 2010) and considering the hormonal profile of canine proestrus and estrus, It has been devised to use LH (5µg/ml), FSH (5µg/ml), E2 (1µg/ml) and P4 (1µg/ml) at the same time for the base maturation media of this study. Reviewing other similar studies this treatment seems to be closest hormonal supplementation to *in vivo* concentrations (Concannon *et al.* 2001, Willingham-Rocky *et al.* 2003, Kim *et al.* 2005, Lee *et al.* 2007a, Vannucchi *et al.* 2009, Concannon 2011). Throughout the 3 years of this study many changes were molded into this basic maturation media to improve the maturation rates and developmental potentials.

1.5.3.1. Insulin like growth factor 1 (IGF-1)

One of the key molecules responsible for follicular growth during the gonadotropin independent period is the hepatic insulin like growth factor (1&2)

(Mazerbourg *et al.* 2003). IGF increases the proliferation of granulosa cells via inducing gonadotropic responsiveness specifically toward FSH. Although the IGF levels during the pre-ovulatory period is constant, bio-availability of this hormone changes during the selection of the dominant follicle. IGF binding proteins regulate (inhibition IGFBP2, 4, 5; stimulation IGFBP3) the bio-availability of this hormone via management of IGF ligand/receptor interactions (Mazerbourg *et al.* 2003). IGF-1 plasma and follicular fluid levels are simultaneously matched and it's bio-availability has direct effect on late/antral follicular changes. The IGFBPs are also degraded by a 400kDa pregnancy associated plasma protein (PAPP-A) found in high concentrations within the follicular fluid of the dominant/antral follicles (Reynaud *et al.* 2010).

1.5.3.2 Epithelial growth factor (EGF)

EGF family (Amphiregulin [AREG], Epiregulin [EREG], Beta-cellulin [BTC], Tumor growth factor α [TGF-α], epigen and neuregulin) are all integral membrane precursors sharing a EGF like domain (Conti *et al.* 2006). These peptides are shed through proteolytic (ADAM protease family) cleavage of the ecto-domain from theca and mural granulosa cells in order to influence the COCs cell-cell interaction and oocyte maturation (Conti *et al.* 2006). There is a general observation among different species that LH receptors (LHR) on cumulus cells and oocytes are either absent as in mice (Conti *et al.* 2006) or hardly expressed like dogs(Song *et al.* 2010b). The EGF receptors (EGFR) have also very weak expression signal in the canine COCs (Song *et al.* 2010b). However the presence of LHR on the theca cells and mural granulosa population within the follicle along with EGFR has been found in a paracrine functional system (Conti *et al.* 2006). It also has been proven that during pre-ovulatory stage when oocyte and cumulus cells are almost insensitive to any LH signal, the maturating promoting message of LH surge is translated through EGF signaling pathway and gap conjunctions (Park *et al.* 2004). Briefly LH signal activates the G protein couple stimulatory component of LHR via activation of adenylate cyclase and production of cAMP. The cAMP as the second messenger regulates the pathway to up regulation of

EGF like peptides shedding in to the follicular fluid and cumulus cells gap junctions. This paracrine effect promotes cumulus expansion (through FSH receptor activation) and nuclear maturation of the oocyte (MAPK phosphorylation) (Conti *et al.* 2006). There is hard evidence for up-regulation of AREG, EREG and BTC just 3 hours after the administration of LH analogues (Park *et al.* 2004). Phosphorylation of EGF receptors during the first 6hours of LH treatment is also gradually increasing (Conti *et al.* 2006).

1.5.3.3 Optimal Protein Source (Serum vs. BSA)

According to the literature addition of fetal bovine serum (FBS) is not a good source of protein due to reduction of GSH level and increase of oxidative stress over oocyte maturation (Concannon *et al.* 2009, Rodrigues & Rodrigues 2010). The best replacement for FBS is either canine estrus serum (Concannon *et al.* 2009) or 0.3%-4% BSA (Hewitt & England 1999). The Addition of the canine estrous serum due to its unknown hormonal ingredients and indeterminate collection time had been utilized in different studies with controversial outcomes. Some reports are in favor of serum addition up to 10% (Otoi *et al.* 1999, Oh *et al.* 2005) and the rest question the essentiality of its presence (Bolamba *et al.* 2002, Salavati *et al.* 2012). However; serum enrichment of the maturation media is one of the oldest and most conventional supplements in oocyte and embryo culture.

1.5.3.4. Porcine growth hormone (PGH)

The presence of the GH in the cytoplasm of canine oocytes and the cumulus cells has been confirmed in previous studies (Chigioni *et al.* 2008). Supplementation of GH in oocyte maturation media accelerates the meiotic resumption and promotes developmental potential (pronucleus formation) in cattle (Izadyar *et al.* 1996) and rat (Apa *et al.* 1994)IVM. The GH receptor (GHR) is a cytokine/hematopoietin receptor super family activating cytosolic tyrosine kinase JAK2 signaling pathway and It's believed that GHR activation is transduced through IGF-1 downstream reactions (Bevers & Izadyar 2002). Moreover; cAMP

signalling system has also been suggested as an eligible candidate for GH ligand binding to GHR (Bevers & Izadyar 2002). Canine and porcine growth hormones (cGH,pGH) have identical amino acid sequences and high level of similarity (Chigioni *et al.* 2008). Thus recombinant PGH has been utilized in this study within the maturation media.

1.5.3.5. Melatonin

Melatonin is an indolamine hormone secreted from pineal gland, retina, extra-orbital lacrimal glands, bone marrow and ovaries (Adriaens *et al.* 2006). Physiological concentrations of 1nM melatonin have been reported in the follicular fluid of porcine (Shi *et al.* 2009) and bovine (Takada *et al.* 2010) species even higher than plasma (Nakamura *et al.* 2003). Main sources of ovarian melatonin are granulosa cells and COCs (Reiter *et al.* 2007). Melatonin influences the reproductive cycle and hormonal changes in uterus and ovaries through its dedicated G- protein coupled receptors (MTNR-A1&B1) (Reiter *et al.* 2007). MTNRA1 and acetyl serotonin O-methyltransferase (ASMT; melatonin producing enzyme) mRNA expression have been reported in both oocyte and cumulus cells; however MTNRB1 is only present in cumulus cells (Kang *et al.* 2009, El-Raey *et al.* 2011). This highly lipophilic hormone has chemically direct and potent antioxidant effects, beside supporting the expression of reactive oxygen species (ROS) repairing enzymes (Adriaens *et al.* 2006, Takada *et al.* 2010). Although melatonin could be the best natural antioxidant in maturation media, it has dose dependent toxicity level and most studies reported 1nM concentration to have improving effect in IVM and embryo production (Shi *et al.* 2009, Rocha *et al.* 2012). Besides antioxidative effects, melatonin has also proven to differentially regulate estradiol and progesterone production regarding the time point of follicular/luteal phase (Chuffa *et al.* 2011, Pedreros *et al.* 2011, Wang *et al.* 2012)

1.5.4. Developmental potentials (PA/IVF)

1.5.4.1. Parthenogenetic activation (PA)

One of the methods of obtaining pluripotent embryonic stem cells which has been developed during recent years, is activation of oocytes or Parthenogenesis (Brevini *et al.* 2008, Brevini *et al.* 2009, Brevini *et al.* 2010). Parthenogenetic activation (PA) is the procedure in which the resumption of second meiosis is carried out without the penetration of the sperm or any male genomic content (Lee *et al.* 2007c). The parthenotes produced by PA are the fittest model for study of genetic imprinting in humans and animals (Brevini *et al.* 2008). PA is conducted via several methods which promote anaphase II (AII), Telophase II (TII) progression under the same principles: 1. the utilization of free Calcium concentration (intra cytoplasmic or extra cellular) to mimic the Ca^{2++} influx of sperm penetration and cytoplasmic signaling that follows. 2. To disturb the MII maintaining signals (high MPF activity and phosphorylated MAPKs) in order to push the oocyte through AII-TII transition accompanied by cortical reaction and separation of the second polar body (Lee *et al.* 2007c). As examples of chemicals used for PA, Ionomycin releases Ca^{2+} from cytoplasmic reservoirs and electric stimulation or ethanol induce the calcium influx from the extracellular microenvironment. Protein kinase/synthase inhibitors such as cyclohexamide or 6DMAP perturb the protein machinery in charge of MII maintenance (Lee *et al.* 2007c). The blastocyst formation rate between 20% -50% have been reported from (Azuma *et al.* 2001, Lee *et al.* 2004, Sato *et al.* 2005) porcine, bovine (Campbell *et al.* 2000) and ovine (Loi *et al.* 1998) parthenotes. Previous attempts have been made to integrate PA in to canine IVM (Song *et al.* 2010a)and *in vitro* embryo production (IVEP) (Lee *et al.* 2007c, Lee *et al.* 2009, Kim *et al.* 2010) systems of which results are not satisfactory.

1.5.4.2. In vitro fertilization (IVF)

Introduction of male and female gametes in order to produce *in vitro* cultured embryos is a renowned ART technique in human and domestic animals. *In vitro*

fertilization of dog oocytes due to previously discussed reasons has also been one of the most complicated procedures for reproductive biologists in this field (Luvoni *et al.* 2005, Luvoni *et al.* 2006a, Rodrigues & Rodrigues 2010). IVF not only requires matured competent oocytes, but also consists of multiple steps of sperm quality/motility test, sperm capacitation, penetration and male pronuclear formation (MPN) (Brewis *et al.* 2001). The capacitation and acrosomal reaction of dog sperm are still under investigation as of diverse reports of it occurrence during oviductal accommodation (Brewis *et al.* 2001) or in contact with ZP binding sites (Kawakami *et al.* 2004). Not only the cumulus cells behavior but also the sperm penetration patterns are unlike other mammals. The fertile oocyte can remain up to 6 days with 100% conception rate and the inner most layers of cumulus cells packed and intact without any mucification (Reynaud *et al.* 2005, Reynaud *et al.* 2006, Tsutsui *et al.* 2009). There were several attempts to produce *in vitro* cultured blastocyst in canine species of which most of them couldn't get pass the 8 cell block (Rodrigues Bde *et al.* 2004, Lee *et al.* 2007b). Except sporadic single blastocyst production reports (Mahi & Yanagimachi 1976, Otoi *et al.* 2000b), the cleavage rates and number of ≥8 cell embryos are drastically low (Renton *et al.* 1991, Yamada *et al.* 1992, Yamada *et al.* 1993, Reynaud *et al.* 2004, De los Reyes *et al.* 2006, Luvoni *et al.* 2006a, Lee *et al.* 2007b, Saikhun *et al.* 2008). Although multiple sperm penetration (polyspermia) is rare *in vivo*, two frequently reported features of canine IVF are as following: 1. High polyspermia and premature sperm penetration 2. High degeneration rates accompanied with 2 and 8 cell blocks (Reynaud *et al.* 2006).

1.6. Summary

To sum up dogs are polytocous, mono-estrous, spontaneous ovulatory, non-seasonal breeders that ovulate oocytes at the germinal vesicle stage (GV; Diplotene of the first prophase of meiotic division) in contrast with other domestic animals which usually ovulate oocytes at metaphase II stage of first meiosis. As previously described, in contrast with all mammalian species , canine oocytes complete their meiotic maturation to metaphase II (M II) stage during 48

– 72 hours within the oviducts (Lane & Lofstedt 1996). *In vitro* maturation rate of bitch oocytes is very low with the highest success rate reported to be about 33 % (Songsasen *et al.* 2002) compared to other domestic species which maturation rate of 80-100% is achieved .

The maturation process generally contains two main steps (Lane & Lofstedt 1996):

1. Nuclear maturation that includes the development of nucleus from GV to MII. Germinal vesicle will go through the membrane break down (GVBD) and condensation of chromatin to resume meiosis and develop to Metaphase I (M I) , Anaphase I (A I), Telophase I (T I) followed by the M II stage. Further development of oocytes occurs only during the process of fertilization. Penetration of sperm into the oocytes activates them to complete meiosis II. Such phenomenon can be mimicked using chemicals or electric current which release Ca^{2+} and cause parthenogenetic activation of oocyte.

2. Cytoplasmic maturation which is a complex of different organelles redistribution and cascades of protein reactions resulting in both structural and functional changes required for cellular division.

The very first elements of an efficient *in vitro* maturation protocol for culture of canine COCs should be established considering their peculiar specifications. Gas composition, media components, protein/energy sources and hormonal supplementations should all be tailored to the requirements of the oocytes during post ovulatory period. Moreover; hormonal priming of the follicular environment should also be considered within the maturation media supplementations.

During meiotic maturation, protein kinases play key roles in the cell cycle regulation. Maturation promoting factor (MPF) , a complex of a catalytic subunit

P^{34}cdc2 activated at GVBD stage, triggers chromatin condensation , nuclear envelope breakdown, and the formation of the first meiotic spindle (Wehrend & Meinecke 2001, Schatten & Constantinescu 2007). In addition to MPF activation , entry into meiotic metaphase coincides with the activation of mitogen activated protein kinase (MAPK) such as serine/threonine kinases of 41-42 (MAPK1[ERK2]) and 43-44 kDa (MAPK3[ERK1]) , which are involved in many signal transduction pathways (Abrieu *et al.* 2001). Among these maturation factors MAPK family also known as Extracellular regulated kinases (ERK) have a unique and gradual increase during the whole maturation process (Wehrend & Meinecke 2001); thus, they have been chosen as credible indicator of maturation development.

Developmental potential and actual performance of the matured oocytes can be evaluated through *in vitro* fertilization using sperm or parthenogenetic activation of the first polar body to act as a male pronucleus.

1.7. Hypothesis

The main objective of this thesis was to establish an efficient *in vitro* maturation protocol including the sample collection, oocyte extraction, culture conditions, staining and staging of the oocytes. In case of successful IVM attempts were made to evaluate developmental potentials of canine oocyte. During the studies of this PhD project the results of chapter 3 onwards were implemented in the experimental design of each following chapters.

In the first experimental chapter (chapter3) the most primitive obstacles of canine oocyte maturation were studied as following:

- Setting up a chemically defined, serum free culture media capable of maintaining prolonged duration of culture required for canine oocyte maturation.
- Establishment of an efficient, accurate and discriminative nuclear staining and staging protocol optimized tailored to unique characteristics of canine oocytes.

- Analysis of the oocyte culture performance under two different gas compositions (Low oxygen = 5% O_2 - 5% CO_2 - 90% N_2; High oxygen = 5% CO_2 in air) in order to clarify the effect of oxygen tension over nuclear maturation.

Within the second experimental chapter (Chapter 4) effects of caffeine as the non specific PDE inhibitor were studied as following:

- Devising a biphasic maturation protocol to mimic the follicular-oviductal transition (peri-ovulatory events) of canine oocytes using caffeine pretreatment under certain time plans
- Studying the effects of caffeine pre-treatment on cell cycle biomarkers such as MAPK1&3, Akt and MPF CDC2 kinase activity
- Utilization of BCB non invasive staining as a screening test for better selection of developmentally competent canine oocytes prior to IVM

Considering the literature mining and the physiological hormonal profile series of experiments were conducted in the 3rd experimental chapter (chapter5) to:

- Study the effect of hormonal (IGF-1, EGF, PGH and melatonin) and serum supplementation (FBS) treatments on the nuclear maturation of canine oocytes.
- Further elucidate the immuno localization of MTN receptors in canine COCs.

In the 4th experimental chapter (chapter 6) production of canine embryos was studied. To assess the developmental potential of *in vitro* matured oocytes, via optimized culture conditions devised in previous experimental chapters, attempts were made:

- To fertilize *in vitro* matured oocytes using parthenogenetic oocyte activation.

- To fertilize *in vitro* matured oocytes using chilled dog semen and further optimization of this IVF method
- To utilize estrus bitch serum supplementation for improvement of IVM, IVF, and IVC outputs.

Canine ovarian tissue as an invaluable source of follicular cells (granulosa and theca) was also being processed throughout the sample collection of this study. Studies presented within the last experimental chapter (chapter 7) were carried out in the pursuit of canine follicular cells primary culture. Limited number of studies are available (Abdel-Ghani *et al.* 2012) concerning theca/granulosa primary cell culture in dogs. In order to characterize and further investigate the unique physiological profile of canine follicular cells experiments were designed. Main objectives of these experiments were:

- Establishment of primary canine follicular cell culture and maintaining them up to 96h in order to study cell morphology, culture behavior and original characterization.
- Study of the steroidogenic profile (E2 & P4) and response of these cells to physiological (hormonal) and pharmaceutical compounds.

2. General Materials and Methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company (Poole, UK), unless otherwise mentioned.

2.2. Sample collection

The sample collection procedure was carried out after routine ovariectomy of bitches by approval of the ethics committee of the Royal Veterinary College from a small animal hospital under supervision and consent of dog's owners. (Refer to appendix 1) The ethical approval was also verified by the University of Bedfordshire Ethical Scrutiny committee. Due to the nature of this study and also previously published reports (Otoi *et al.* 2000a, Otoi *et al.* 2001, Otoi *et al.* 2002, Songsasen & Wildt 2005), sample collection were blind to reproductive stage of the animal, breed, age, weight or size. Ovaries were collected immediately after ovariectomy and placed in a 60ml container (VWR international, USA) half full of warm (37^0C) sterile Phosphate Buffer Saline (PBS) and transferred to the lab within 2hours post operation. The container was immersed in a Thermos flask containing warm PBS.

2.3. Dissection and oocyte retrieval

Prior to dissection of follicle and oocytes, the ovaries were washed by warm PBS and trimmed of the ovarian bursa and other debris by a scalpel blade (Fig. 2.1).

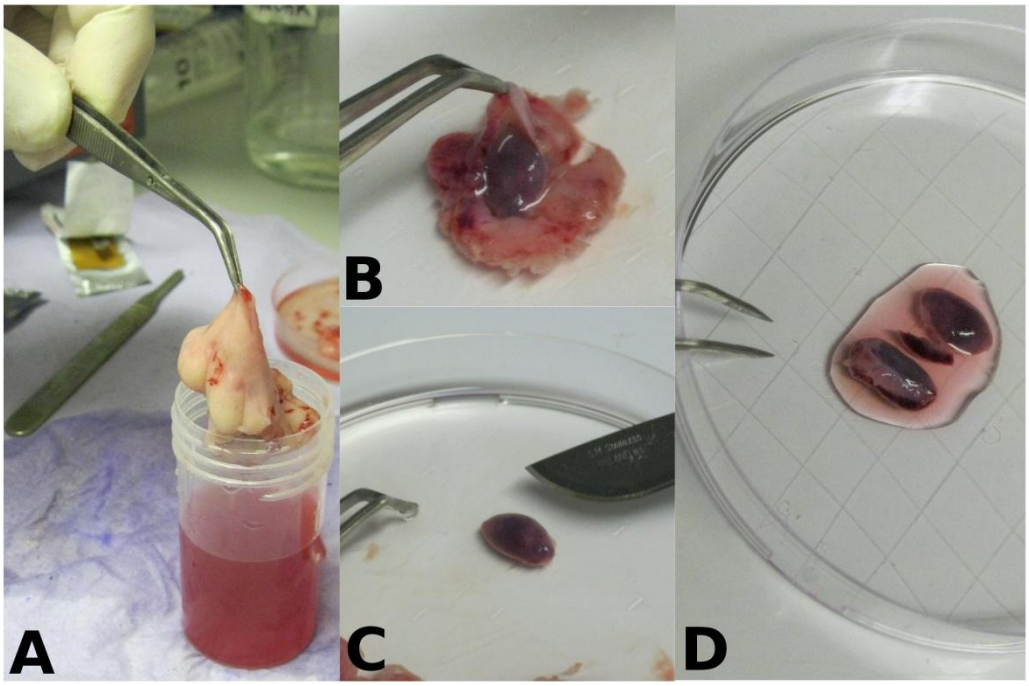


Figure 2.1. Dissection steps of canine ovaries collected immediately after ovariohisterectomy. Picture shows the steps of dissection required for extraction of COCs. A) Transport container half filled with warm PBS 1X. B) Locating the ovarian bursa while the ovaries are emerged in PBS 1X within a 90mm petri dish. C) ovary dissected and cleared of debris. D) A pair of ovaries in the new petri dish containing washing media before mechanical extraction of the COCs.

The ovaries were washed with a sterile filtered (0.2µm microbial filter [Anachem, Bedfordshire, UK]) washing media (TCM199 + HEPES buffer 20mM + 10% Fetal Bovine Serum [PAA Laboratories, USA]) and then sliced gently using a set of multiple blades (Fisher Scientific, United Kingdom) as previously described (Alhaider & Watson 2009) (Fig. 2.2).

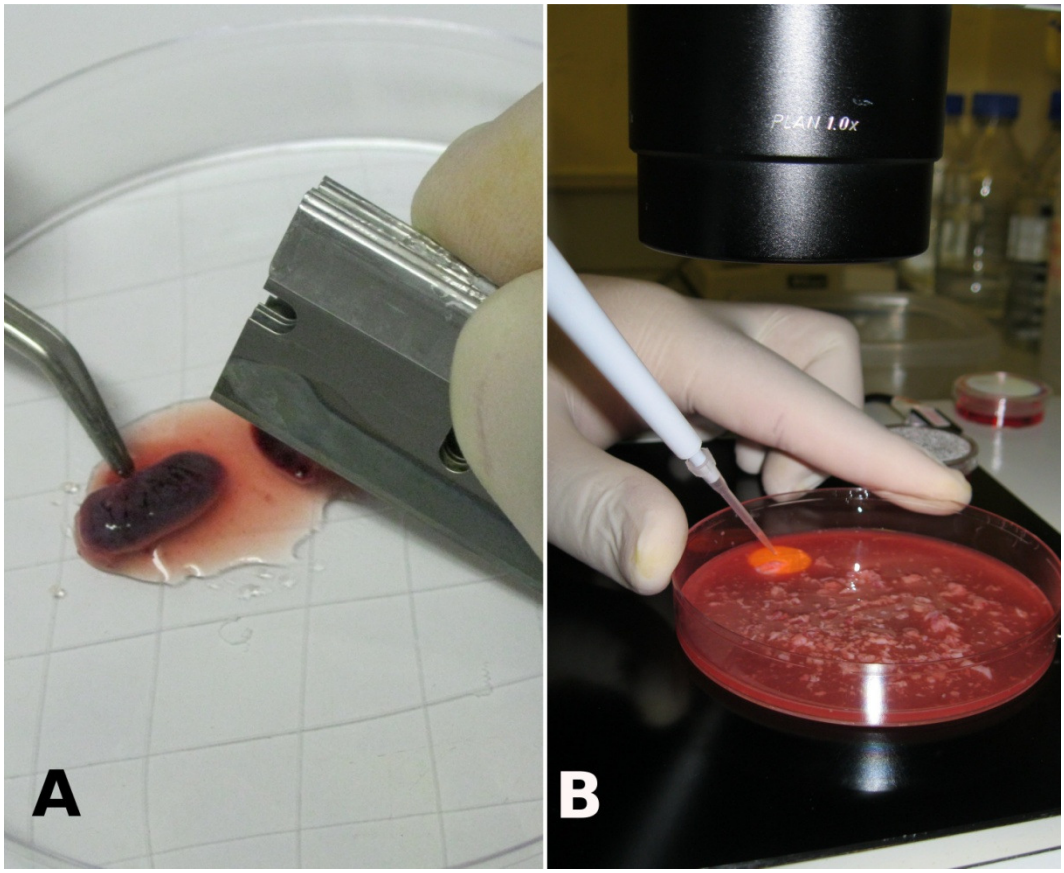


Figure 2.2. Mechanical extraction of canine COCs and selection process. Pictures show the mechanical extraction of the COCs from the ovaries using multiple blades technique (A) followed by selection of COCs under a light microscope on the 38°C warm plate (B).

Cumulus oocyte complexes (COCs) with an oocyte diameter over 100 µm (surrounded by at least 3 layers of cumulus cells) having dense and homogenous lipid yolk in the ooplasm were selected and washed twice before culture (Fig. 2.3).

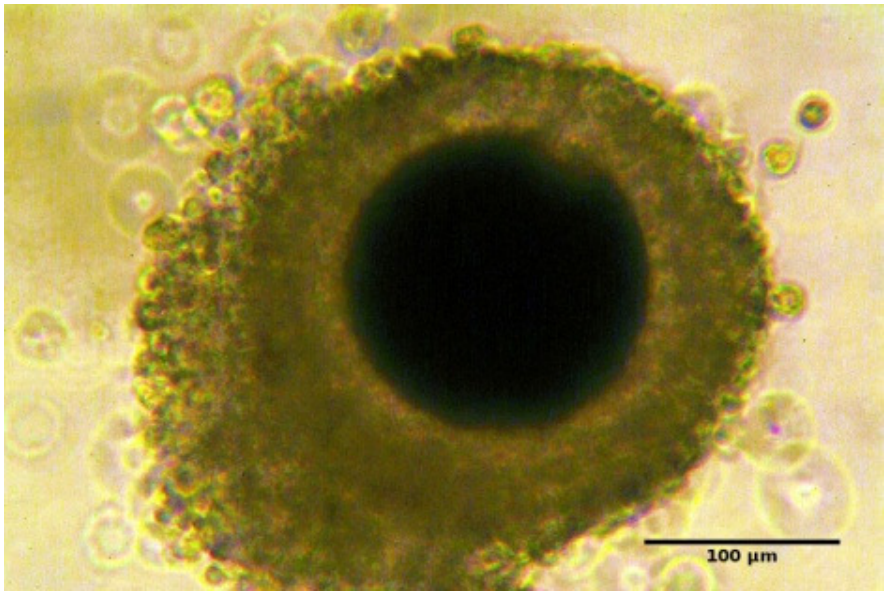


Figure 2.3 – Canine cumulus oocyte complex. Canine COC freshly fixed beneath a cover slip. (40X)

COCs selection criteria for canine IVM protocols used to be quite controversial (Songsasen & Wildt 2005, Concannon *et al.* 2009). Recently it has been accepted that age (older than 6 months), breed, weight and the stage of estrous cycle are less likely to affect the maturation rate of oocytes to MII stage or their degeneration rate (Concannon *et al.* 2009). Instead cellular criteria have been introduced for oocyte quality including; condense and homogenous lipid yolk, oocyte diameter above 100µm excluding the ZP and being surrounded with at least 3 layers of cumulus cells (Otoi *et al.* 2000a, Otoi *et al.* 2001, Otoi *et al.* 2002). Oocyte diameter above 100µm is one of cellular criteria during selection stage which is highly recommended by many reports (Songsasen & Wildt 2005). Nevertheless large size of the oocyte is not necessarily a guarantee for neither their competency nor the cumulus expansion(Rodrigues & Rodrigues 2010). During all of the experiments of this study oocytes were collected from ovaries according to the cellular criteria to maintain the unity.

2.4. In vitro Maturation

Oocytes were cultured in maximum 25 oocytes groups (Salavati *et al.* 2012). Selected oocytes were cultured in four-well culture dishes (NUNC, VWR international, Milan, Italy). The base maturation media was modified Synthetic oviductal fluid (SOFaaci; refer to appendix 2) (Holm *et al.* 1999, Marei *et al.* 2009) supplemented with Luteinizing Hormone 5 μg/ml (LH) [Leutropin; Bioniche Animal Health, Belleville, ON], Follicle Stimulating Hormone 5 μg/ml (FSH) [Follitropin; Bioniche Animal Health, Belleville, ON), 17β estradiol 1 μg/ml (E2), Progesterone 1 μg/ml (P4), 50 μg/ml Gentamycin and 6 mg/ml Bovine Serum Albumin (BSA). Culture was terminated according to the time table devised for each experiment and samples were collected for determining the stage of oocyte maturation. However; extrapolating the results of the first experimental chapter suggested that the optimal culture time set to 72h for the rest of the study.

2.5. Assessment of cumulus cell expansion

Cumulus expansion (mucification) was described by the partial or complete loosening of cumulus cells into extra cellular matrix surrounding the oocyte (Lee *et al.* 2007a, Marei *et al.* 2009, Silva *et al.* 2009). Cumulus expansion in canine oocyte unlike other species has not been a reproducible and forthcoming indicator of oocyte maturation according to literature mining (Reynaud *et al.* 2006)and results presented in this study (Salavati *et al.* 2012). Morphological changes of cumulus cells were observed and captured during the culture using a digital camera (Fig. 2.4). The mucification was compared to bovine oocyte maturation as a gold standard of the cumulus expansion after 24h of oocyte maturation (Fig. 2.5).

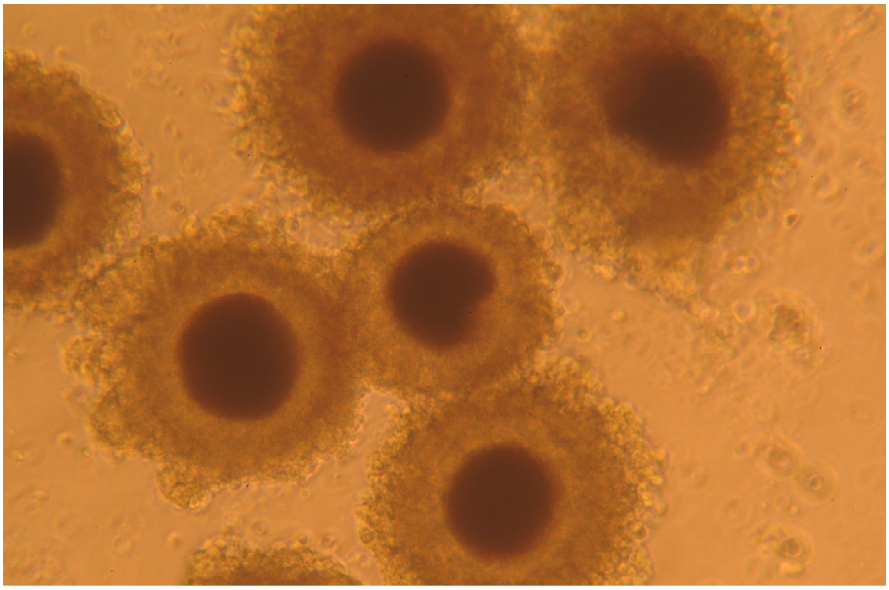


Figure 2.4. Cumulus disintegration in canine COCs after 52h of culture. Picture shows the cumulus cells unorthodox expansion pattern after 52h of culture which does not include mucification.

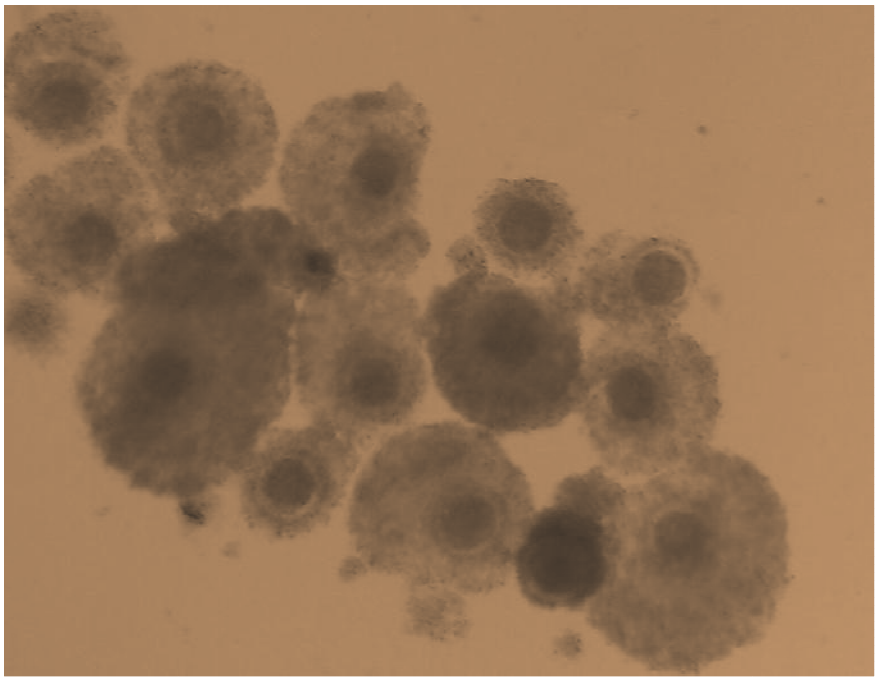


Figure 2.5. Cumulus expansion in bovine COCs after 24h of culture. Picture shows the cumulus cells expansion and mucification after 24h of culture.

2.6. Denuding oocytes and assessment of nuclear Maturation

Oocytes were denuded by incubation in 1% (W/V) Tri-Sodium Citrate in PBS buffer for 3-5 minutes and vortexed for 2 minutes on maximum agitation inside a 15 ml conical centrifuge tube (VWR international, Westchester, PA, USA). Remained cumulus cells were denuded using an oocyte holding needle with gauge of 135µm (Yellow EZ-Strip® Research Instruments limited, Cornwall, UK) (Fig. 2.6).

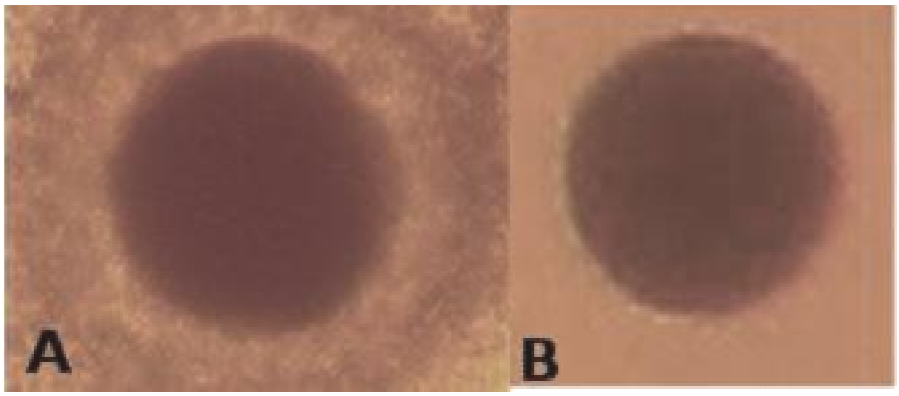


Figure 2.6. Denuding canine COCs. Picture shows canine oocyte before (A) and after (B) denuding process in order to remove the cumulus cells and facilitate visualization of the oocyte nuclear content.

The oocytes were placed in a drop on superfrost slides (VWR international, USA) and air dried in room temperature for 20 minutes. Afterwards the oocytes were fixed in cold (-20^{0}C) 99% Acetone (MERK, Germany) for 10 minutes. Hoechst 33342 fluorescent DNA dye (excitation/ emission = 350/461 nm) was prepared in PBS at the concentration of 10µg/ml for simultaneous staining and rehydration of slides for 5 min at 4^{0}C. Hoechst and PBS were dried with a stripped filter paper as much as possible prior to mounting the oocytes. Oocytes were mounted using Vectashield mounting media (Vector laboratories, Burlingame, CA, USA) beneath a cover slip fixed with four paraffin/wax drops (1/40 ; w/w) at the corners of the cover slip. Nuclear stage of the oocytes was assessed under Olympus BX60 fluorescent microscope (Olympus, UK).

During maturation oocytes go through different stages between prophase I and metaphase 2 (MII). By the time they had reached the end of culture period they were distributed among 7 groups regarding their nuclear stage. The number of oocytes at each stage was recorded according to the morphology of nuclear content (Hewitt *et al.* 1998). Degenerated oocytes with undetermined, disappeared or morphologically abnormal nuclear material were categorized as a separated group (Degen) in all experiments. A panel of stained oocytes at different meiotic stages shown in Fig. 2.7 was used as guidance for all experiments.

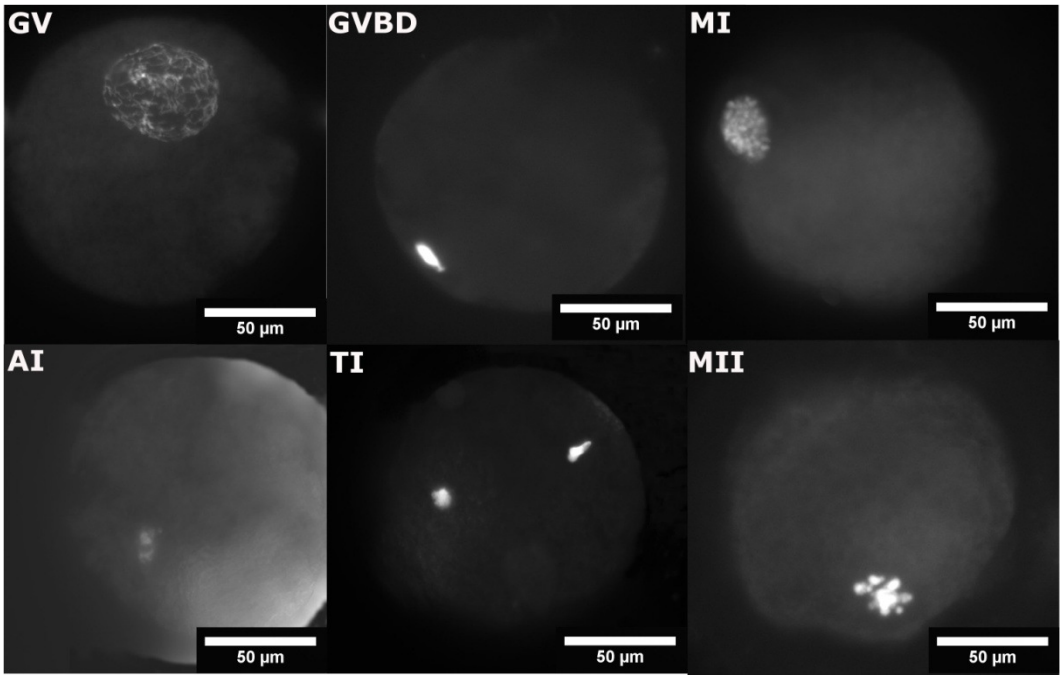


Figure 2.7. Canine oocytes at different stages of meiotic division: Oocytes were stained with Hoechst 33342 (10µg/ml) and visualized by Fluorescent microscope at 40X. Prophase 1 (Germinal Vesicle=GV), Germinal Vesicle Break Down = GVBD accompanied by chromatin condensation, Metaphase 1 (MI), Anaphase 1 (AI), Telophase 1 (TI), Metaphase 2 (MII.

2.7. Statistical analysis:

All experiments in this thesis were repeated at least 3 times. The Proportional average of oocytes in different stages of meiotic resumption was calculated at the end of the culture period in comparison to the total number. The oocytes were categorized in to 7 groups of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (M I), anaphase I (A I), telophase I (T I) , metaphase 2 (M II) and Degenerates (Degen). Statistical analysis was carried out in PAWS statistics 18: Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA) using binary and ordinal logistic regressions via Generalized linear model. P value < 0.05 was regarded as the level of significance.

3. Canine *in vitro* maturation setup

3.1. Introduction

Lack of universally accredited assisted reproductive techniques (ART) for canid species, has driven researchers to investigate this specific area in canine reproductive biology (Rodrigues & Rodrigues 2010). Unlike other domestic animals (cattle, sheep and pigs) *in vitro* maturation of oocytes in dogs results in very low nuclear maturation rate accompanied with high degeneration. Furthermore presumptuously matured oocytes have non-satisfactory fertilization capabilities which lead into either polyspermia or further developmental blocks (8 cell blockage)(Luvoni *et al.* 2005, Chastant-Maillard *et al.* 2010). Poor developmental potential of the presumptive zygotes, primarily originate from poor quality and developmentally incompetent oocytes. However; *in vitro* fertilized oocytes after *in vivo* maturation have synchronized nuclear development, normal sperm decondensation and consequently higher developmental potentials (Kim *et al.* 2007, De los Reyes *et al.* 2012). Thus during this study attempts were made to identify factor(s) perturbing the maturation profile of canine COCs and optimize sustainable *in vitro* maturation. In order to have a competent IVM system several aspects of this culture method should have been probed and adjusted. Selection criteria of oocytes have also shared equal challenges as the culture conditions and the duration of culture for canine IVM (Reynaud *et al.* 2006). As a well-established examination tool, the stage of nuclear development during meiotic maturation is a performance indicator for oocytes. In order to determine the stage of nuclear progression throughout meiotic resumption, the chromatin content of the nucleus should be visualized. Visualization of nuclear content can be carried out after denuding the oocytes followed by either staining with specific dyes followed by light microscopy or by using a fluorescent DNA binding dye and observation under a fluorescent microscope. Visualization of chromatin content for detection of meiotic stage has also been an issue in canine IVM (Reynaud *et al.* 2006). High lipid content (lipid

yolk) impairs fixation and staining process and obscures chromatin and makes it difficult to stage nuclear maturation of the oocytes by light microscopy. There have also been reports concerning the predisposition of canine oocytes and cumulus cells to oxidative stress due to high lipid content (Hossein *et al.* 2007, Silva *et al.* 2009). This weakness of canine oocytes *in vitro* could also be related to their lower glutathione (GSH) content, as a major intracellular antioxidative compound, compared to oocytes matured *in vivo* (Kim *et al.* 2007).

This chapter presents the results of the attempts which have been made to achieve a reliable and efficient technique for staining and staging of canine oocytes. In addition, the effects of maturation media, gas composition of the incubator and the duration of the culture on nuclear maturation of the oocytes were assessed and changes in the oocytes H_2O_2 level as a bi-product of the reactive oxygen species (ROS) repairing enzymes were determined.

3.2. Materials and Methods

3.2.1 *In vitro* maturation

The IVM culture was carried out following the protocol described in the section 2.2., 2.3. and 2.4.

3.2.2. Denuding isolated and cultured COCs

There were several technical challenges during the staining and fixation processes which were optimized through trying different methodologies. Denuding oocytes in mammalian COCs is normally carried out by incubation (up to 5min) in 300 IU/ml Hyaluronidase at 37^0C followed by 2 min vortexing. The same protocol was applied for canine oocytes after the culture periods (Fig. 3.1):

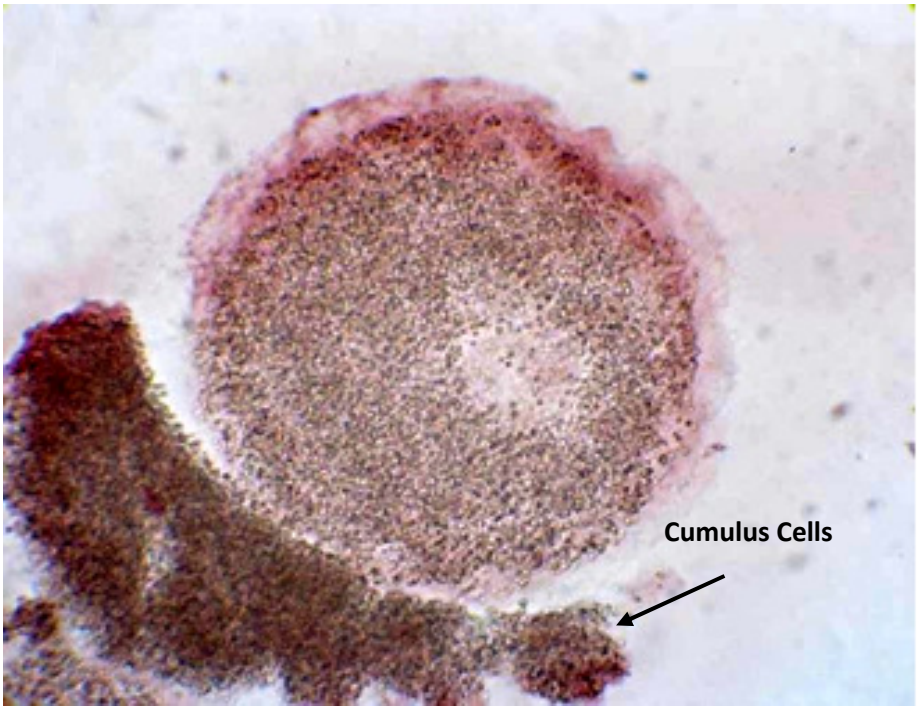


Figure 3.1. Light microscope picture of a canine oocyte stained by 2% Aceto-Orcein stain.

Hyaluronidase digestion was tried with different incubation/vortex durations in order to examine the efficiency of this denuding technique (e.g. 2-10 min incubation and 2-5 min vortex; Fig. 3.2).

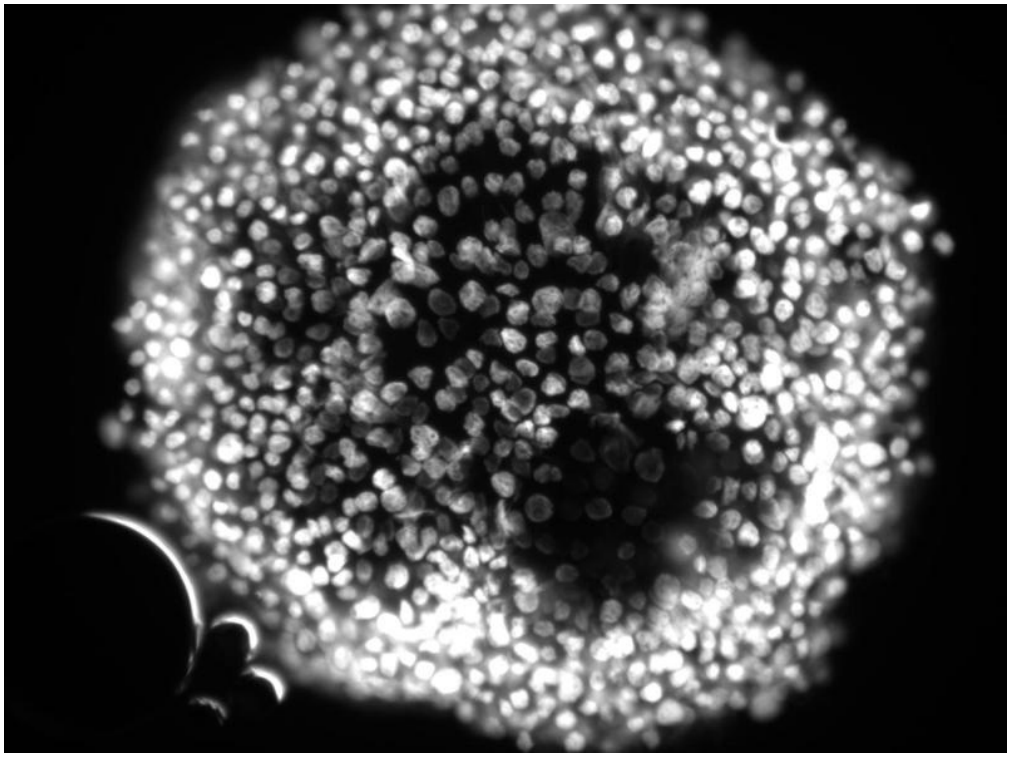


Figure 3.2. Fluorescent microscope picture (40X) of an oocyte stained with 10 µg/ml Hoechst in PBS1X. Oocyte is still surrounded by the most inner 2 or 3 last layers of cumulus cells and the nuclear material is not visible

After unsuccessful denuding with Hyaluronidase, I utilized an aggressive methodology by using proteases to break intercellular matrix and dissolve ZP. *In vitro* cultured canine COCs were treated with a stripping solution containing Pronase 0.1µg/ml + Poly Vinyl Alcohol (PVA) 5 µg/ml + Hyaluronidase 300 IU/ml in TCM 199. The denuding was carried after 2-10min incubation followed by 3-5 min vortexing in maximum agitation. The main problem with this method was fragility of the oocytes after ZP removal which often resulted in rupture. (Fig. 3.3)

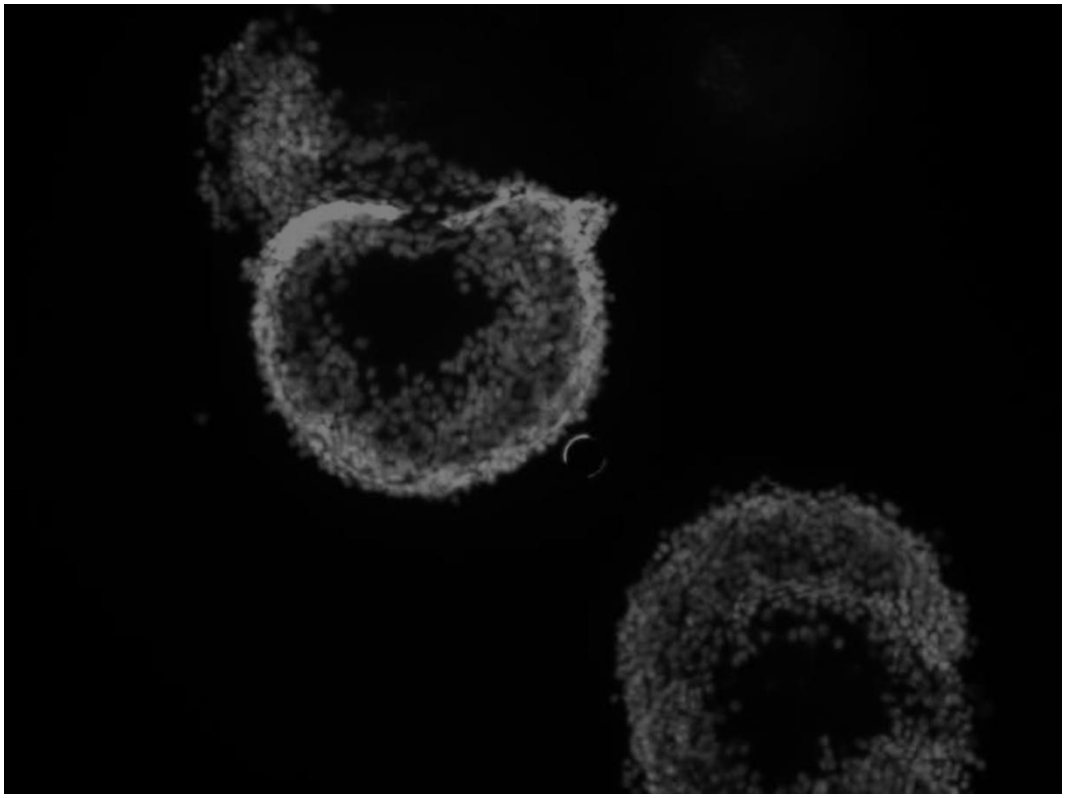


Figure 3.3. Fluorescent microscope picture (10X) of an oocyte treated with Pronase. The oocyte was stained by 10 µg/ml Hoechst in PBS1X. Please note that the oocytes are still surrounded by last layers of cumulus cells and some of them are ruptured.

Alongside the denuding issue, fixation and staining of the oocytes had to be optimized. Routine staining methods (visible dyes e.g. Aceto-Orcein) due to high lipid yolk content of the oocyte were not time beneficial. The Aceto-Orcein staining procedure was performed after 48-72hr fixation of oocytes beneath a cover slip in the 3:1 v/v solution of methanol (MERCK, Germany) and glacial acetic acid (GAA) (MERCK, Germany), followed by staining with 2% Orcein in 45% GAA. Beside the Aceto-Orcein method, 2% Lacmoid acid in 45% GAA and 10 µg/ml Hoechst in PBS 1X were also used in these pilot experiments.

In order to achieve optimal fixation and staining resolution, different fixative solutions were tested as detailed below:

GAA: Methanol: Chloroform (v/v = 3:6:1)	**48h**
GAA: Meth: Chloroform (v/v = 3:6:1) followed by **GAA: Methanol (v/v = 1:1)**	3min 2h
Paraformaldehyde 4 %	10min
Methanol 99% -20^{0}C	10 min
Methanol 99%: Acetone 99.9% (v/v = 1:1) -20 ^{0}C	10min
Acetone 99.9% -20 ^{0}C	10min

In long duration fixation methods the amount of distortion and artefacts were high and the method itself was time consuming (Fig. 3.4 A and B). Thus fixation approach was modified toward cold and fast fixatives. Overall cold Acetone 99.9% (MERCK, Germany) showed to be the best fixative with maximum fluorescent resolution (Fig. 3.5).

In addition, I tried to achieve a very high resolution, through elimination of lipid granules artefact by polarization of lipid yolk (Hara *et al.* 2005) via centrifugation at 8200g for 30min (Alvarez *et al.* 2009) after denuding. Results of this approach were not satisfactory (Fig. 3.4 C).

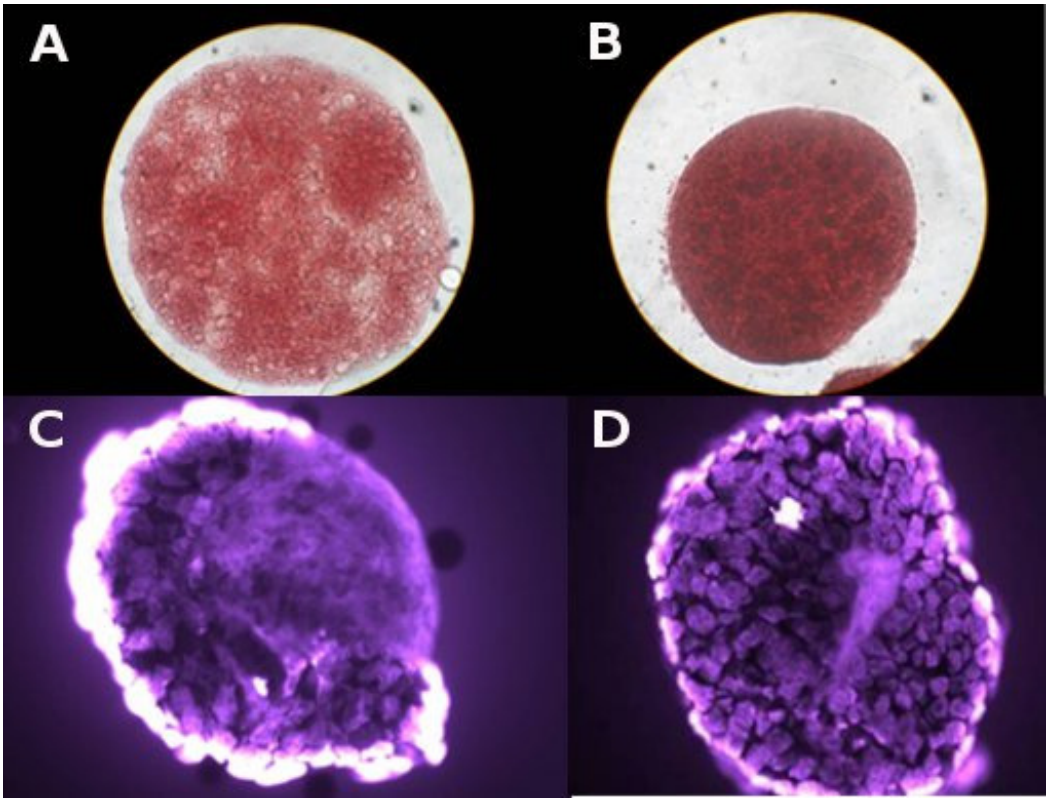


Figure 3.4. Optimization of fixation and staining of canine oocytes: A) Oocyte fixed for 48hours in Methanol: GAA solution and stained with Orcein. B) Oocyte fixed in the Methanol: GAA for 2h and stained with Lacmoid acid. C) Oocyte fixed in 4% Paraformaldehyde for 10min and stained with Hoechst 33342. Polarized lipid content by centrifugation of the oocyte is visible by the left side of the ooplasm. D) Oocyte fixed in 4% Paraformaldehyde for 10min and stained with Hoechst 33342.

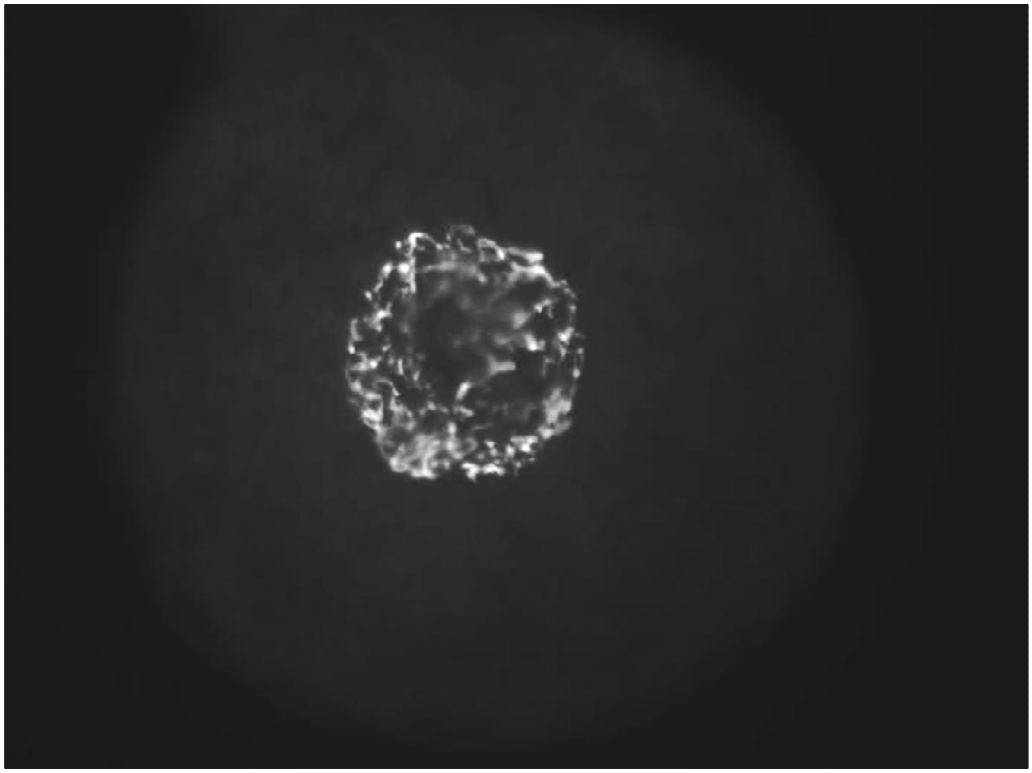


Figure 3.5. Canine oocyte (40X) stained following the methods described in section 2.6. COC was denuded using 1% sodium citrate in PBS1X followed by fixation in cold acetone and staining with 10 µg/ml Hoechst.

3.2.2. ROS staining using DCHFDA

Oocytes were denuded after 72h of culture and stained using 2',7' – dicholorodihydrofluoroescein diacetate (DCHFDA). This substance hydrolyzes via intracellular esterase to produce 2', 7' – dicholorodihydrofluoroescein (DCHF), and the latter metabolite will be oxidized via H_2O_2 to 2', 7' – dichlorofluorescein (DCF) (Nasr-Esfahani *et al.* 1990, Wakefield *et al.* 2008). With DCF excitation at 470nm wavelength, the emission at 522-530nm could be captured by fluorescence microscopy. Thus the oocytes were washed twice in 0.04 % PVP in PBS and then incubated for 30min at 38.5^0C in the dark in the 0.04%PVP-PBS buffer containing 10 µM DCHFDA. Afterward oocytes were washed two times in the same buffer and mounted on slides using Vectashield mounting media (Vector Labs, UK) under a cover slip. Oocytes were visualized by 470nm LED lamp Olympus BX60 fluorescence microscope, and photographs were obtained from

10 oocytes of the two groups in each repeat. The intensity of fluorescent signal was quantified using ImageJ software particle analysis plugin (Abràmoff *et al.* 2004).

3.2.3. Real-Time PCR and expression profile of ROS repair enzymes

3.2.3.1. Primer design

Coding sequences (CDS) of target genes were obtained from NCBI PubMed nucleotide database (http://www.ncbi.nlm.nih.gov) and their exon mapping was confirmed by the intron/exon recordings available on Ensemble Genome Browser (http://www.ensembl.org/index.html). Gene specific primers were designed on intron spanning positions (forward and reverse placed on different exons) in order to avoid genomic DNA amplification, using the Primer3 web-based software (Rozen & Skaletsky 2000). The following criteria were in place for primer design:

Primer size (bp)	20-24 (optimum 22)
Guanidine & cytosine content (GC %)	40-60 %
Maximum self-complementary	4
Maximum 3' self-complementary	2
Maximum Poly X (A,T,G or C)	3
Preferred amplicon size (bp)	<250

Primers were then further analysed for specificity and thermal defects using separate web-based softwares. The sequences of the forward and reverse primers were aligned with whole dog genome using the Basic Linear Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov). Forward primers as they were but reversed and complementary sequence of the reverse primers were analysed in the nucleotide BLAST section. For thermal defects of primers such as hairpins, loops and self/hetero dimers the primer sequences were also tested by Oligo Analyzer 3.1 web-based software following the manufacture's instruction (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/). The primers were then

ordered as lyophilized, salt free and HPLC purified oligonucleotides through EUROFINs (MWG-Operon, London, UK). Upon arrival primers were reconstituted at 100µM concentration using nuclease free water and aliquoted in 10µl to be kept in -80^{0}C freezer. The working concentrations of forward and reverse primers (20µM) were produced by mixing 20µl of each primer (F+R =40µl) in 60µl of nuclease free water. The details of the designed primers can be found in the table 3.1:

Table 3.1 – Sequence of the designed primers and accession numbers of the ROS repairing enzyme genes.

Canine Genes	**Accession number**	**Oligos (5'⇨ 3')**	**Product size (bp)**
GPX1	NM_001115119.1	F: GACACCACTGCGCTAATGAC R: AGGGAAAGGAGGGTTGCCTA	215
SOD1	NM_001003035.1	F :ACCATTACAGGGCTGACTGAAG R: TGGACAGAGGATTAAAGTGAGGA	115
SOD2	XM_533463.3	F: AGAAGGGTGACATTACAGCTCA R: AATCACGTTTGATGGCTTCC	153
CAT	NM_001002984.1	F: CCCATTGCAGTTCGATTCTC R: CTATGGATAAAGGACGGAAACA	179
GSR	XM_532813.3	F: CTACGTGAGCCGCCTAAATAC R: CTGTGGCAATCAGGATGTGAG	155
GAPDH	NM_001003142.1	F: GTGATGCTGGTGCTGAGTATGT R: ATGGATGACTTTGGCTAGAGGA	233

GPX1 (Glutathione peroxidase); Superoxide dismutases (Cytosolic SOD=Cu-Zn SOD1, Mitochondrial SOD= Mn-SOD2); Catalase (CAT); Glutathione reductase (GSR); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) the house keeping gene.

3.2.3.2. RNA extraction

In vitro matured oocytes were snap-frozen after 72h of culture in PBS-PVP 0.4% (polyvinylpyrrolidone) using liquid nitrogen and kept at -20^{0}C until analysis. RNA extraction was done using a QIAGEN RNA-easy kit (QIAGEN, UK). Briefly 20 COCs were lysed in 350µl lysis buffer (RLT) buffer, mixed with equal amount of 70% ethanol, and transferred to the RNA-easy spin columns. Washes and centrifugation were carried out according to the manufacturer's instruction, and RNAs were finally eluted in 30µl of nuclease free water and the concentration was measured by a TECAN plate reader (TECAN, Switzerland). RNA concentration was normalized among samples by dilution to 50ng in 8µl volume. In order to eliminate any genomic DNA contamination, DNA digestion was carried out using RQ1 RNase-free DNase kit (Promega, UK; M6101). Reaction tubes were prepared by mixing the following:

Total RNA (50ng)	8µl
RQ1 RNase-free DNase 10x buffer	1µl
RQ1 RNase-free DNase	1µl

After 30 min incubation at 37^{0}C, reaction was terminated by addition of 1µl RQ1 DNase stop solution and 10min incubation at 65^{0}C.

3.2.3.3. Reverse transcription

Reverse transcription of RNA samples was conducted by QIAGEN Sensiscript RT kit (QIAGEN, UK) in an equal amount of 50ng of RNA in a total reaction volume of 20µl. The master mix was prepared according to the manufacturer's instructions:

RT buffer 10X	2µl
dNTP mixture 10mM	2µl
Oligo(dt)$_{15}$ Primer	2µl
Random primers	2µl
RNasin® Ribonuclease Inhibitor (20unit/µl)*	1µl
Sensiscript® Reverse Transcriptase (15unit/µl)	1µl

**Promega, UK*

Prepared master mix was distributed among tubes containing DNase treated RNA followed by 1h of 37^{0}C incubation. The final complementary DNA (cDNA) product was diluted to 2.5ng/µl prior to utilization in PCR. Constructed cDNA was either kept at 4^{0}C to be consumed within the next 24h or transferred to -80^{0}C for later analysis.

3.2.3.4. Conventional PCR

PCR reactions were carried out using Qiagen Multiplex PCR kit (QIAGEN, UK) in 12.5ng cDNA per reaction in duplicate tubes and 4 sets of repeats. Conventional PCR was conducted on every batch of cDNA including the negative control for reverse transcription for every primer prior to temperature gradient optimization. PCR reactions were prepared as following:

QIAGEN Multiplex PCR Master mix 2X	10µl
Q-Solution 5X	2µl
2µM F+R Primers*	2µl
12.5 ng cDNA	5µl
NFW**	1µl

** Target genes: GPX1, SOD1, SOD2, GSR, CAT and GAPDH*

*** Nuclease free water*

After through mixing of the tube content, reactions were transferred to the PCR thermal cycler machine (G storm, GRI Ltd, Essex, UK). The following program was set as the template for the conventional PCR:

1	Taq activation	95^0C	5min
2	Denaturation	95^0C	30sec
3	Annealing temperature	*^{0}C	90sec
4	Extension	72^0C	30sec
5	Repeat steps 2-4	38 times	
6	Final extension	72^0C	10min

** Different for every set of primers*

PCR products were visualized using 1.5% (w/v) agarose gel electrophoresis. Briefly 10µl of the sample was mixed with 2µl of loading dye and loaded in to the gel containing 1µl of ethidium bromide and previously submerged in Tris-acetate-EDTA (TAE) buffer. Molecular weight (bp) of the target amplicons were confirmed for every gene (e.g. GAPDH; Fig. 3.6).

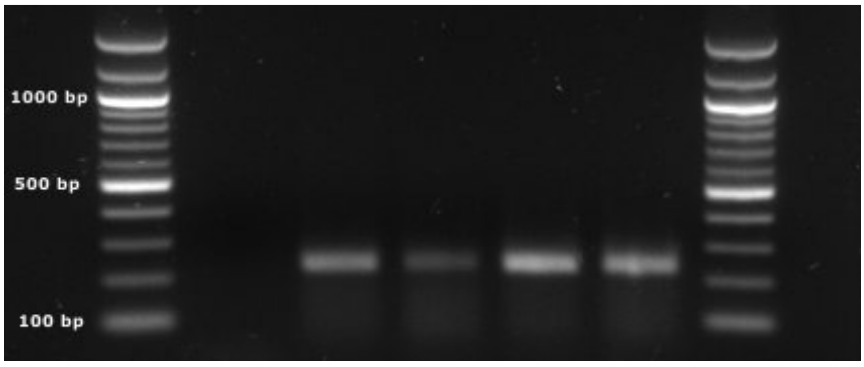


Figure 3.6. Visualization of conventional PCR products of GAPDH gene on agarose gel. PCR product size of 233bp was confirmed for this set of primers in 4 repeats (different batches of cDNA). Lanes from left to right: 100bp DNA ladder, -ve Ctrl, COCs +ve Ctrl repeats 1-4 and 100bp DNA ladder.

3.2.3.5. Melting curve analysis of temperature gradient qPCR (optimization)

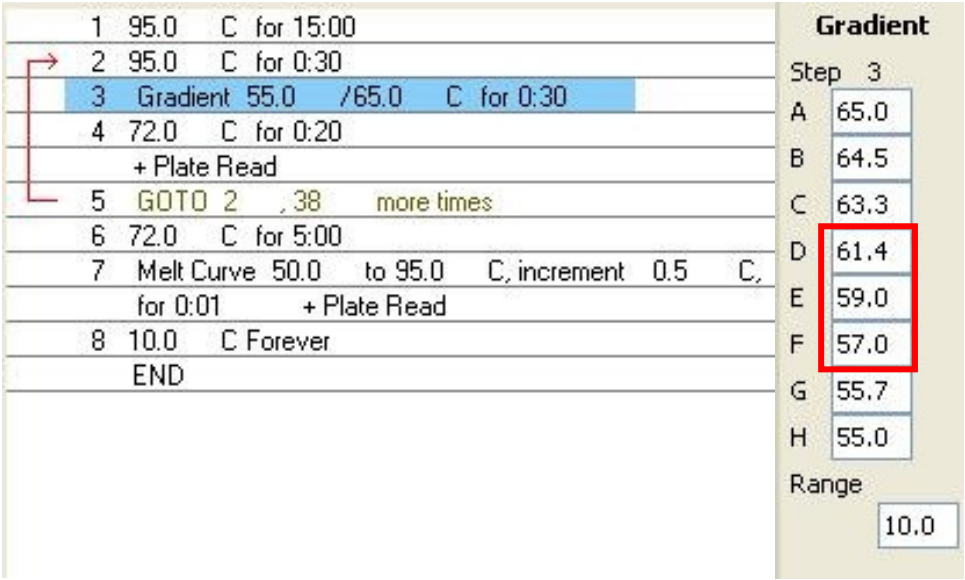
Quantitative PCR was done using KAPA SYBR® FAST qPCR Kits (KAPABIOSYSTEMS, UK) and 20µM primers in 20µl volume and 38 cycles via Bio-Rad CFX96 real-time machine (Bio-Rad, UK) using CFX manager 1.7 software (Bio-Rad, UK). The temperature gradient real-time PCR was carried out in the range of 57 - 61.4 ^{0}C using a set of 3 identical tubes for each gene (57, 59 and 61.4). The temperature range was calculated according to the annealing temperature provided by the manufacturer. The reaction tubes were prepared as following:

KAPA SYBR green	10µl
20µM F+R Primers*	0.5µl
10 ng cDNA	4µl
NFW**	5.5µl

** Target genes: GPX1, SOD1, SOD2, GSR, CAT and GAPDH*

*** Nuclease free water*

The Bio-Rad CFX 96 machine was programmed as following:



Tubes were placed in row positions of D, E & F within the 96 well frame for 38 cycles and the optimal annealing temperature was determined regarding the

earliest signal (lowest Cycle of threshold = Ct) accompanied with highest RFU at melting curve analysis (e.g. GAPDH; Fig 3.7).

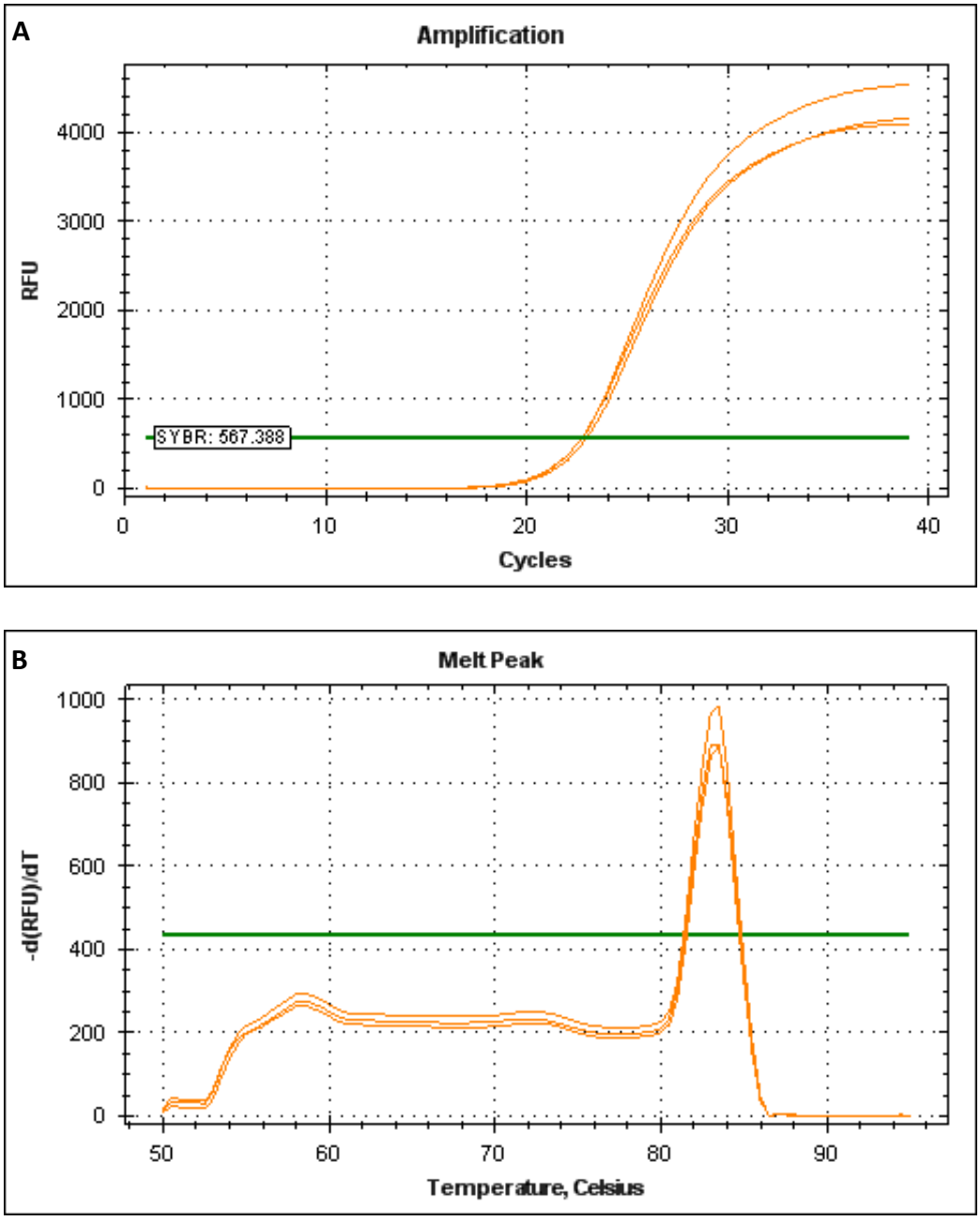


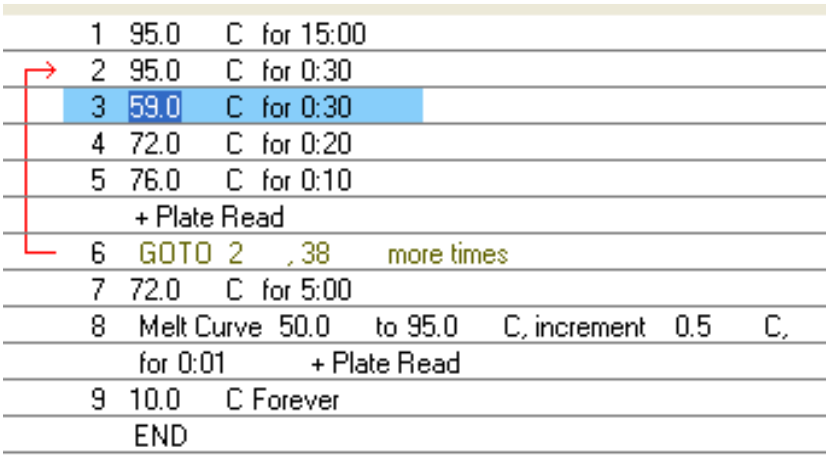
Figure 3.7. Amplification and melting curve analysis of GAPDH primers. A) Amplification curves of 57, 59 and 61.4 ^{0}C tubes shows the Ct of 22.71, 22.69 and 22.98 respectively. B) Melting peak of the PCR product is 83.5^{0}C. The optimal annealing temperature was considered as 59 ^{0}C and cycle reading temperature 76^{0}C.

Except CAT (57^{0}C), the rest of primers had an optimal melting temperature of 59^{0}C. All the reaction was read at 76^{0}C (plate) after each cycle.

The program for the rest of the qPCR on CFX machine was set as following:

1	KAPA activation	95^{0}C	15min
2	Denaturation	95^{0}C	30sec
3	Annealing Temperature	57/59^{0}C*	90sec
4	Extension	72^{0}C	30sec
5	Reading plate temperature	76^{0}C	10sec
6	Repeat steps 2-5	38 times	
7	Final extension	72^{0}C	5min
8	Melting curve from 50-90^{0}C reading every 0.5^{0}C and holding for 1sec		

** GPX1, SOD1, SOD2, GSR,GAPDH = 59^{0}C; CAT=57^{0}C*



3.2.3.6. Real time PCR (Livak method)

Relative Real-time qPCR was carried out using canine *GAPDH* as the house keeping gene, which had a stable expression among 3 groups (control [0h] and the two treatments) in 4 replicates. (Levene's test P = 0.086; one way ANOVA [equal variances assumed, LSD post-HOC]; p value = 0.227).

The fold induction (expression) of target genes was analyzed using Livak method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen 2001). In Livak analysis cycle of threshold (Ct) in the treatment group is normalized against the control in target genes (TG) and House Keeping Gene [HKG](e.g. GAPDH) following these equations:

$$\Delta Ct = \text{Average Ct [TG]} - \text{Average Ct [HKG]}$$

$$\Delta\Delta Ct\ [TG] = \Delta Ct \text{ Treatment 72h [TG]} - \Delta Ct \text{ Ctrl 0h [TG]}$$

$$\text{Normalized expression ratio} = 2^{-\Delta\Delta Ct\ [TG]}$$

3.2.4. Statistical analysis:

All experiments were repeated at least 3 times. The Proportional average of oocytes in different stages of meiotic resumption was calculated at the end of the culture period in comparison to the total number. The oocytes were categorized in to 7 groups of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (M I), anaphase I (A I), telophase I (T I) , metaphase 2 (M II) and Degenerates (Degen). Statistical analysis was carried out in PAWS statistics 18: Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA) using binary and ordinal logistic regressions via generalized linear model. Analysis of qPCR data was carried out using CFX manager software (Bio-Rad, UK) and one way ANOVA (LSD Posthoc multiple comparisons) in PAWS statistics 18: Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA).

3.3. Experimental design

Experiments were repeated at least 3 times. COCS were cultured in groups about 20 and specific time points were devised for each experiment. Summary of experiments and number of the COCs utilized are described in Table 3.2.

3.3.1. Age & weight distribution among samples collected

During the first year of this PhD project age and weight of the dogs were recorded up on collection of the ovaries. Within that period 73 cases (COCs = 5753) were collected and the age/weight data was analyzed.

3.3.2. Denuding, fixation and staining setup

In order to optimize an efficient and time worthy protocol for staining and staging of canine oocytes after maturation, a total number of 1488 COC was utilized. Fresh and cultured COCs in numerous attempts were processed by different denuding and fixation buffers in combination with 3 staining methods: 1. Aceto-Orcein staining 2. Lacmoid Acid staining 3. Hoechst33342 fluorescent staining (Refer to section 3.2.2).

3.3.3. Cumulus expansion and nuclear maturation after 52h

During this experiment, 118 COCs were cultured in humidified incubators under two different gas compositions for 52h at 38.5^0C: High O_2 [(5% CO_2 in air (approximately 20% O_2)] or Low O_2 (5% CO_2 5%O_2 90%N_2) in order to study the pattern of cumulus expansion. COCs were photographed twice during the 52h of culture (at 24h and 52h) using a digital camera and an inverted microscope to record pattern of cumulus expansion within different oxygen levels. At the end of culture (52h) COCs were denuded, fixed and stained to assess nuclear maturation of the oocytes.

3.3.4 Culture duration

From results of the nuclear maturation rates, a subsequent experiment was designed to compare nuclear maturation rates of canine oocytes in longer

incubation periods. A total number of 460 COCs were cultured in the low oxygen for 72 and 84h time periods; oocytes maturation and degeneration rates were analysed after staining.

3.3.5 ROS staining using DCHFDA

This experiment was designed to assess the role of oxidative stress as a detrimental factor which may contribute to the high degeneration and low maturation rates in canine IVM oocytes. A total number of 130 oocytes were cultured for 72 h in the low or high oxygen gas atmospheres. The H_2O_2 concentration in oocytes as the product of ROS was quantified after DCHFDA fluorescent staining. Photographs of stained oocytes were analyzed via ImageJ software to quantify the spot density of fluorescence which is directly correlated to amount of H_2O_2 produced inside each oocyte (Nasr-Esfahani *et al.* 1990, Wakefield *et al.* 2008). An average of total intensity was compared between the Low and High oxygen groups.

3.3.6. ROS repairing enzymes relative expression

For investigating the mRNA expression profile of ROS defensive enzymes, 340 oocytes in 4 repeats were cultured in low and high oxygen incubators (groups of 20). At the beginning of the experiment 20 COCs were snap-frozen as the 0 h control group. At the end of the 72h culture period, oocytes were snap-frozen as Low and High oxygen treatment groups. PCR reactions were carried out in duplicates and 4 repeats. In Livak analysis, cycles of threshold (Ct) of the target genes (TG) were first deduced by the Ct of Housekeeping gene (HKG; *GAPDH*): ΔCt = Average C(t) [TG] - Average C(t) [HKG]. Resulting values for treatment groups (72h) were normalized against the 0 h control using the following equation: ΔΔCt [TG] = ΔCt Treatment 72h [TG] - ΔCt Ctrl 0h [TG]; normalized expression ratio = $2^{-\Delta\Delta Ct\ [TG]}$

3.3.7. Antioxidative effects of Vit. E

This experiment assessed the protective effects of α-tocopherol (Vit.E) over oxidative stress. A total of 201 oocytes were cultured in low and high oxygen incubators in 2 groups in absence (control) and presence of 100µM Vit.E. Nuclear maturation rates and meiotic resumption of oocytes were analysed after 72h of culture using fluorescent staining.

Table 3.2. The summary of experiments in this chapter.

No. Exp.	No. COCs	Treatment	Measurements
3.3.1	**5753**	**Age, weight and COCs**	**analysis**
3.3.2	**1488**	**Cumulus expansion 52h IVM**	**Staging**
3.3.3	**118**	**52h Oxygen comparison**	**Staging**
3.3.4	**460**	**52 vs. 72 vs. 84 Low oxygen**	**Staging**
3.3.5	**130**	**ROS (H2O2) Oxygen Comparison**	**DCHFDA**
3.3.6	**340**	**ROS repairing enzymes**	**qRT-PCR**
3.3.7	**201**	**Vit. E vs. Oxygen comparison**	**Staging**

3.4. Results

3.4.1. Effect of age and weight on available COCs

During the first year of this PhD a total number of 73 sample collections were carried out and on average 79 ± 5 COCs were collected in each batch of ovaries (per animal). There has been slightly positive correlation between the weight of bitch and number of COCs collected (Correlation coefficient [CC] = 0.11) which was not statistically significant. However there was strong negative correlation between animals age and the collected COCs (CC = - 0.55; *P* = 0001) which is in agreement with theories in aging and oocyte reservoir of the ovaries. The scatter of collected data has been illustrated in Fig. 3.8.

It's noteworthy that during the sample collection several Staffordshire terrier cross ovaries (around and above 1 year old) were collected with depleted ovarian cortex from follicles and COCs. They were omitted from this study.

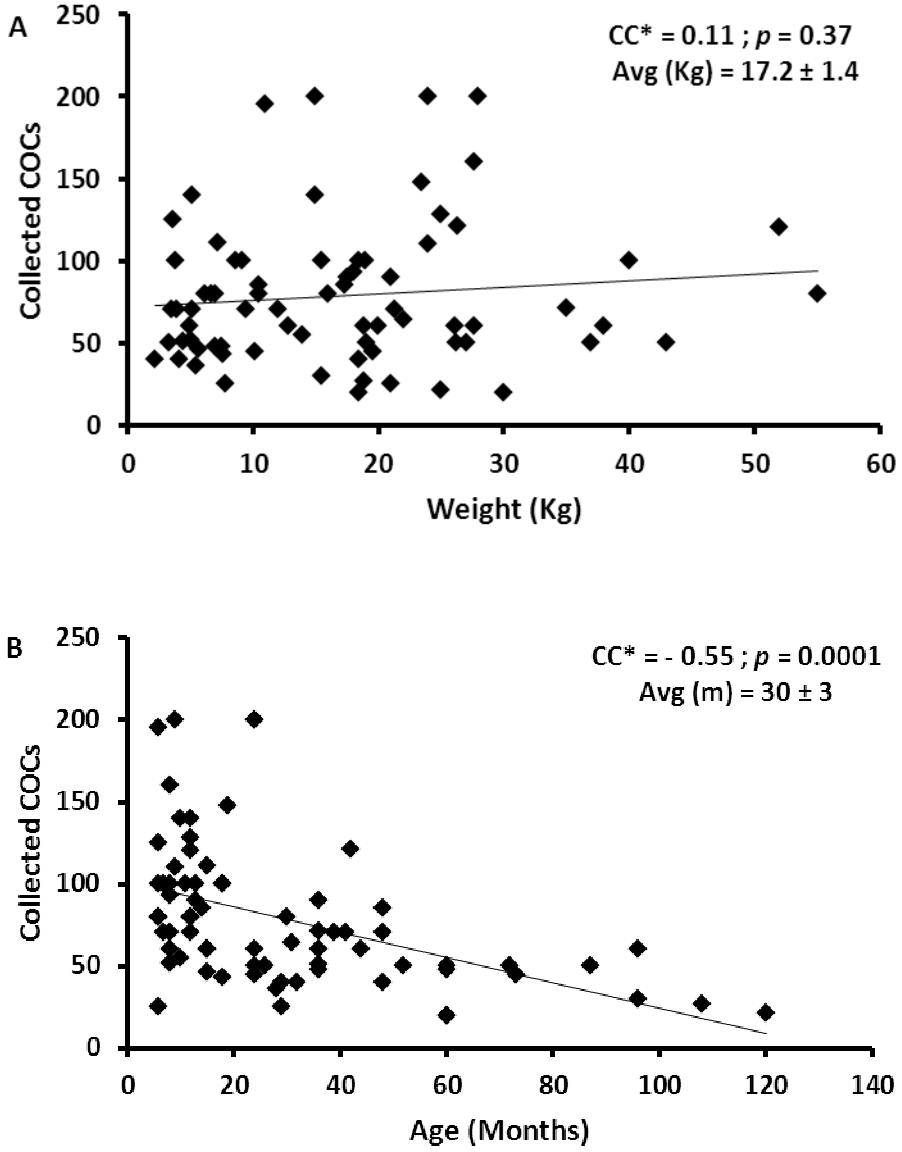


*Figure 3.8. The scatter distribution of Collected COCs from 73 cases (n= 5753). A) Graph shows the scattered correlation between weight (Kg) of bitch and number of COCs collected. Dataset has been analyzed using spearman test (non-parametric correlation test) due to its skewness. B) Graph shows the scattered correlation between age (in months) of bitch and number of COCs collected. Dataset has been analyzed using spearman test (non-parametric correlation test) due to its skewness. * CC = Correlation Coefficient.*

3.4.2. Different stages of meiotic resumption

Nuclear development of the canine oocytes during oocyte maturation process was captured using the methods described in section 2.6. The results were as following:

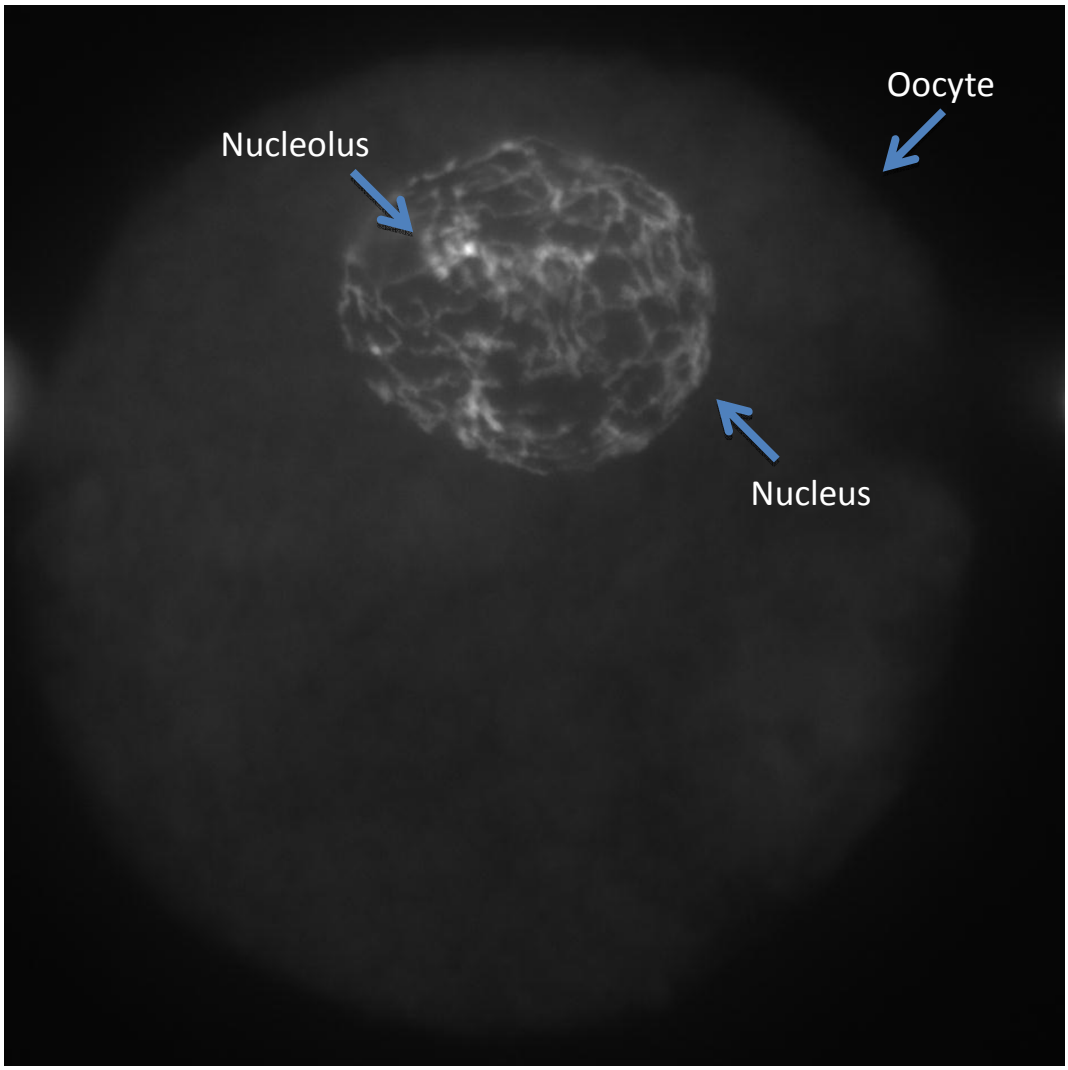


Figure 3.9. Canine oocyte at Germinal Vesicle (GV) stage. Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification.

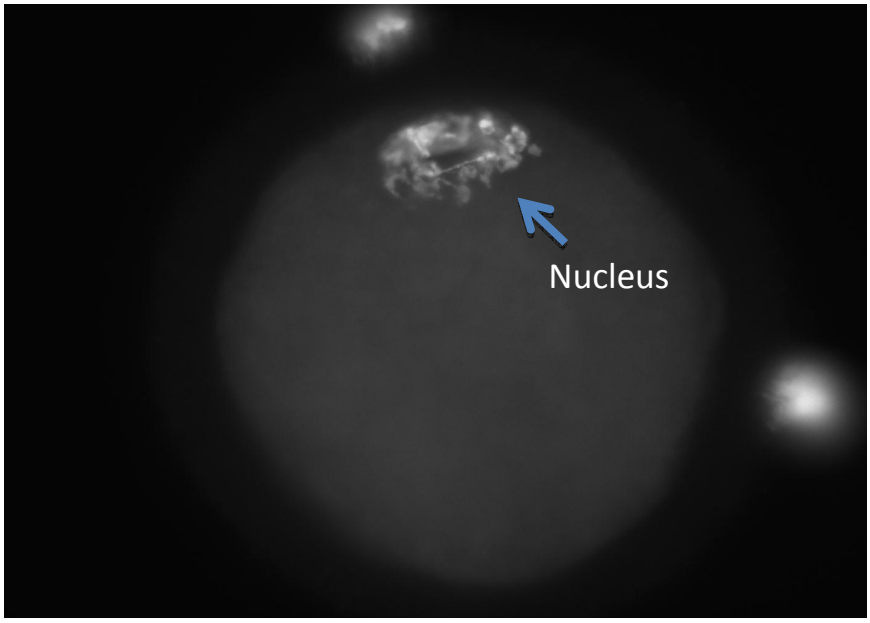


Figure 3.10. Canine oocyte at late Diakinesis. Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification.

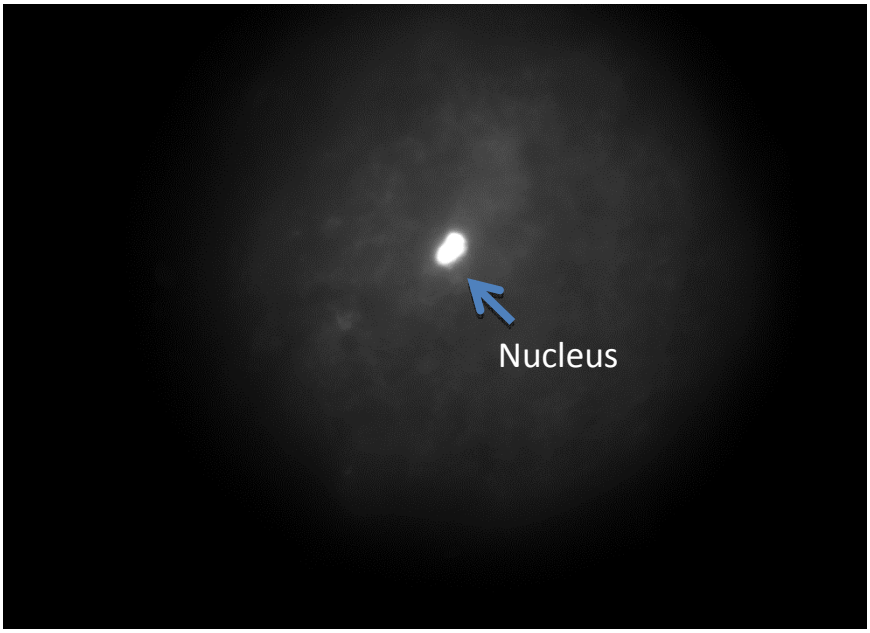


Figure 3.11. Canine oocyte at complete Germinal Vesicle Break Down (GVBD). Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification.

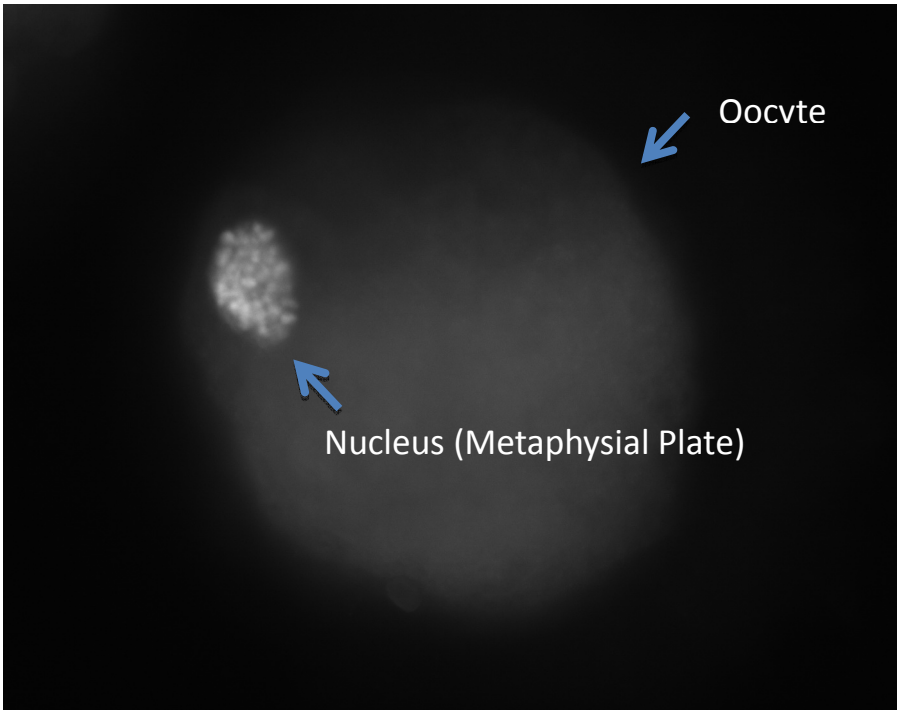


Figure 3.12. Canine oocyte at metaphase I (MI). Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification.

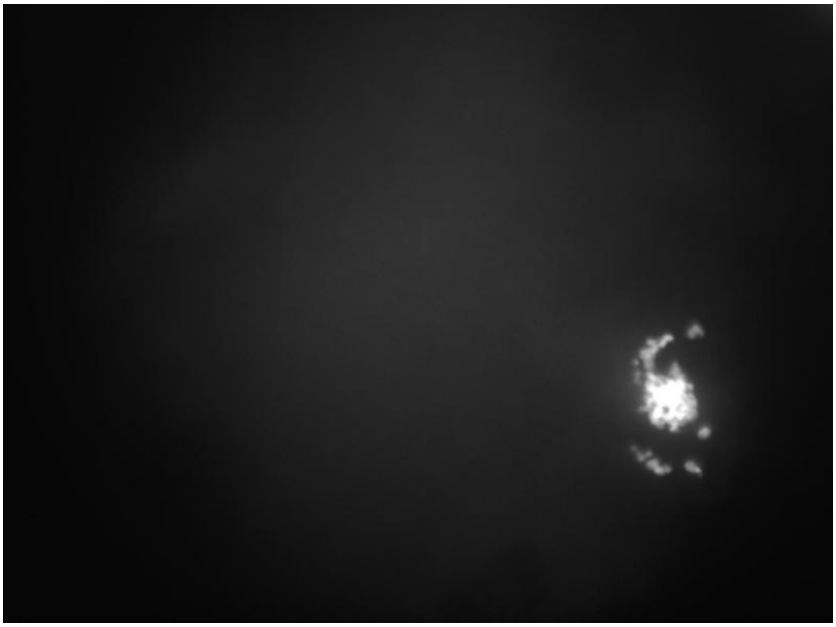


Figure 3.13. Canine oocyte at anaphase I (AI). Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification.

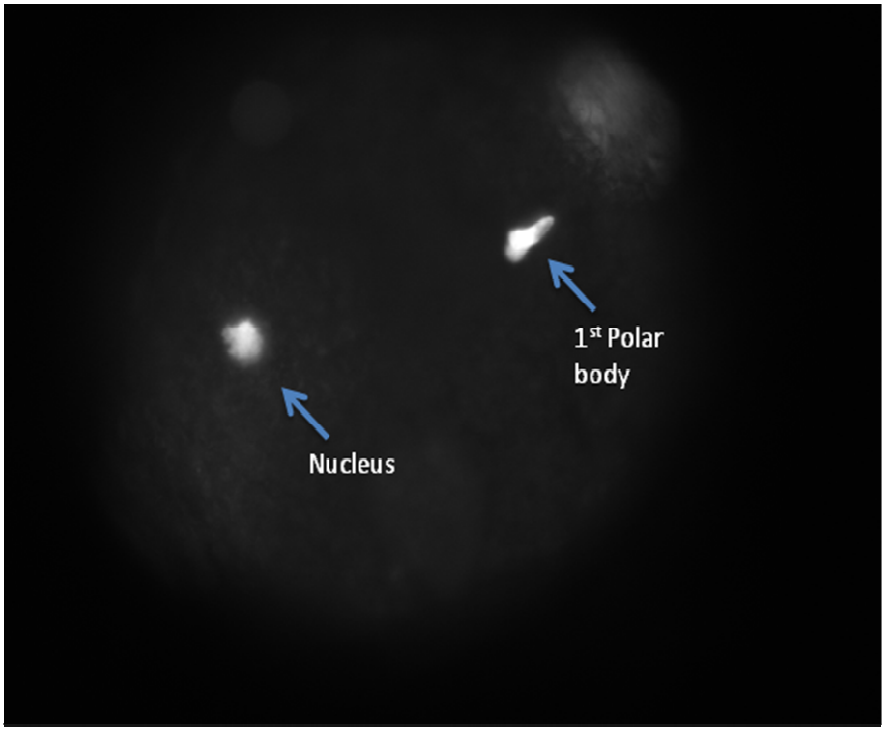


Figure 3.14. Canine oocyte at telophase I (TI). Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification.

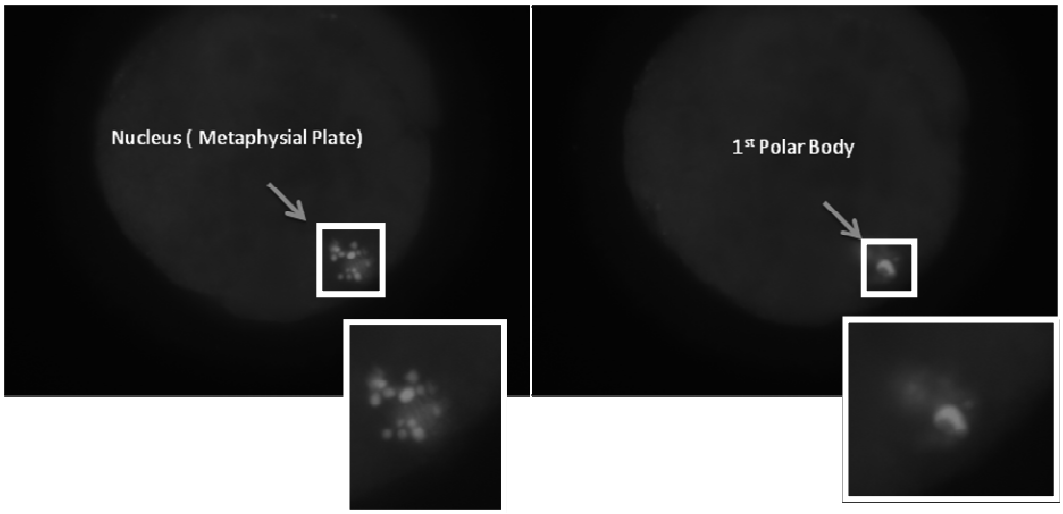


Figure 3.15. Canine oocyte at metaphase II (MII). Graph shows canine oocyte stained using Hoechst 33342 method and visualized under fluorescent microscope at 40X magnification. Both pictures were taken from the same oocyte but at different focus levels. This was due to the fact that MII nucleus and polar body were positioned vertically above each other.

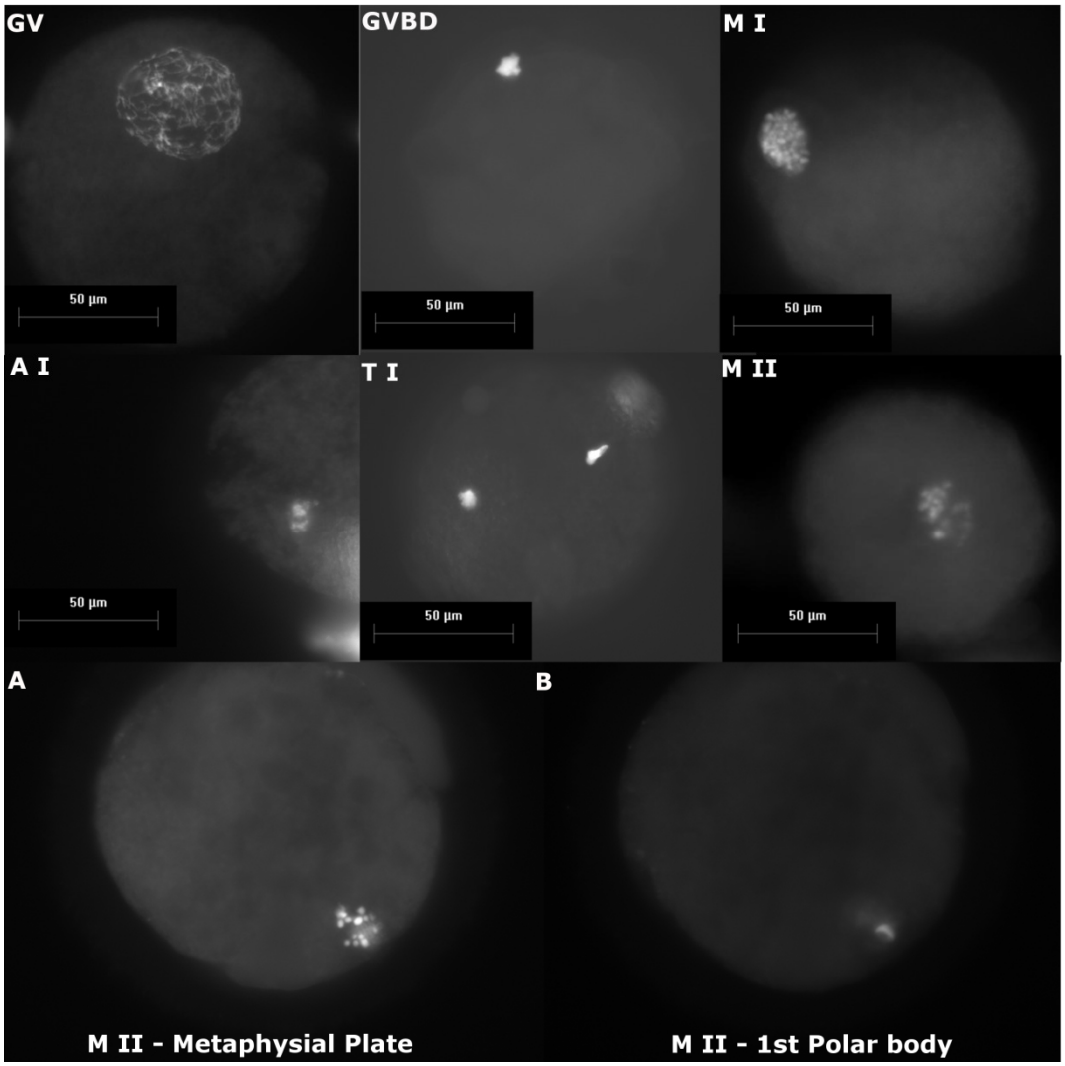


Figure 3.16 .Canine oocytes at different stages of meiotic division: Oocytes were stained with Hoechst 33342 (10µg/ml) and visualized by Fluorescent microscope at 40X. Prophase I (Germinal Vesicle=GV), Germinal Vesicle Break Down = GVBD + chromatin condensation, Metaphase I stage (MI): bipolar meiotic spindles [kinetochore microtubules] from two centriols attach to their respective partners and homologous chromosomes will be arranged equatorially at metaphysial plate that bisects the spindles, Anaphase I (AI): in which kinetochore microtubules shorten and pull the bivalents apart from each other to the opposite end of spindle. Telophase I (TI): 1st polar body formation and arrival of chromosome sets at opposite poles, Metaphase II (MII): characterized by the margination of the tightly packed first polar body and the rearrangement of the chromosomes back to metaphysial plate formation.; A & B pictures of the same oocyte with two sets of chromosomes in different levels of Y stack.

3.4.3. Cumulus cell expansion and oxygen concentration

Expansion of cumulus cells was assessed in COCs cultured under low or high oxygen conditions. No full mucification was observed throughout *in vitro* maturation in either group (absence of extracellular matrix cloud) at 52 h (0.0% Full expansion in both groups; Not expanded oocytes 97.1 ± 1.6% in low O_2 and 95.0 ± 2.7% in high O_2 [P >0.05]). However, after 52h limited numbers of cumulus cells were disintegrated from the COCs, with higher disintegration in COCs cultured in the high oxygen incubator (5.0 ± 2.7% in high O_2 vs. 2.9 ± 1.6% in low O_2; [P >0.05]) . Moreover in high oxygen culture, disintegrated cells were less attached to the culture dish (Fig. 3.17).

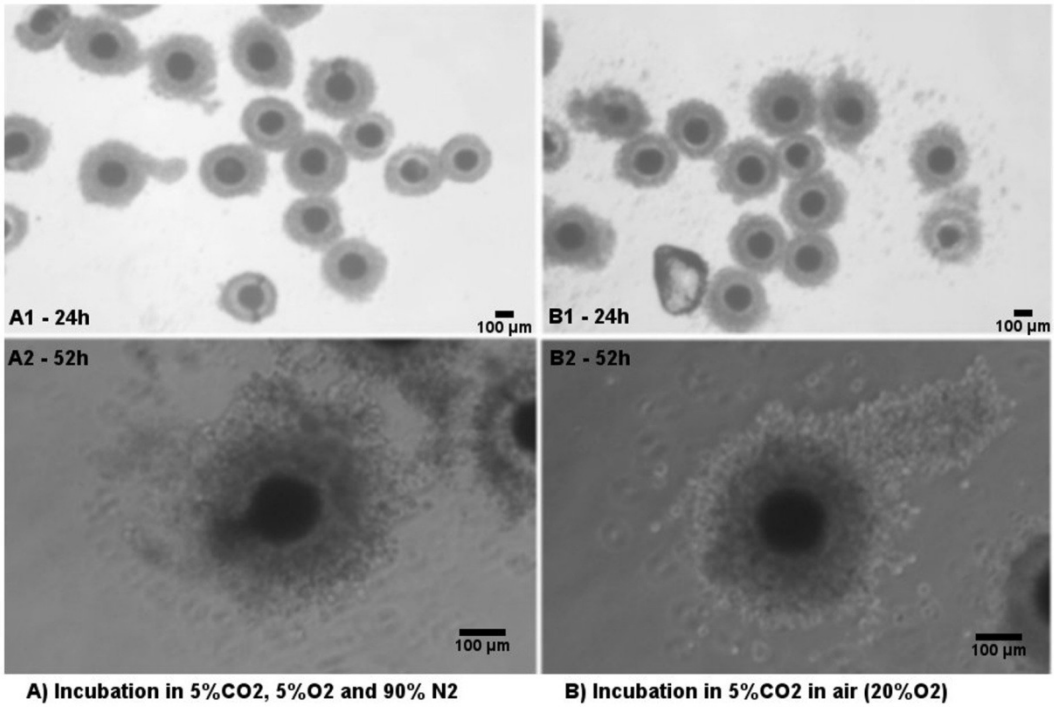


Figure 3.17. Effect of oxygen level during oocyte culture on cumulus expansion of canine oocytes at 24h & 52h. Canine oocytes cultured in low (A) or high (B) levels of oxygen (5% vs 20%). Pictures were taken under light microscope in order to visualize cumulus expansion pattern. [A1 & B1 = oocytes at 24h of culture (magnification 4X)], [A2 & B2 = oocytes at 52h of culture (magnification 10X)]

3.4.4. Nuclear maturation

A total of 118 oocytes from 3 different repeats were cultured in low or high O_2 conditions. After 52h of culture (Table 1), there was no significant difference in degeneration rates of the oocytes cultured under two gas compositions (P>0.05). However, a higher percentage of oocytes resumed meiosis after germinal vesicle breakdown towards MII stage in low O_2 (41.8 ± 13.1%) as compared to the oocytes cultured in high O_2 incubator (15.8 ± 8.1%) (*P* <0.05). The meiotic resumption from MI-MII was also higher in low oxygen group (14.6±7.9 vs. 3.0 ±3.0; *P>0.05*) (Table 3.3).

Table 3.3. Effect of oxygen concentration during oocyte culture (52h) on nuclear maturation of canine oocytes. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV) ~ Germinal Vesicle Break Down (GVBD) ~Metaphase 1 (M I) ~Anaphase 1 (A I) ~Telophase 1 (T I) ~Metaphase 2 (MII). Meiotic resumption of oocytes (total number of oocytes between GVBD and MII) was significantly higher in low O_2 condition. Significance marked by star.

	Percentage of oocytes (Average ± SEM) in 52h Oxygen comparison								
	GV	GVBD	MI	AI	TI	MII	Degen	Meiotic resumption (MI-MII)	(n)
Low O_2	35.0±13.4	27.1±12.1	4.0 ±4.0	4.8±2.5	3.8±2.4	2.0±2.0	23.2±6.6	14.6±7.9	66
High O_2	52.3±9.9	12.7±6.4	3.0 ±3.0	0.0±0.0	0.0±0.0	0.0±0.0	31.9±2.2	3.0±3.0	52

* P value < 0.05, high O_2 compared to low O_2

3.4.5. Extended *in vitro* maturation

A total number of 460 oocytes were cultured in base maturation media for 52h (n=66), 72h (n=231) and 84h (n=163). The number of oocytes that remained at GV stage in the 52h group was higher ($P<0.05$) than the other two and the lowest MII stage percentage occurred within this group. Highest meiotic resumption (GVBD-MII) was in the 72h cultured group (64.1±6.0%) which was higher than 52h ($P<0.05$). On the other hand the 84h group resulted in the highest rate of degeneration with 43.2 ±4.5% compared to 52h ($P<0.05$) (Table 3.4). Although there were no significant difference in MI-MII percentages among the three groups, the highest MII maturated oocytes 7.5 ± 4.0% occurred at 84h ($P<0.05$) . (Table 3.4)

Table 3.4. Effect of the duration of culture period from 52-84hours on the nuclear maturation of canine oocytes (5%O_2, 5%CO_2 and 90%N_2). Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV) ~ Germinal Vesicle Break Down (GVBD) ~Metaphase 1 (M I) ~Anaphase 1 (A I) ~Telophase 1 (T I) ~Metaphase 2 (M II). Statistically significance has been marked by the asterisks (P value < 0.05)

	Percentage of oocytes (Average ± SEM) in 52h vs. 72h vs. 84h & Low oxygen							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
52h	35.0±13.4	27.1±12.0	4.0±4.0	4.8±2.4	3.8±2.4	2.0±2.0	23.2±6.6	14.6±7.9	66
72h	4.0±1.1*****	37.2±8.4*****	16.5±2.5	2.9±1.8	3.7±0.8	3.8±0.8	31.9±5.4*****	26.9±3.5	231
84h	0.7±0.7*****	22.5±4.2	20.6±4.8*****	2.8±1.4	2.5±1.3	7.5±3.9*****	43.2±4.4*****	33.5±3.2	163

* P value < 0.05, within the column compared to 52h

3.4.6. Reactive oxygen species and oxygen level

To analyze the effects of oxygen level during IVM, oocytes (n= 130) were stained for H_2O_2 levels using DCHFDA after 72h culture. Densitometry using ImageJ software showed the overall intensity of fluorescence was greater for high O_2 oocytes higher than low O_2 oocytes (P = 0.004) (Fig. 3.18).

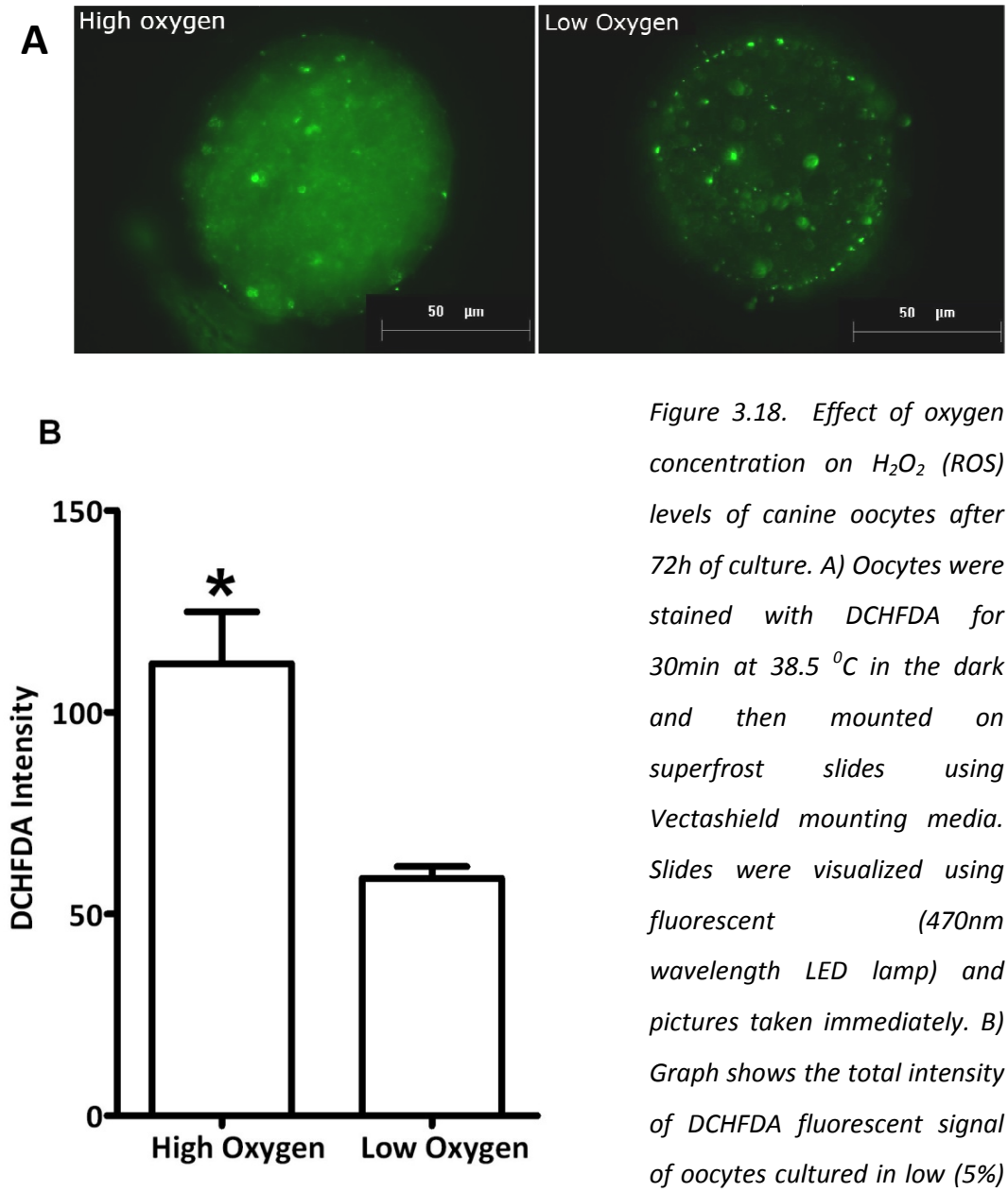


Figure 3.18. Effect of oxygen concentration on H_2O_2 (ROS) levels of canine oocytes after 72h of culture. A) Oocytes were stained with DCHFDA for 30min at 38.5 ^{0}C in the dark and then mounted on superfrost slides using Vectashield mounting media. Slides were visualized using fluorescent (470nm wavelength LED lamp) and pictures taken immediately. B) Graph shows the total intensity of DCHFDA fluorescent signal of oocytes cultured in low (5%) and high (20%) oxygen levels in the base maturation media after 72h. DCHFDA intensity is a direct indicator of H_2O_2 level.

3.4.7. ROS repair enzymes mRNA expression

Analyses of the fold induction of target genes (*GPX1*, *SOD1*, *SOD2*, *GSR* & *CAT*) at 0h and 72h in culture showed significant end point differences between COCs cultured in low and high oxygen incubators after 72h ($p<0.05$). The expression of mitochondrial *Mn-SOD2* among other enzymes involved in GSH metabolism was more prominent in the high oxygen group. (Fig. 3.19)

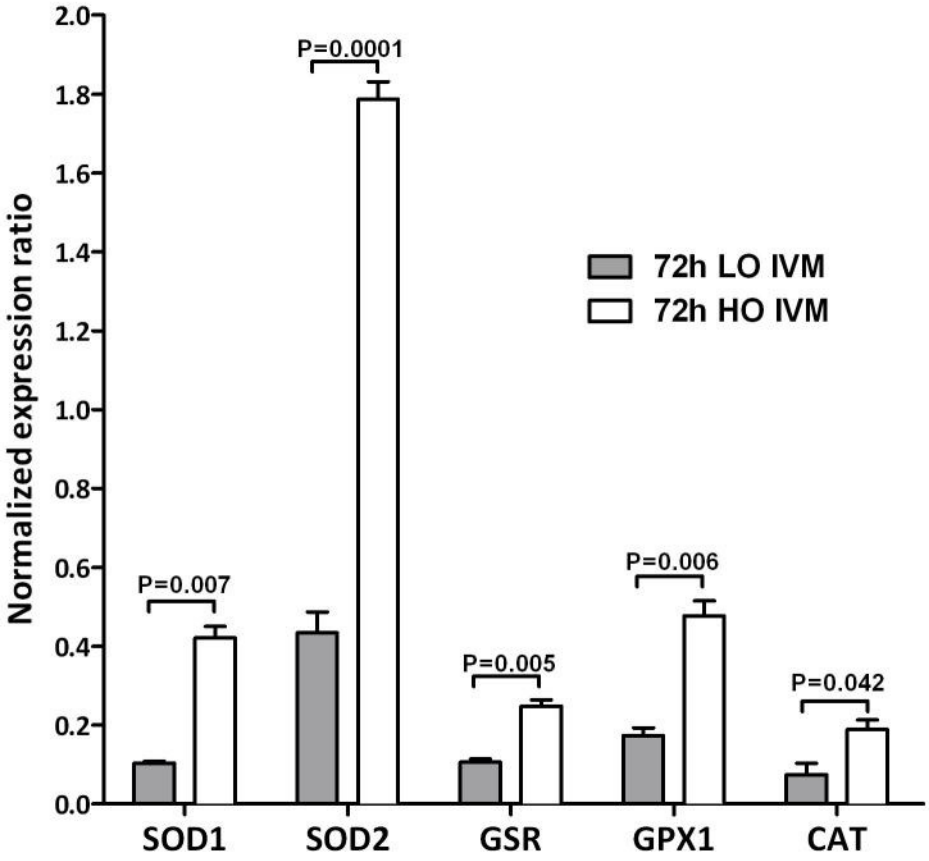


Figure 3.19. Effect of oxygen concentration on mRNA expression profile of ROS repairing enzymes in canine oocytes. Graph shows the fold induction of ROS repairing enzymes after 72h of IVM compared to the 0h and normalized by the GAPDH house keeping gene. (SOD1= Cytosolic Superoxide dismutase; SOD2= Mitochondrial Superoxide dismutase; GSR= Glutathione reductase; GPx1= Glutathione peroxidase and CAT= Catalase; LO=low oxygen; HO= high oxygen)

3.4.8. α-tocopherol (vitamin E) and oxidative stress

COCs cultured in low and high oxygen incubators were supplemented with α-tocopherol during 72h of IVM. α-tocopherol at 100μM reduced degeneration in the high oxygen group compared to its control (41.7 ± 7.6% down to 30.4 ± 2.3%; p=0.008) (Table 3.5). Meiotic resumption in control (p=0.0001) and α-tocopherol (p=0.007) treated oocytes in the low oxygen group was higher than their high oxygen counterparts (Fig.3.20). Also α-tocopherol also has no significant effect on the percentage of MII matured oocytes in both control groups (low = 13.1 ± 3.1% and high= 4.6 ± 2.5%; P=0.072)(Table 3.5). However, in the presence of vitamin E the MII maturation rate in the low oxygen group was higher than in high oxygen group (p=0.008). (Fig. 3.20)

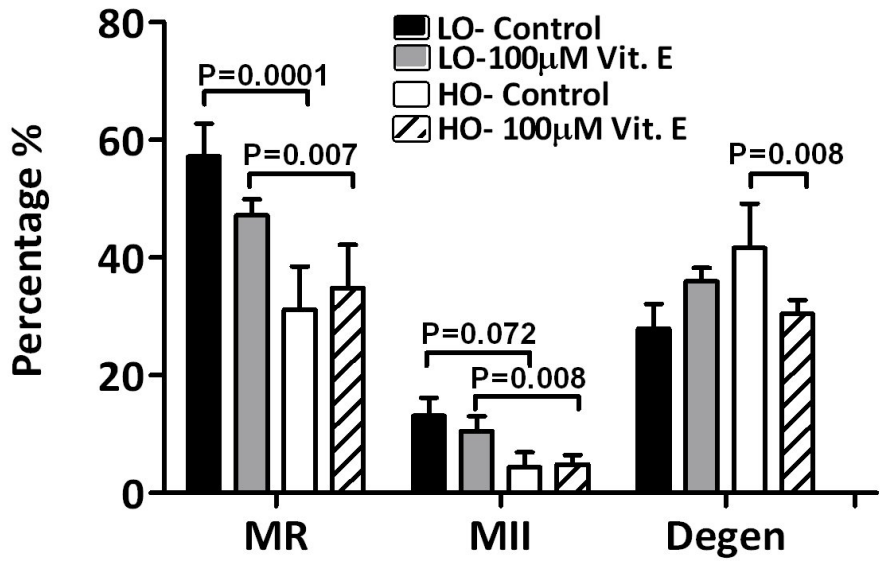


Figure 3.20. Effect of vitamin E on the nuclear maturation of canine oocytes in low and high oxygen. Graph shows the percentages of meiotic resumption (MR; GVBD-MII), metaphase II (MII), degenerated (Degen) oocytes cultured in the presence or absence of Vit. E (LO = low oxygen; HO = High oxygen)

Table 3.5. Effect of Vit. E on the nuclear maturation of canine oocytes in low and high oxygen. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV) ~ Germinal Vesicle Break Down (GVBD) ~Metaphase 1 (M I) ~Anaphase 1 (A I) ~Telophase 1 (T I) ~Metaphase 2 (M II).

	Percentage of oocytes (Average ± SEM) in Vit. E experiment (72h)							Meiotic resumption (GVBD-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Ctrl Low	14.9±4.1	31.5±5.4[a]	7.6±2.6	2.3±2.3	2.8±2.8	13.1±3.1	27.9±4.2	57.2±5.5 [a]	39
Vit. E Low	16.9±2.9	24.9±4.7[b]	8.0±4.8	0.0±0.0	3.6±2.2	10.5±2.5[b]	35.9±2.3	47.2±2.7[b]	51
Ctrl High	27.2±2.2[a]	16.2±3.1	8.6±3.7[*]	2.1±2.1	0.0±0.0	4.6±2.5	41.6±7.5[*]	31.2±7.3	45
Vit. E High	34.7±5.7[b]	16.5±3.6	7.6±1.5	1.3±1.3	4.6±2.9	4.8±1.6	30.4±2.3	34.8±7.3	66

* P value < 0.05 within the column in low or high oxygen compared to their controls
[ab] P value < 0.05 within the column between control groups [a], between Vit. E groups [b]

3.5. Discussion

Establishment of an efficient *in vitro* maturation system for canine oocytes, which is complicated by unique and complex canine reproductive physiology, is still unsolved. During this study, attempts were made to establish a chemically defined and time wise optimally modified *in vitro* maturation protocol for canine oocytes. The majority of *in vitro* maturation protocols are based on serum enriched culture media (Otoi *et al.* 1999, Luvoni *et al.* 2005, Oh *et al.* 2005) in which the exact beneficial effects of serum are unknown and in most cases unpredictable (Bolamba *et al.* 2002, Lee *et al.* 2007b). In addition, results from our preliminary experiments indicated that presence of foetal bovine serum tends to have detrimental effects on nuclear maturation of canine oocytes and significantly increases degeneration rates. Thus in this study, all the experiments were carried out in a chemically defined medium and without serum supplementation.

3.5.1. Staining and staging

Nuclear staining and staging of canine oocytes after IVM is one of the most critical issues in canine assisted reproductive techniques. Therefore, it is worth mentioning that we have optimized a protocol which allows precise assessment of oocyte nuclear stage during meiotic maturation. It includes a procedure for complete denuding of cumulus cells and fixation followed by staining of oocytes and visualization of oocyte chromatin under a fluorescence microscope. Denuding canine oocytes due to their highly inter-digitized cumulus- ZP attachment (Blackmore *et al.* 2004, De los Reyes *et al.* 2009a) after culture is challenging (Hewitt *et al.* 1998). In addition, the lipid droplets inside the oocyte make it very difficult to visualize the chromatin content by simple Aceto-Orcein staining unless oocytes are fixed for 5-7 days (Song *et al.* 2010a). After trying several denuding buffers with different timetables of incubation and vortexing (Reynaud *et al.* 2004), incubation in sodium tri-citrate 1% (Hewitt & England 1999) and denuding using an oocyte holding needle with gauge of 135µm (Yellow

EZ-Strip® Research Instruments limited, Cornwall, UK) resulted in complete removal of the cumulus cells. For nuclear staining, the combination of 10 min fixation with acetone at -20^{0}C followed by 5 min staining in 10µg/ml Bisbenzimide (Hoechst 33342) solution provided a quick and reliable solution. Utilizing this method we were able to provide a panel of images which can be used as guidance for investigators (Fig. 3.16).

3.5.2. Oxygen level and *in vitro* maturation

Expansion of cumulus cells did not differ after 52 h of culture of COCs in low (5%) or high (20%) oxygen incubators; nevertheless, the disintegration of cumulus cells differed in high oxygen (Fig. 3.17). The oviductal oxygen level is almost one forth to one third of the normal air oxygen level (e.g. 5% vs. 20%) (Rodrigues & Rodrigues 2010). Despite the presence of anti-apoptotic intracellular mechanisms acting via GSH against ROS (Silva *et al.* 2009), having high lipid content in canine COCs can reduce GSH level and predispose them to oxidative damage and induce apoptosis in the cumulus cells (Silva *et al.* 2009). This can contribute to lowered oocyte maturation rates (Kim *et al.* 2007, Rodrigues & Rodrigues 2010). Expansion of cumulus cells *in vivo* or during culture of COCs *in vitro* occurs through production of hyaluronan by cumulus cells under the influence of LH. Cumulus cells must be viable and express hyaluronan synthase II in the cell membrane (Marei *et al.* 2012). Induction of apoptosis in COCs through oxidative stress during the extended culture period is expected to hamper production of hyaluronan and cumulus cells expansion. Albeit that cumulus cell expansion is not a convincing marker of canine oocyte maturation (Reynaud *et al.* 2005, Otoi *et al.* 2007, Rodrigues & Rodrigues 2010), there were significantly higher number of maturing oocytes (GVBD-MII) after 52h culture in the low oxygen (41.8 ± 13.1%) group compared to the high oxygen group (15.8 ± 8.2%). This was accompanied by lower degeneration rate in the low oxygen group (23.2 ± 6.6%) compared to the high oxygen group (31.9 ± 2.2%) ($P<0.05$) confirming the detrimental effects of high oxygen tension on dog oocytes.

Duration of culture period for *in vitro* maturation of canine oocytes ranges from 48 to 96h in different studies (Luvoni *et al.* 2005, Rodrigues & Rodrigues 2010). *In vivo*, oocytes need 2-4 days for completion of this stage (Concannon 2011). Therefore it was decided to extend the culture period up to 84h in order to find the optimal culture period for canine oocytes which results in highest maturation and lowest degeneration rates in the low oxygen atmosphere. Extension of culture period to 72h resulted in increased meiotic resumption to 64.1 ± 5.9% (Table 3.4). However, further extension of the culture period to 84h resulted in elevation (7.4%) in the percentage of MII oocytes (Table 3.4) but increased degeneration rate. Similar findings were reported by other research groups (Otoi *et al.* 2002, Rodrigues Bde *et al.* 2004). Therefore, for the remainder of experiments presented here including H_2O_2 comparison, ROS and Vit. E experiments, 72h incubation period was used to avoid high degeneration but achieve highest meiotic resumption.

3.5.3. Oxidative stress and *in vitro* maturation

In the H_2O_2 staining (DCHFDA) experiment a comparison was done between low and high oxygen levels and the total amount of H_2O_2 produced inside ooplasm of canine oocytes after 72h of culture. Oocytes stained with DCHFDA (Fig. 3.18 - A) produced significantly higher levels of H_2O_2 ($P = 0.004$) in the high oxygen group (Fig. 3.18 - B). It is noteworthy that H_2O_2 is a bi-product of the ROS protection system in which SOD enzymes transform damaging oxygen free radicals into less aggressive H_2O_2 molecules (Whitaker & Knight 2008). Since oxidative stress is introduced to oocytes immediately after mechanical extraction till the end of the culture period via various sources of free radicals, light and physical trauma (Guerin *et al.* 2001, Menezo *et al.* 2010), it is necessary to provide these cells with least amount of stressors. Reduction of oxidative stress can profoundly contribute to improved nuclear and cytoplasmic maturation.

Results of the real-time qPCR showed that mRNA of ROS repair enzymes are significantly higher in oocytes cultured in high oxygen (Fig. 3.19) which is

concomitant with high H_2O_2 level, lower meiotic resumption and higher degeneration rates. Superoxide dismutase, present in mitochondria (*SOD2*), showed remarkable increase in mRNA expression in the high oxygen group, which is in agreement with previous reports (De los Reyes *et al.* 2011). Involvement of mitochondrial mobilization during cytoplasmic maturation of canine oocytes in terms of perturbation due to oxidative pressure could be the main retarding factor in canine IVM. Moreover oocytes cultured in high oxygen showed increased *GPX1* and *GSR* expression in response to high oxygen tension (Fig. 3.19), indicating that the mitochondrial oxidative profile requires further investigation.

Supplementation of vitamin E in the maturation medium did not prevent the detrimental effects of high oxygen tension on degeneration rates of canine oocytes during 72h culture. Meiotic resumption of oocytes cultured in low oxygen tension was higher in both control and 100µM vitamin E-treated oocytes than in the high oxygen group. Vitamin E in the presence of high oxygen was able to decrease degeneration (P=0.008) but did not improve the percentage of oocytes in MII. The beneficial effect of vitamin E on oocyte maturation and embryo development was previously reported in animal species regardless of lipid content, including porcine cells (Tao *et al.* 2010) which contain high lipid or in ovine oocytes (Natarajan *et al.* 2010) which contain relatively low lipid contents.

The studies presented here concerned culture of canine oocytes in a serum free and chemically defined maturation media. Culture period of 72 h in low oxygen level (5%) was the optimal condition for canine COCs. Oxidative stress and the level of ROS in canine ooplasm affected maturation efficiency, particularly involving mitochondrial activity.

To sum up, results of this chapter elucidated:

- Number of COCs available for collection from each bitch significantly decreases by aging.
- There is no significant difference in expansion or disintegration of cumulus cells in a serum free culture system between low (5%) and high (20%) oxygen incubators.
- Among three extended culture times (52, 72 and 84h), canine oocytes had optimal nuclear maturation and degeneration rates after 72h of culture.
- COCs cultured in high O_2 condition significantly produce higher levels of H_2O_2 (ROS) than the ones cultured in low O_2.
- Relative mRNA expression of ROS repairing enzymes such as *SOD1/2, GPX1*, *CAT and GSR* was higher in COCs cultured in high O_2 conditions. *SOD2,* mitochondrial isoform of superoxide dismutase, was affected the most by exposure of the COCs to high O_2.
- Addition of Vit.E 100µM as an antioxidant to the culture media during the 72h of IVM, did not promote meiotic resumption but significantly reduced the degeneration rate in the high O2 condition.

4. Effects of caffeine on nuclear and cytoplasmic maturation canine oocytes

4.1. Introduction:

For *in vitro* maturation, the bitch oocytes are mainly collected after spaying which is usually done during anoestrus when the animals have basal plasma levels of ovarian steroids to avoid post-operational complications. *In vivo*, canine oocytes are ovulated at the germinal vesicle stage (GV= diplotene of prophase I), then maturation resumes during oviductal transition 48-72h after ovulation (Concannon 2011). Therefore, collection of ovaries during anoestrus will provide immature oocytes arrested at the GV stage and from secondary or preantral follicles which were not recruited by preovulatory surge of gonadotropic hormones (Vannucchi *et al.* 2009). Studies in mammalian species have shown that the high concentration of cyclic adenosine monophosphate (cAMP) is the major mechanism for this meiotic arrest through several pathways (Bornslaeger *et al.* 1984, Bornslaeger & Schultz 1985, Guixue *et al.* 2001, Luciano *et al.* 2004, Takeda *et al.* 2006). The LH mediated changes during the pre-ovulatory period govern the initial changes of cAMP concentration within the oocyte and cumulus cells (Mehlmann 2005, Zhang *et al.* 2009) leading to resumption of meiosis in the oocyte. As described previously the changes in cAMP level have been hypothesized to mainly act through LH receptors (GPRs, AD), PDEs and intracellular calcium (Ca^{2+}) pathways (Mehlmann 2005). Phosphodiesterases within the COC complex are the most affected molecules during this chain of events. Therefore the usage of PDE inhibitors such as methylxanthines (caffeine, theobromine, 3-Isobutyl-1-methylxanthine [IBMX]) (Lee & Campbell 2006, Barretto *et al.* 2007) and cAMP analogues (e.g. dibutyryl cyclic AMP [dbcAMP]) (Guixue *et al.* 2001) have been applied in maturation media in order to manipulate the nuclear changes of the oocytes *in vitro*.

Methylxanthines, a family of PDE inhibitors, such as caffeine, IBMX (1-methyl-3-isobutylxanthine) and theophylline can be utilized to sustain cAMP concentration (Minelli & Bellezza 2011). Caffeine has been used to improve male gametes fertilization and female gametes developmental potential as an established culture supplement within a specific range of concentration (Abeydeera *et al.* 1994, Kren *et al.* 2004, Lee & Campbell 2006, Choi *et al.* 2010). Temporal exposure of COCs to caffeine was proposed to slow down/or arrest the nuclear changes and preserves the intercellular gap junction in the designated time period. However; to our knowledge, there has been no report of caffeine treatment on canine oocytes to date.

During the oocyte maturation, MPF and MAPK pathways accompanied by changes in cAMP concentration trigger a chain of events resulting in the meiotic resumption (Katayama *et al.* 2005, Han *et al.* 2006, Liang *et al.* 2007, Tripathi *et al.* 2010). The molecular cascade leading to activation of MPF within the oocyte, is formed of series of protein kinases (PKC, PKB[PI3K/AKT]) and phosphorylating MAPK1&3 in the cumulus cells (Tripathi *et al.* 2010). Changes in the phosphorylation of MAPK1&3, AKT phosphorylation and MPF activity could indicate the molecular progression of meiotic resumption within the COCs.

The molecular events leading to nuclear changes of the oocytes during meiotic progression are spanned to time period between preovulatory stage up to 2-5 days after ovulation and during oviductal transition (Concannon 2011). Thus a biphasic IVM protocol is required to compensate the gonadotropic induction and oviductal transition. During this critical follicular-oviductal transition of the oocytes, the relative changes in the concentration of cAMP are precedential (Luciano *et al.* 2004, Saint-Dizier *et al.* 2004). Moreover; manipulation of the cAMP concentration by substances such as caffeine could be critical in optimizing the maturation time course.

The unique premature status of canine oocyte prior to IVM concerned several studies (Otoi *et al.* 2001, Kim *et al.* 2007, Rodrigues *et al.* 2009a). BCB staining is

a non-invasive screening test used recently in oocyte selection in other species (Pujol *et al.* 2004, Wu *et al.* 2007, Catala *et al.* 2011). Growing oocytes at the beginning of S-phase perceive high glucose-6-phosphate dehydrogenase (G6PD) activity which metabolizes dark blue/purple BCB in to a colorless compound (Rodriguez-Gonzalez *et al.* 2002). The studies presented here examined the impact of oocyte selection based on BCB staining as a screening method in combination with caffeine pre-treatment on canine oocytes nuclear maturation. Dog oocytes were cultured using a biphasic protocol involving 12h of culture in the presence of it followed by 60h of culture in the absence of caffeine. Rate of oocyte development through stages of meiosis I was assessed.

4.2. Materials and Methods

Chemicals and reagents:

All chemicals were purchased from Sigma-Aldrich chemical Co. (Poole, UK) unless otherwise stated.

4.2.1 *In vitro* maturation

The IVM culture and oocyte staining/staging was carried out following the protocols described in the sections 2.2.-2.6.

4.2.2. BCB staining

Prior to staining the COCs, a working buffer was prepared containing 1mg/ml glucose, 0.036mg/ml Na-pyruvate and 0.5mg/ml BSA in 0.5 liter of PBS 1x (Rodrigues *et al.* 2009a, Catala *et al.* 2011). This buffer is referred to as modified PBS (mPBS). BCB was freshly prepared in mPBS at 26µM just before each experiment. Within a 60mm culture dish, 100µl drops of mPBS and BCB were placed separately and the dish was equilibrated at 38.5^{o}C in the incubator 2h prior to staining of the oocytes. Freshly collected COCs were washed for 10 sec in the mPBS drop and transferred immediately to the BCB drops and incubated for 30 min (Fig. 4.1).

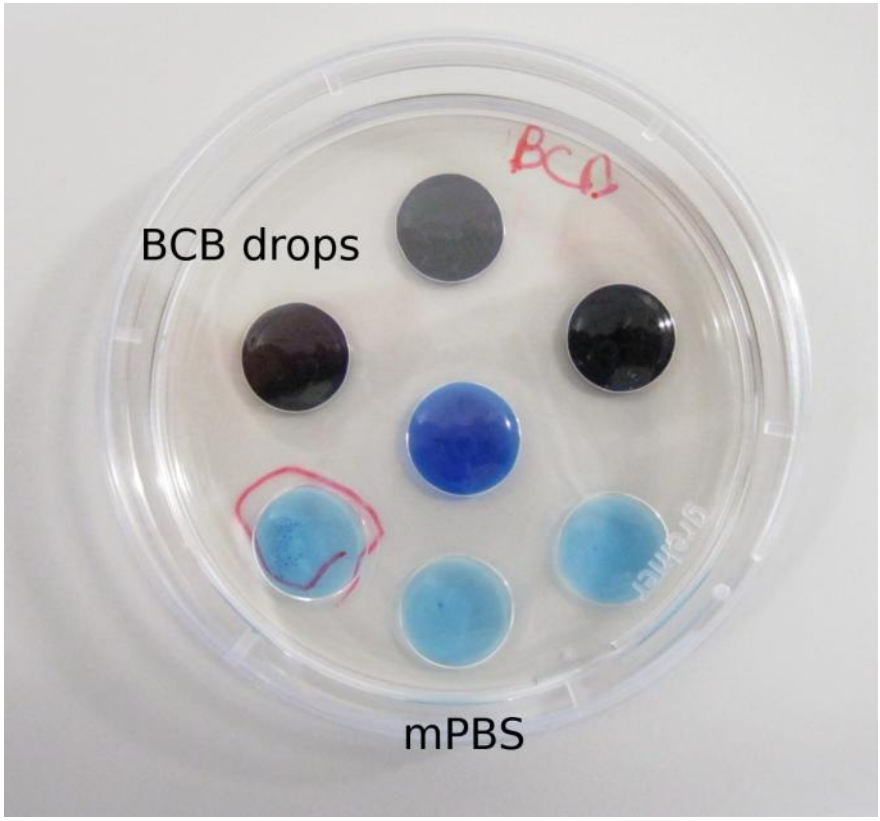


Figure 4.1. Image of a 60mm culture dish containing BCB working droplets. Picture shows the BCB staining dish with 3 BCB 2µ6M (dark droplets on the top) and mPBS washing droplets .

COCs were then assessed using a stereo light microscope. Developmentally competent oocytes which have reached their maximum size and mitochondrial capacity contain low level of G6PD remain purple (BCB +). The growing oocytes with low developmental potential containing high levels of G6PD will metabolize the BCB and become colorless (BCB-) (Fig. 4.2). After separation of COCs into BCB+ and BCB– groups, they were washed 3 times in mPBS prior to transfer to the maturation media.

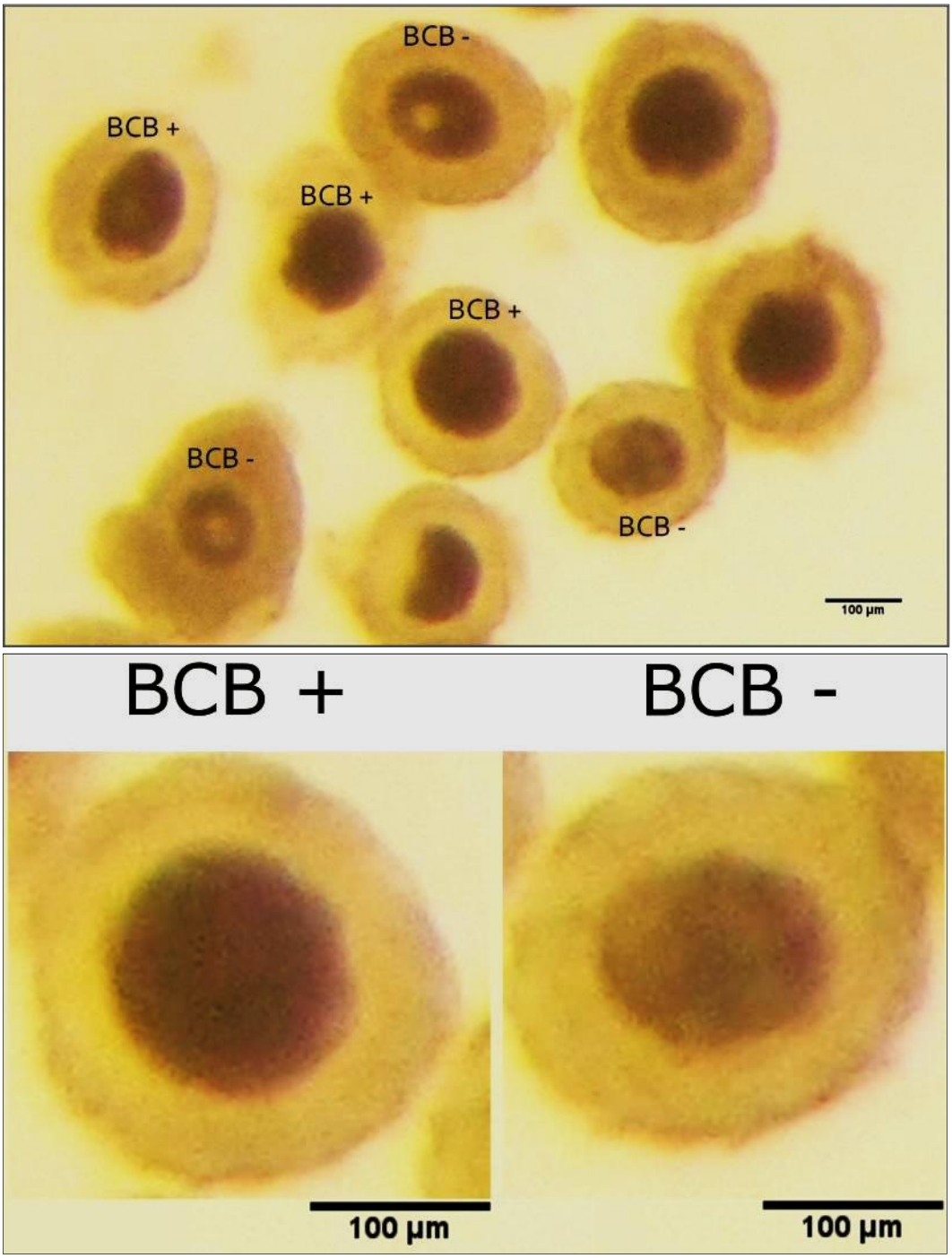


Figure 4.2. BCB staining of freshly collected canine COCs. Images taken after BCB incubation for detection of BCB+ and BCB - COCS according to the dark purple (blue) colour of cytoplasm.

4.2.3. Western blotting for determination of AKT and MAPK 1&3

Buffers and reagents used for western blotting were prepared as following:

Reagents	Instructions
Polyvinylpyrrolidone (PVP 4%)	4g of PVP in 100ml of PBS 1x
Tris-base 76.5 mM (pH 6.8)	0.9267g in 100ml DDW*
Tris - Glycerol 10% (v/v) -SDS 4% (Tris-Glycerol-SDS)	83 ml of 76.5mM Tris pH 6.8 10ml glycerol 4g SDS (sodium dodecyl sulphate) DDW up to 100ml volume
Protease inhibitor cocktail (x100 stock) Calbiochem # 539131	Reconstituted in 1ml DDW
Phosphatase Inhibitors mix [200mM Sodium orthovanadate (Na_3VO_4); 20mM Sodium fluoride]	36.7 mg of Na_3VO_4 10mg of NaF In 1ml of DDW
Lysis buffer (Tris 63.5mM , pH = 6.8)	942µl Tris-Glycerol-SDS 38µl DDW 10µl Protease inhibitor cocktail 10µl Phosphatase inhibitor mix
Tris Glycin SDS PAGE Buffer (10X) National Diagnostics #EC-870	
Transfer buffer 20X (1 litre)	288gr Glycin 60 gr Tris 1 litre of DDW
PBS –Tween 0.05% (PBS-T)	0.5ml Tween20 995ml PBS1X

*Deionized distilled water

COCs were washed in PBS-polyvinylpyrrolidone (PVP) 4% (w/v) and snap frozen in liquid nitrogen at each time point according to the experimental design. The COCs were lysed in 20µl lysis buffer containing Tris (63.5mM, pH= 6.8), 10% Glycin and 4% SDS. To preserve the target protein during lysis a combination of protease and phosphatase inhibitors was utilized; sodium orthovanadate 2mM, sodium fluoride 200µM, protease inhibitor cocktail 1X (Calbiochem, Darmstadt, Germany). Electrophoresis buffers were prepared as following:

Reagent	Weight	DDW*
Tris 1.5M (pH 8.4$^{\$}$)	45.43g	250ml
Tris 0.5M (pH 6.8$^{\$}$)	15.4g	250ml
SDS (10%)	10g	100ml
Ammonium persulphate (10%)	0.1g	1 ml

*Deionized distilled water

$^{\$}$ pH alteration was carried out using 5M HCl

Buffers were mixed in 50ml conical tube, in order to obtain 10% resolving and 4 % stacking gel:

Reagent	10% Resolving (12.5ml)	4% Stacking (5ml)
Tris 1.5M (pH 8.4$^{\$}$)	3.13ml	-
Tris 0.5M (pH 6.8$^{\$}$)	-	1.25 ml
DDW	5.0ml	3.0 ml
Protogel 30% (29:1 acrylamide :bis-acrylamide)	4.18ml	0.67 ml
SDS (10%)	125µl	50 µl
Ammonium persulphate (10%)	125µl	50 µl
TEMED 0.1%*	12.5µl	5 µl

* Chemicals were added in the same descending order.

Samples were boiled at 100^{o}C for 10 min (reduced using 2-mercapto ethanol) then loaded on a SDS-PAGE. As a protein marker, 10µl pre-stained protein ladder (Fermentas; Loughborough, UK) was loaded in the first lane. Electrophoresis was carried out at 170V ~ 24mA for 1h35m (Fig. 4.3).

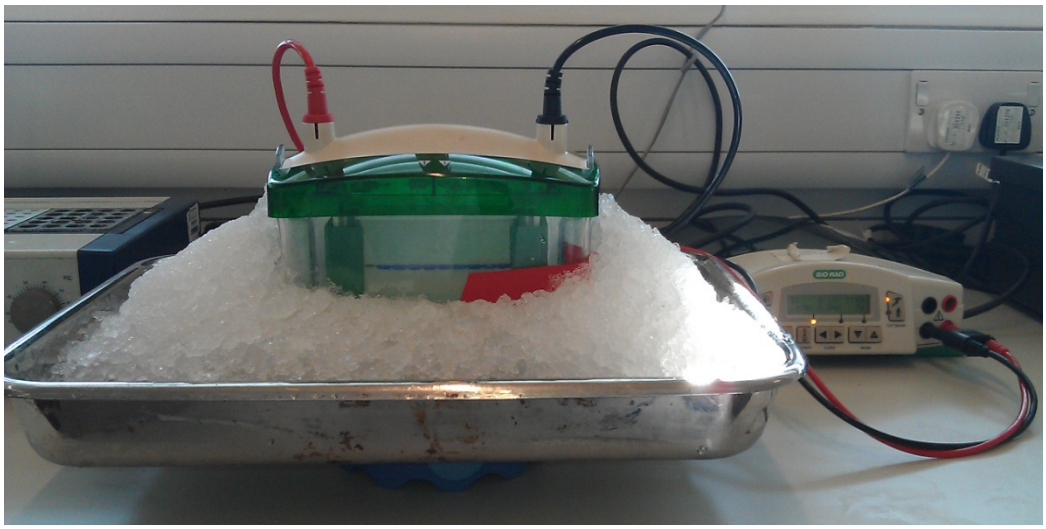


Figure 4.3. Picture shows electrophoresis of a SDS-PAGE in progress. Samples were loaded in to the gel and electrophoresis tank was assembled then submerged in to a tray of ice.

Afterwards the proteins were transferred from the gel to PVDF membrane (GE healthcare, Hatfield, UK) at 100 V ~ 51mA for 1h 40m. The transfer was carried out using Bio-Rad Transfer module (Bio-Rad, UK). The order of items in the transfer basket is shown in Figure 4.4. Negatively charged proteins were transferred from the gel (green layer) to the PVDF membrane (pink layer) within the transfer casket. The transfer tank was also submerged in to ice during the transfer process (Fig. 4.4).

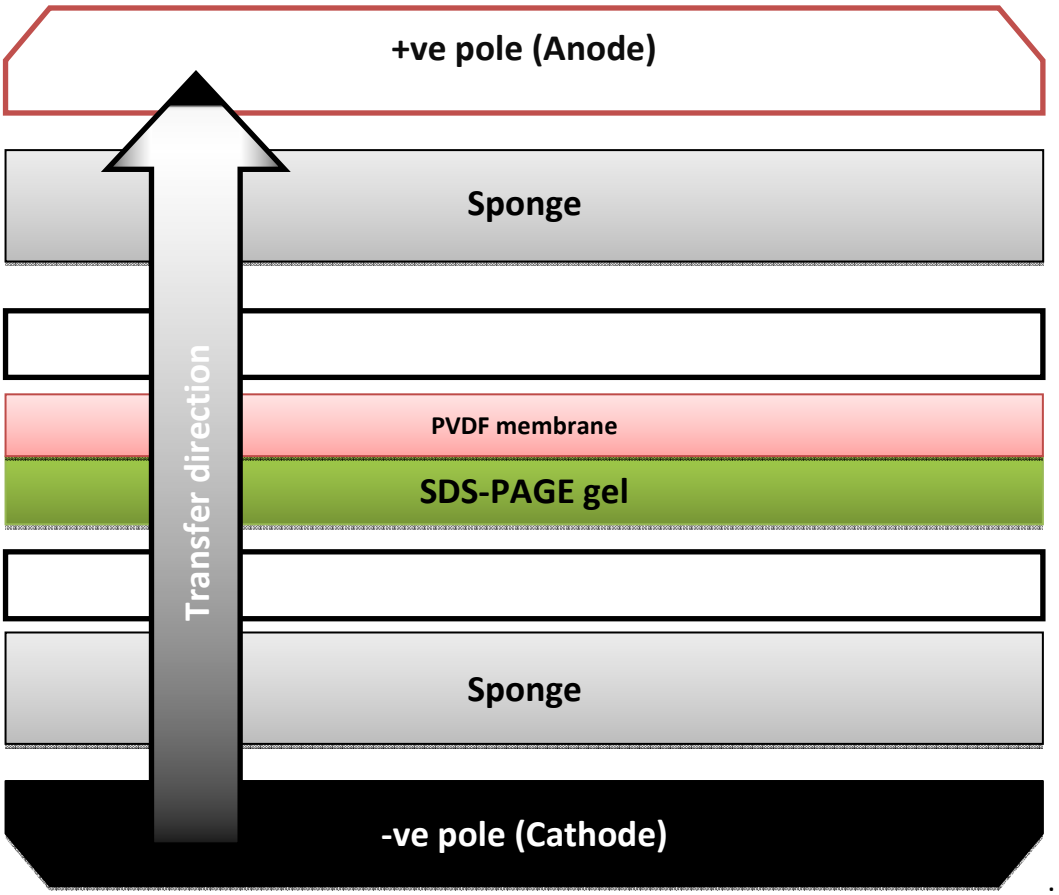


Figure 4.4. The order of different layers of a transfer casket for Western blot. The picture shows the order of the layers within a transfer casket prepared via Bio-Rad transfer module and tank. Filter papers (4mm thickness) and sponges were used to cover the gel membrane structure within the casket.

The membranes were then blocked in 10% Skimmed milk for 4 h at room temperature. Preceded by a 5 min wash in PBS-Tween 20 (0.05% v/v), the membranes were blotted with primary antibodies against target proteins; total and phosphorylated AKT (1:1000 dilution; Cell Signaling, Boston, MA, USA) and total and phosphorylated MAPK (1:2000 dilution; Cell Signaling, Boston, MA, USA). Donkey anti rabbit IgG-HRP conjugated secondary antibody (SantaCruz, CA, USA) was used for all probing. The membranes were first probed for phosphorylated protein target. As the molecular weight of AKT (60 kDa) and MAPK3&1 (44-42 kDa) are different, the membranes were cut at 50 kDa according to the protein ladder. Each half was probed with a separate primary

antibody. The membranes were then developed using ECL Select (GE healthcare, Hatfield, UK) and a gel documentation facility (SYNGENE, Cambridge, UK). Obtained images from phosphorylated bands, the membranes were stripped by washing in the stripping buffer (Tris-HCL pH=6.8, 2% SDS, 1% Tween20, 0.1% 2-mercaptoethanol) for 5 min with maximum agitation. Then, the membranes were blocked and re-probed with the total protein primary antibodies.

Pictures obtained from the developed membranes were analyzed using Alpha Ease FC software v.3.1.2 (AlphaInnotech; Protein Simple, CA, USA) for band densitometry (Integrated density value; IDV). Bands were selected manually and the background value was deduced automatically from the analysis. The phosphorylated proteins were calculated as a percentage of total protein of the same sample.

4.2.4. MPF cdc2 kinase ELISA assay

COCs were processed using the MESACUP cdc2 kinase assay kit (MBL, Woburn, USA) following manufacturer's protocol. COCs (n=10; each treatment) were lysed using the freezing (liquid nitrogen) and thawing technique in 5µl PBS-PVP 4%. The extract was mixed with equal volumes of 10x cdc2 reaction buffer, biotynilated MV peptide and 30 µl of distilled water in 1.5ml micro centrifuge tubes. The phosphorylation reaction was initiated by addition of 10 µl of 1mM ATP (adenosine tri-phosphate). After 30 min of incubation of the tubes at 30^{o}C the reaction was terminated by addition of 200 µl of phosphorylation stop reagent. The final mixture was transferred to 8 well strips previously coated with monoclonal antibody against the phosphorylated peptide in 100 µl volume and duplicates. After primary incubation and washing, the POD substrate-streptavidin conjugate was added to provide a blue colour reaction which was stopped by phosphoric acid and resulted in orange color solution. At the end of the ELISA process the amount of phosphorylated peptide were detected by TECAN ELISA reader machine (TECAN, Switzerland). Optical density of the phosphorylated MV peptide is a direct indicator of the CDC2 kinase (Suzukamo *et al.* 2009).

4.2.5. Statistical analysis:

All experiments were repeated at least 3 independent times. The proportional average of oocytes in different stages of meiotic resumption was calculated at the end of the culture period in comparison to the total number of cultured oocytes. Statistical analysis was carried out using PAWS statistics 18: Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA) using binary and ordinal logistic regressions via generalized linear model. Analyses of western blot IDV and MPF ELISA data were carried out using one way ANOVA and differences between parameters were compared by LSD posthoc test if main effects were significantly different. Differences among treatment groups were considered significant if *P* values were < 0.05. Data are presented as mean ± SEM.

4.3. Experimental design

A total of 1622 COCs were used in these studies. COCS were cultured in groups of about 20 and specific time points were devised for each experiment. Summary of experiments and number of the COCs utilized are described in Table 4.1.

4.3.1. Caffeine pretreatment (12+60) 72h

To study the effect of caffeine pre-treatment on oocyte maturation, a total number of 192 COCs were cultured in a humidified incubator at 38.5^{o}C containing 5% CO_2, 5% O_2, and 90% N_2 gas atmosphere. The COCs were treated with increasing concentrations of caffeine (0, 5, 10, 20 mM) for 12h and then washed twice and transferred to a maturation media without caffeine for an additional 60 h (72 h overall). The COCs were then stained to determine nuclear stage of maturation. Each stage of meiotic resumption was reported as a percentage of the total number of the oocytes cultured.

4.3.2. Caffeine pretreatment (12+72) 84h

The second experiment was designed to investigate whether the extended culture of COCs after caffeine pre-treatment will improve the maturation rates. A total number of 234 COCs were cultured in 0 and 10 mM caffeinated maturation

media for 12 h followed by 72 h of basic maturation media (84 h overall). Oocytes maturation and degeneration rates were assessed after staining.

4.3.3. AKT and MAPK1&3 expression profiles (12+60) 72h

Analyzing time course of molecular changes associated with oocyte maturation following 0 (Ctrl) and 10 mM caffeine pre-treatment. In this experiment (n=720) COCs were collected in groups of 20 at 0, 12, 24, 48 and 72h of culture. The collected COCs were snap frozen in liquid nitrogen and used for western blotting analyses of MAPK1&3 and AKT immediately after the culture period. Integrated density value (IDV) of phosphorylated and total protein bands were calculated using AlphaEase FC (AlphaInnotech; Protein Simple, CA, USA) and the (phosphorylated/total) ratio was reported as percentage.

4.3.4. MPF kinase activity (12+60) 72h

In order to study temporal changes in MPF kinase activity, a total number of 308 COCs were cultured in 0 (Ctrl) and 10mM caffeine pretreatment. Samples were obtained at 0, 12, 24, 48 and 72h of culture and snap frozen using liquid nitrogen. COCs were lysed and processed for MPF kinase activity immediately after the culture period following the manufacturer's instruction (MESACUP cdc2 kinase assay kit [MBL, Woburn, USA]).

4.3.5. Brilliant cresyl blue screening of oocytes (12+60) 72h

For assessment of the efficiency of BCB staining as a screening test to select oocytes with better maturation properties and to elucidate whether BCB staining can provide additional improvement to caffeine pre-treatment. A total number of 168 freshly collected (uncultured) COCs were stained with BCB to study population distribution. Overall 137 COCs (BCB+ and BCB-) were also cultured for 12 h in caffeine pre-treatment (10mM) followed by 60 h of culture in the absence of caffeine. Maturation rates of the oocytes were compared at the end of the culture period (72 h) between the BCB+ and BCB- groups.

Table 4.1. The summary of experiments in this chapter.

No. Exp.	No. COCs	Treatment	Measurements
4.3	**1622**	**Total COCs**	**Analysis**
4.3.1	**192**	**Caffeine (12+60) 72h**	**Staging**
4.3.2	**234**	**Caffeine (12+72) 84h**	**Staging**
4.3.3	**720**	**AKT&MAPK1,3 72h**	**Western blot**
4.3.4	**308**	**MPF 72h**	**ELISA**
4.3.5	**168**	**BCB 72h**	**Stain/Staging**

4.4. Results

4.4.1. Effect of caffeine pre-treatment on oocyte maturation (72h)

COCs were cultured in increasing concentration of caffeine for 12 h before continuation of culture to 72 h in the absence of caffeine. Oocytes cultured in 10 mM caffeine had higher MII rate (16.9 % ± 2.4; $P < 0.05$) as compared to the control group (2.2% ± 2.2). At 5 and 20 mM concentrations of caffeine pre-treatment there were no significant changes in the percentage of MII matured oocytes (4.4% ± 4.4 and 5.7% ± 3 respectively). Degeneration rate of the oocytes was significantly lower than control group (37.6% ± 4.3) in all concentrations (5, 10, and 20 mM) of caffeine pre-treatment (32.1% ± 5.4, 25.9% ± 5.2 and 29.3%±8.0, respectively ($P < 0.05$)]. Meiotic resumption (MI-MII) was numerically highest in 10 mM group (26.7% ± 3.5; $P > 0.05$) and lowest in 5mM group (13.3% ± 6.7; $P > 0.05$) (Table 4.2).

*Table 4.2. Effect of 12 h caffeine pre-treatment on nuclear maturation of canine oocytes after 72 h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Mean ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Control	21.1±3.6	25.1±7.4	9.2±5.8	2.6±2.6	2.2±2.2	2.2±2.2	37.6±4.3	16.2±9.6	46
Caff 5mM	20.2±13.3	34.4±13.9	2.2±2.2	6.7±3.8	0.0±0.0	4.4±4.4	32.1±5.4*	13.3±6.7	44
Caff 10mM	31.6±4.2	15.8±5.7*	8.1±1.2	0.0±0.0	1.8±1.8	16.9±2.4*	25.9±5.2*	26.7±3.5	48
Caff 20mM	34.0±25.5	14.8±8.7*	11.4±5.9	2.4±2.4	2.4±2.4	5.7±3.0	29.3±8.0*	21.9±11.1	54
* *P*< 0.05 within the column compared to control , Caff = caffeine pre-treatment (12h)									192

4.4.2. Caffeine pre-treatment and extended culture (84h)

COCs were cultured for 72h after 12 h pre-treatment with 10mM caffeine. Most oocytes were found degenerated in both treatment groups. Degeneration rate in the control group was 54.9% ± 3.8 on average higher than caffeine treated group with 45.5% ± 0.9 ($P < 0.05$). No differences were found in meiotic resumption (MI-MII) between control and caffeine group, 13.2% ± 3.4 and 14.9% ± 7.6 respectively ($P > 0.05$). MII matured oocytes were still higher than the control group (4.4% ± 2.2 vs. 0.8% ± 0.8; $P < 0.05$), (Table 4.3).

*Table 4.3. Effect of 12h caffeine pre-treatment (10mM) on nuclear maturation of canine oocytes after 84h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); ~ Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Average ± SEM) in different meiotic stages at 84h							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Ctrl	8.9±4.5	23.0±3.5	9.1±2.3	3.3±1.6*	0.0±0.0	0.8±0.8	54.9±3.8*	13.2±3.4	71
Caff10mM	14.7±2.0	24.8±8.1	8.8±5.4	0.8±0.8	1.0±1.0	4.4±2.2*	45.5±0.9	14.9±7.6	163
* *P*< 0.05, within the column ; Ctrl = control group, Caff = caffeine pre-treatment									234

4.4.3. MAPK1&3 and AKT western blotting and IDV analysis

Specific bands at 42-44 kDa of MAPK1&3 were identified for both the phosphorylated and the total proteins (Fig. 4.5).

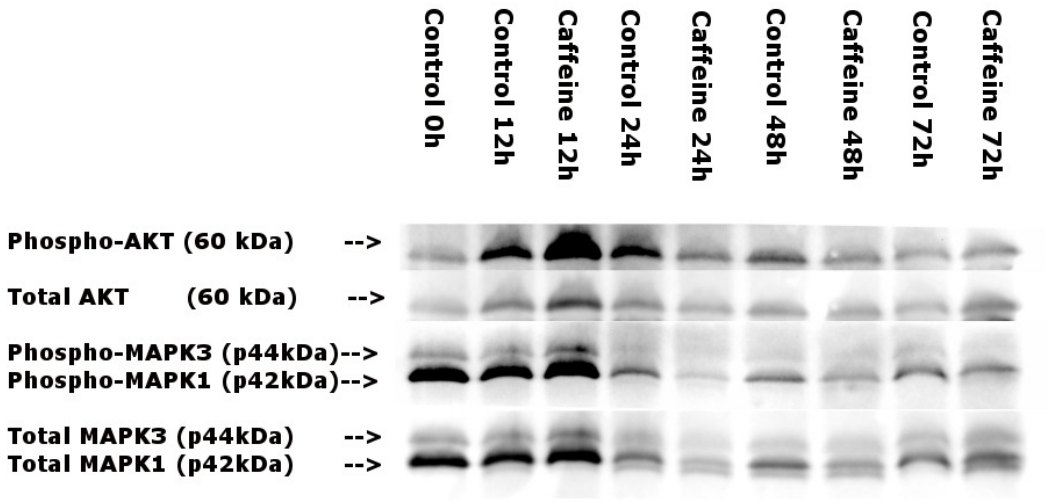


Figure 4.5. Western blot of phosphorylated and total AKT, MAPK1& 3 in canine COCs. Examples of images showing the temporal expression of phosphorylated and total form of AKT (60 kDa) and MAPK3 &1 (44-42 kDa) at 0, 12, 24, 48 and 72h after culture in the control and caffeine treated groups.

Integrated density value (IDV) of the bands were analysed and the phosphorylation ratio of MAPK1&3 during the 72h of culture was calculated (Fig. 4.6). Caffeine treated COCs had higher levels of phosphorylated/total ratio of MAPK1 at 12h, 48h, and 72h as compared to the control group. Significant differences were found at 12h (Fig. 4.6A). The same pattern was observed for MAPK3 phosphorylation but only significant difference was at 48h (Fig. 4.6B).

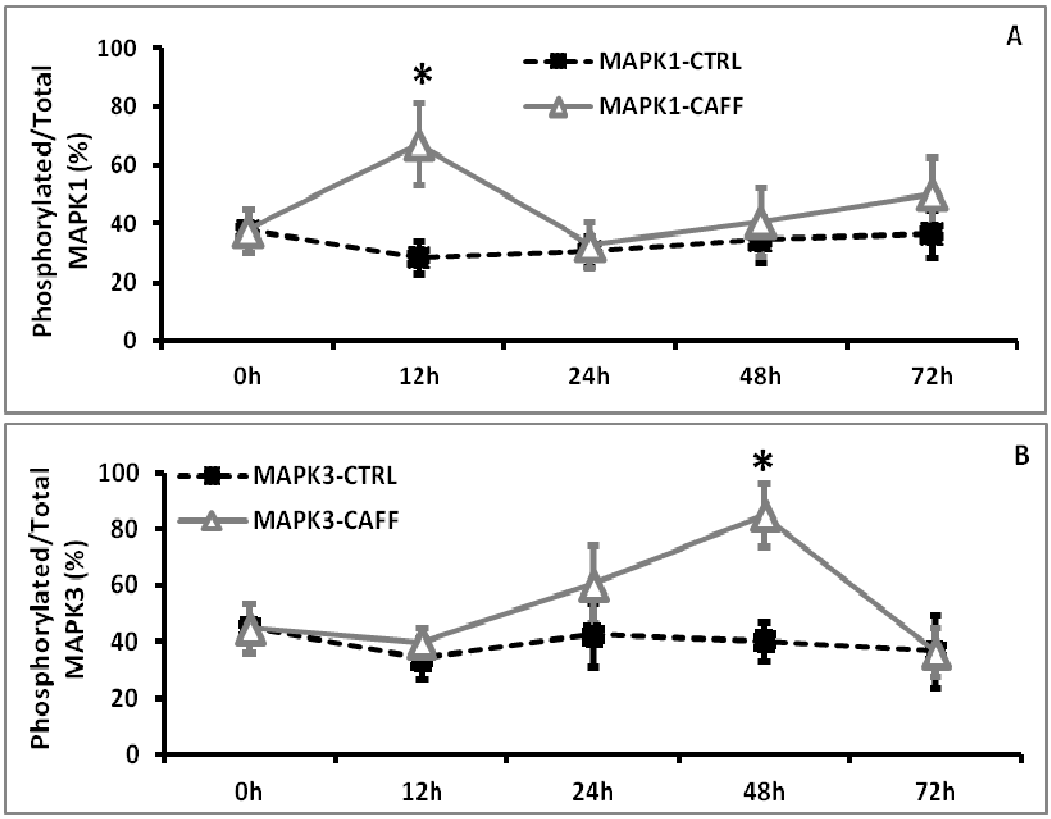


*Figure 4.6. Phosphorylated/total ratio of MAPK1&3 kinases during 72h of IVM. Phosphorylation level of (A) MAPK1, (B) MAPK3 in the caffeine and the control groups from 0-72h as the IDV ratio of phosphorylated to the total protein. * = P<0.05*

The AKT band at 60 kDa in total and phosphorylated form was detected as shown in Fig. 4.5 Phosphorylation of AKT during 72 h of maturation was closely similar between the control and the caffeine treated oocytes throughout the time points 12h, 24h, 48h and 72h. (Fig. 4.7)

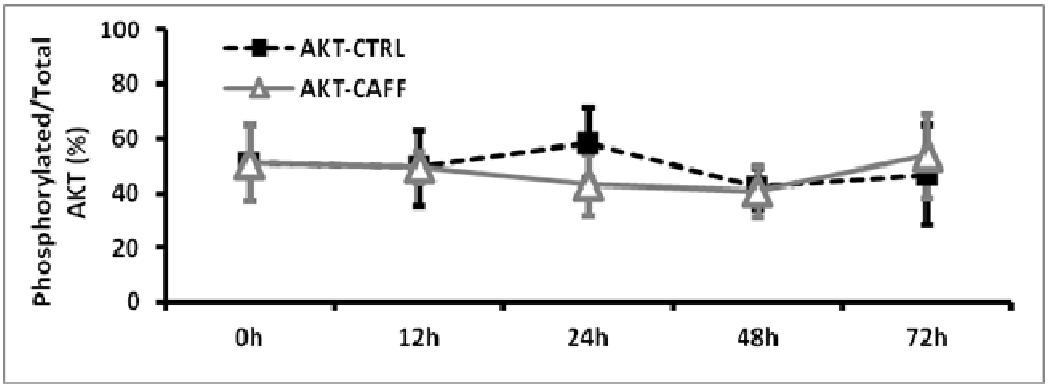


Figure 4.7. Phosphorylated/total ratio of AKT (PI3K/PKB) kinase during 72h of IVM. Graph shows the phosphorylation of AKT in the caffeine and the control groups from 0-72h as the IDV ratio of phosphorylated protein to the total form. No significant difference was observed.

4.4.4. MPF CDC2 kinase ELISA analysis

COCs were cultured in the previously described condition and analysed for MPF phosphorylation activity. MPF ELISA kit was run in duplicates and an average optical absorption was measured (Fig. 4.8).

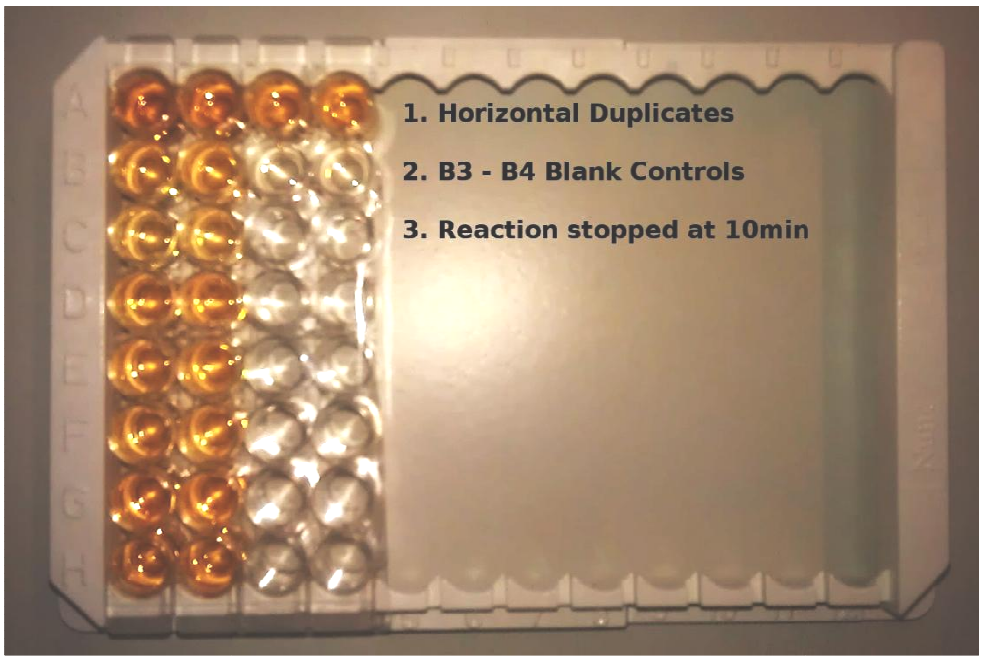


Figure 4.8. A sample of MPF CDC2 ELISA plate. Picture shows four strips of the MESACUP kit after termination of reaction prior to spectrophotometry by ELISA reader machine.

Optical density of the phosphorylated MV peptide which is a direct indicator of the CDC2 kinase activity was significantly higher in the caffeine treated oocytes compared to the control group at 12h and 48h during the culture (P<0.05). The CDC2 kinase activity had a double peak pattern in caffeine treated group as shown in Fig. 4.9.

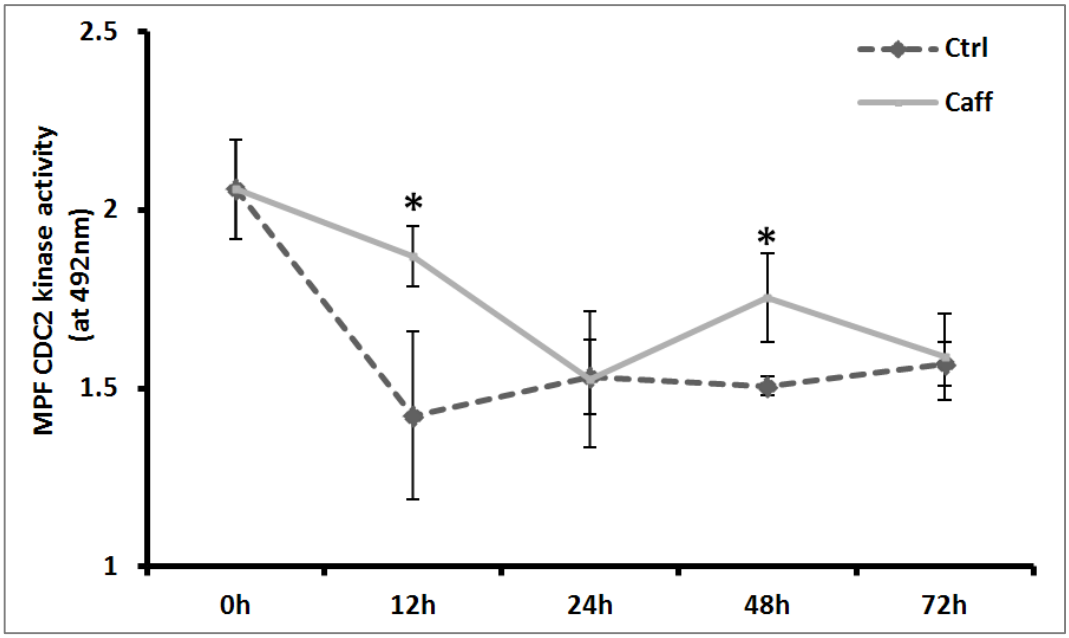


*Figure 4.9. Phosphorylation activity of CDC2-kinase (MPF) during 72h of IVM. Graph shows the phosphorylation of AKT in the caffeine and the control groups from 0-72h as the IDV ratio of phosphorylated protein to the total form. * = P<0.05*

4.4.5. Brilliant cresyl blue (BCB) staining

From the pool of freshly collected COCs (n=168) 29.93% ± 6.08 were BCB + and 70.07% ± 6.08 BCB - (Fig. 4.10). Distribution of BCB screened oocytes (n=137) at different stages of meiotic division after 12h caffeine pre-treatment followed by 60h of culture is presented in Table 4.4.

Meiotic resumption of BCB + oocytes after 72h of culture was 16.11% ± 5.5 on average and 9.86% ± 5.0 for BCB - oocytes (*P* <0.05). MII matured oocytes in BCB + and BCB - groups were 10.2% ± 2.9 and 1.1% ± 1.1 respectively (*P* < 0.05). Degeneration rate was 51.3% ± 3.5 in BCB + group and 49.6% ± 4.7 in BCB - group (*P*> 0.05).

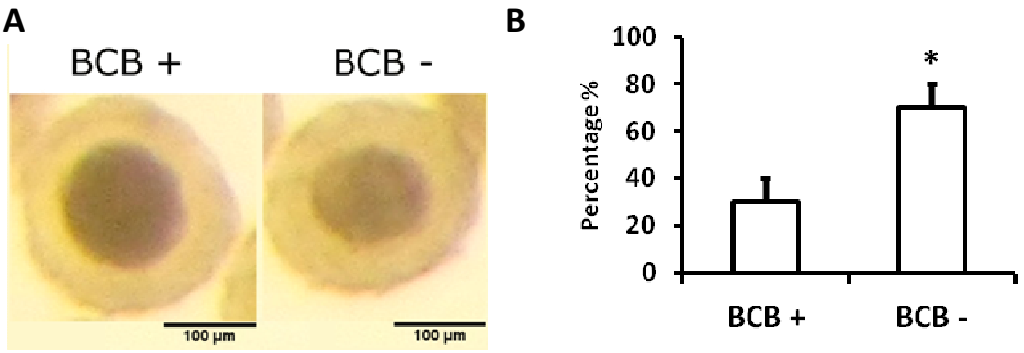


*Figure 4.10. BCB staining of freshly collected canine COCs. A) Image taken after BCB incubation for detection of BCB+ and BCB - COCS according to the dark blue (purple) colour of cytoplasm. B) Graph shows the distribution of freshly collected canine COCs after BCB screening. * = P<0.05*

Table 4.4. Combined effects of BCB screening and 10mM caffeine pre-treatment on nuclear maturation of canine oocytes. Table shows distribution of oocytes in different stages of meiotic division. After BCB staining the BCB+ and - COCs were pre-treated with 10mM caffeine for 12h separately and cultured for 60h in a caffeine free maturation media. (Overall 72h culture time). Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII).
** = P<0.05*

	Percentage of oocytes (Average ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
BCB +	21.3±6.9	11.2±6.7	3.3±3.3	0.0±0.0	2.6+2.6	10.2±2.9*	51.3±3.5	16.11±5.5*	42
BCB -	28.6±12.6	11.9±4.4	5.8±3.1	0.9±0.9	2.0±1.0	1.1±1.1	49.6±4.7	9.86±5.0	95
* *P*< 0.05, within the column									137

4.5. Discussion

Improving the maturation rate of canine oocytes requires complex and unprecedented culture conditions which are still under optimization. In the studies presented in this chapter the effects of caffeine as a non-selective competitive PDE inhibitor was studied on canine oocyte meiotic resumption and the temporal activation of four oocyte maturation markers: MAPK1&3, AKT and MPF cdc2 kinase. Results of these studies showed that 12 h caffeine pre-treatment at 10mM concentration improved MII maturation rate to 16.9% and lowered the degeneration rate down to 25.9%. Caffeine pre-treatment had significant effects on MAPK1and MPF kinase activity at 12h compared to the control group. Caffeine treated COCs also had higher MAPK3& MPF activity at 48h ($P < 0.05$).

Resumption of meiosis in canine oocytes unlike other mammals is not timely characterized and due to its delayed progression is of great complexity (Saint-Dizier *et al.* 2004). The nuclear development of oocyte is strongly controlled by changes in cAMP level provided by different follicular compartments (Luciano *et al.* 2004). In mammalians, stable and high cAMP concentration within the oocytes during meiotic arrest supports the activity of inhibitory mechanisms leading to inactivation of MPF (Sirard & First 1988, Mehlmann 2005, Smitz *et al.* 2011). After removal of COCs from the follicular environment, spontaneous resumption of GVBD (Sirard & First 1988) can also be prevented via provision of sustained cAMP concentration within the oocyte (Luciano *et al.* 2004) . Nevertheless, the slight increase in the cAMP level of COCs in respond to FSH or LH promotes the meiotic resumption and changes in connexon's distribution and function (Luciano *et al.* 2004). These controversial phenomena are due to involvement of cAMP as the second messenger in several maturation promoting pathways (Zhang *et al.* 2009). The cAMP levels that are required for initiation of meiotic resumption and cumulus disruption have been reported to be different and critical (Luciano *et al.* 2004). In the studies reported here caffeine was utilized in order to improve the maturation quality and serve three important

objectives: First to maintain the cAMP level within the oocyte to mimic the preceding gonadotropic resumption condition and reducing the pace of nuclear changes during GVBD (Luciano *et al.* 2004). Second to promote the extrinsic pathways like PKAII, PKC and PKB (PI3K/AKT) to initiate MPF activity through MAPK activation via the same second messenger cAMP (Zhang *et al.* 2009). Third to simulate the biphasic follicular-oviductal transition through 12h pre-treatment followed by 60h of culture in the absence of caffeine. During caffeine pre-treatment it was expected for the COCs to have enough time to acquire sufficient developmental competence before any disruption of gap junctions occur. The duration of caffeine pre-treatment and dose respond were analysed in preliminary experiments for 6, 12, 24, 48 and 72h with increasing concentrations (5, 10 and 20mM) of caffeine . The optimal time and concentration window was devised according to these results, as less than 12h of caffeine treatment had no effect on maturation rates and more than 12h exposure resulted in terminal GV or GVBD arrest. Caffeine at 2.5mM concentration has been reported to increase cAMP and maintain meiotic arrest in porcine oocytes (Kren *et al.* 2004). In the same study the removal of caffeine resulted in resumption of meiosis and development to MII stage after 48h culture. Similarly PDE inhibitors have been able to delay bovine (IBMX) (Barretto *et al.* 2007) and pause murine (caffeine) (Miao *et al.* 2007) oocytes meiotic resumption without having detrimental effects on developmental potential of the oocytes. Caffeine treatment has also been reported to maintain healthy meiotic spindle morphology even in aged murine oocytes (Ono *et al.* 2011). Considering the improving evident of this substance, caffeine pre-treatment was assessed during the first experiment to narrow down the optimal concentration required for improved maturation rate. Compared to the control group, 12 h exposure of canine COCs to 10mM caffeine significantly increased MII maturation rate and decreased degeneration (Table 4.2). This was in agreement with previous reports optimal required caffeine concentrations (Lee & Campbell 2006, Choi *et al.* 2010). In order to examine the effect of extended culture period the same concentration of caffeine (10mM)

was utilized in the second experiment. After 84h of culture MII maturation rates were lower than first experiment in both control and caffeine group (Table 4.3). Although the degeneration was significantly lower in caffeine treated oocytes, it was higher than the 72h culture (Table 4.2). These results were in agreement with previous studies (Suzukamo *et al.* 2009, Vannucchi *et al.* 2009, Salavati *et al.* 2012) concluding that optimal culture period for canine oocyte maturation is 72h.

Investigating the meiotic progression markers in caffeine pre-treated COCs, the present studies analysed the molecular changes which corroborate the nuclear maturation of canine oocytes. Better MII maturation in caffeine pre-treatment was studied retrospectively in temporal phosphorylation of AKT, MAPK1, MAPK3 and CDC2 kinase activity of MPF at 5 time points during maturation. Caffeine pre-treated COCs at 12h (released from caffeine treatment) contained higher MAPK1 and MPF kinase activity than the control group. The same pattern was observed at 48h for MAPK3 and MPF activity. AKT phosphorylation maintained a constant level in both the control and the caffeine groups throughout 72h of culture (Fig. 4.7). Improving effects of 10mM caffeine treatment on MPF/MAPK activity and expression of developmentally essential genes was previously reported in ovine enucleated oocytes during somatic cell nuclear transfer (Lee & Campbell 2006, Choi *et al.* 2010). Higher MPF and MAPK activities followed by caffeine treatment was also reported in previous reports studying porcine (Kren *et al.* 2004) and bovine oocytes (Barretto *et al.* 2007). The MPF/MAPK activities observed in this study were in agreement with previous canine reports (Saint-Dizier *et al.* 2004, Suzukamo *et al.* 2009).

Caffeine pre-treatment altered the MAPKs and MPF activity patterns in the COCs. The fact that caffeine cannot prevent GVBD but only slows it down is in agreement with previous reports (Kren *et al.* 2004, Barretto *et al.* 2007, Smitz *et al.* 2011). Relatively constant AKT phosphorylation and subsequently maintained MPF CDC2 kinase activity during the first 12h of IVM could be interpreted as

healthy cytoplasmic and nuclear maturation progress. MPF activity at 12h was significantly higher in the caffeine pre-treated group and declined at 24h similar to the control group (Fig. 4.9). It could be interpreted that after the caffeine withdraw, the higher phosphorylation levels of MAPK1 promoted the oocytes nuclear progression toward MI stage. Considering the previous assumption and the fact that the second significant increase in MPF activity and MAPK3 occurs at 48h, it's incepted that caffeine treated oocytes progress toward MII stage by a better molecular profile. Since the pattern of changes in the MAPKs and MPF activity evolves a bi-peak climax between 12 to 48h, it seems that the two metaphysial plate formations happen closely following these time points. Therefore, having oocytes at MII stage by 72h corroborates the idea that AI-TI changes could drop down to the period between 24 to 48h and caffeine pretreatment simulates the molecular changes associated with early stages of oocyte maturation *in vivo*.

4.5.1. BCB screening

In pursuit of better quality oocyte selection for canine IVM systems BCB staining was utilized. BCB staining has been utilized in sheep, cow, dog, goat and mouse (Rodriguez-Gonzalez *et al.* 2002, Pujol *et al.* 2004, Wu *et al.* 2007, Rodrigues *et al.* 2009a, Catala *et al.* 2011) and was reported to be a useful tool for screening oocytes with higher developmental potential. The results of freshly screened oocytes in this study were in agreement with previous report (Rodrigues *et al.* 2009a) and clearly in favor of incompetency (high G6PD activity) in majority of them (70.07% ± 6.08; $P < 0.05$). After the 72h IVM with a 12h caffeine pretreatment the MII maturation rate was higher in the BCB+ group (10.2% ± 2.9 vs. 1.1% ± 1.1; $P<0.05$) but there was no significant difference in any of nuclear stages between BCB+ and – groups (Table 4.4). This investigation is the first study to speculate the nuclear maturation outcomes of BCB screened oocytes after caffeine pre-treatment. Although the BCB + oocytes resumed meiosis at a significantly higher rate (16.11%±5.5) than the BCB- group (9.86%±5.0; $P<0.05$) the meiotic resumption and MII rates were inferior to the caffeine treated

oocytes without BCB staining procedure. A possible explanation for lower maturation rates after BCB staining could be due to the susceptibility of canine oocytes to oxidative stress (Silva *et al.* 2009, Salavati *et al.* 2012) and environmental trauma (Whitaker & Knight 2008) during the extra handling steps and incubation for BCB screening. After collection of the COCs the changes in cAMP level are completely time dependent and the amount of time consumed by BCB screening process before the caffeine pre-treatment could affect the downstream molecular changes which are critical for completion of meiosis. Nevertheless results of this experiment seem to show that abundant manipulation of the oocyte during the BCB staining and the delay between oocyte collection and *in vitro* maturation cause more damage to canine COCs and reduces the maturation rate.

4.5.2. Conclusion

Caffeine pre-treatment at the beginning of the IVM process as a biphasic method resulted in significant improvement in canine oocytes reaching MII stage at 72h (12h+60h). The caffeine treated COCs also contained significantly higher levels of phosphorylated MAPK1&3 and kinase activity of MPF. Further investigations are required in order to clarify the exact molecular pathway affected by caffeine pre-treatment during that 12h. BCB screening can easily identify the immaturity of canine oocytes before culture but the IVM results were not significantly affected by separating the BCB+ and BCB- oocytes, thus this method needs further optimization.

Summary of results from this chapter is as following:

- Bi-phasic maturation of canine COCs with 12h caffeine pretreatment improved nuclear maturation of oocytes (12h +60h). Extension of culture up to 84h did not increase the MII matured oocytes.
- 12h caffeine treatment led to higher MAPK1 phosphorylation and MPF CDC2 kinase activity at 12h. Moreover MAPK3 phosphorylation at 48h was significantly higher in the caffeine treated oocytes accompanied by MPF activity. This bi-peak profile of MAPK phosphorylation and MPF activity shows the progress of cytoplasmic maturation in canine oocytes toward metaphase II stage. This also elaborates with higher nuclear maturation rates achieved under caffeine pretreatment.
- Majority of oocytes collected for IVM from anestrous bitches has high G6PD activity (BCB+) which correlates with low nuclear maturation rates *in vitro*. Although BCB+ oocytes had higher MII maturation rates, BCB as a screening tool in COC selection requires further optimizations.

5. Effects of hormonal treatments on nuclear maturation of canine oocytes

5.1. Introduction

The synchronized and competent meiotic resumption of canine oocytes is an ensemble of hormonal and physiological changes within the follicular and oviductal fluid. Unlike other mammalian species, nuclear maturation of canine oocyte progresses from prophase I up to metaphase II stage, 2-3 days after ovulation within the oviductal environment (Reynaud *et al.* 2006). The oviductal fluid environment provides a range of hormones and growth factors and other survival factors which supports oocytes nuclear maturation, fertilisation and early embryo development. The programming required for successful *in vivo* maturation is instructed through the molecular changes of paracrine and autocrine microenvironment of the COCs (Fahiminiya *et al.* 2010). Although hormones play the key role in regulation of meiotic changes, protective substances such as antioxidants are vital for the oocytes throughout long journey of oviductal transition up to the point of fertilisation (Kim *et al.* 2004, Tatemoto *et al.* 2004).

It is well known that nuclear and cytoplasmic maturation of oocytes is not resulted from the direct effect of gonadotropins on these cells as they mainly lack the LH and FSH receptors (Bevers *et al.* 1997). The regulatory manifestations of follicular fluid ingredients are mainly transduced through cumulus cells and translated to the oocyte. In case of LH, there is very low or no LH receptor expression in oocytes or cumulus cells (Lee *et al.* 2007a). Growth hormone (GH) as a major regulator of ovarian function is one of the critical members of follicular fluid hormonal set. GH mRNA is expressed in the oocyte and the granulosa cells of bovine follicles but not in the cumulus cells. However the presence of GH receptor (GHR) has been validated in all three cell types at mRNA and protein level in bovine follicles (Bevers & Izadyar 2002). Although GH

receptors are present in the oocyte addition of GH to the denuded oocyte only has delayed effects on maturation (Bevers & Izadyar 2002). As if the acceleratory improvement of GH over meiotic resumption is only effective through sustained communication of cumulus cells and the oocyte (Izadyar *et al.* 1996). GHR is a member of cytokine receptor family which regulates its function through tyrosine phosphorylation (Rotwein *et al.* 1994). This cytosolic tyrosine kinase activates molecular cascades leading into phosphorylation of nuclear proteins such as JAK2 and GHR itself (Gronowski & Rotwein 1994). Beside the tyrosine kinase pathway, GHR utilizes cAMP as a second messenger and transfers the GH induction through involvement of Adenylate cyclase (AD) and cAMP dependent protein kinase A (PKA) (Bevers & Izadyar 2002). The cAMP dependent pathway is commonly shared by many maturation promoting factors such as MAPK1&3 and MPF (Tripathi *et al.* 2010). The inhibition of PKA does effect the function of GH but in presence of tyrosine kinase blocker such as Erbstatin, GHR phosphorylation is not affected at all (Bevers & Izadyar 2002). This unbalanced pathway tendency is accompanied with the fact that GH also mediates its effect through Insulin like growth factor (IGF-1) (Bevers & Izadyar 2002).

IGF-1 controls the gonadotropin-independent folliculogenesis in antral follicles (Mazerbourg *et al.* 2003). It is the main hormone responsible for granulosa cell proliferation and steroidogenic activity of these cells (Mazerbourg *et al.* 2003). It has also been reported that GHR knockout mice suffer from minimal seric and follicular IGF-1 levels accompanied by no ovulation in the ovaries (Bachelot *et al.* 2002). This reproductive profile is similar to IGF -/- mice which have normal folliculogenesis up to preantral stage but there is no antral follicle visible on their ovaries (Huang *et al.* 1997). The bioavailability of IGF-1 is controlled through six IGF binding proteins (hepatic origin) which either prohibit or promote attachment of IGF molecules to its receptors (Mazerbourg *et al.* 2003). IGF-1 is the major amplification molecule within the biological impact of LH and FSH (Reynaud *et al.* 2010).

Epithelial growth factor (EGF) is another growth factor which has been utilized in IVM media recently upon its discovery in downstream molecular cascade of LH induced meiotic resumption (Song *et al.* 2010b). LH receptors are not present in oocyte and cumulus cells but abundantly found in the granulosa cells (Park *et al.* 2004). The LH activation of these receptors results in shedding of EGF like hormones such as Amphiregulin (AREG), Epiregulin (EREG) and Betacellulin (BTC) (Conti *et al.* 2006). EGF receptor is also a tyrosine kinase dependent protein which is regulated through GH molecular cascade. However; activation of EGF receptor is not only limited to GH and will not be affected by inhibition of cAMP dependent PKA (Bevers & Izadyar 2002). The effects of PGH, IGF-1 and EGF were studied in separate experiments in this chapter which has been described later on.

Addition of serum has also been one of the oldest culture supplements to maturation media which due its unknown chemical and hormonal profile has controversial effects over nuclear and cytoplasmic maturation of the oocyte (Bolamba *et al.* 2002, Lopes *et al.* 2011). The Only successful report of serum improvement of canine IVM originated from oestrus bitch serum supplementations to COCs collected from follicular phase of the ovaries (Otoi *et al.* 1999, Oh *et al.* 2005) [14-16% MII maturation rate].

Melatonin is an indolamine hormone, found in the follicular fluid which besides its receptors distributed in COCs has strong antioxidative properties (Takada *et al.* 2010, El-Raey *et al.* 2011). It's been reported that supplementation of this potent antioxidant to the maturation media at certain concentrations (physiological conc. = 1nM within the follicular fluid) can drastically improve cumulus cell survival and oocyte maturation rates (Kang *et al.* 2009, Shi *et al.* 2009, Rocha *et al.* 2012).

In this chapter attempts were made in order to clarify and point out the hormones and substances with improving effect on nuclear maturation of canine oocytes. These supplementations were studied not only to improve MII

maturation rates but rather maintain oocytes with sufficient support and protection throughout the extended *in vitro* culture.

5.2. Materials and methods

Chemicals and reagents:

All chemicals were purchased from Sigma-Aldrich chemical Co. (Poole, UK) unless otherwise stated.

5.2.1 *In vitro* maturation

The IVM culture and oocyte staining/staging was carried out following the protocols described in the sections 2.2.-2.6.

5.2.2. Immunocytochemistry

In order to stain COCs and oocytes for melatonin receptor (MTNR-A1), samples were collected fresh after extraction. Briefly COCs and denuded oocytes were placed on Superfrost coated slide (VWR, UK) and air dried for 15min at room temperature (RT). The COCs were fixed by placing 50µl of cold (kept at -20^{o}C) acetone 99.9% for 10min at -20^{o}C. Immediately after fixation, the COCs were rehydrated at RT using 1X PBS (50µl/slide) for 5min inside a humidified chamber. Non-specific binding was blocked using 8% normal horse serum (SantaCruz biotechnologies, Dallas, USA; sc-2483) in PBS1X for 1h30m at RT within the humidified chamber. Slides were then washed using 50µl PBS1X once and dried with filter paper and probed overnight using polyclonal rabbit anti MTNRA1 (BiOSS Woburn, MA, USA) at 1:100 (v/v) dilution using rabbit IgG as –ve control in the 4^{o}C fridge. The slides were washed again using 50µl PBS1X once and dried with filter paper. Secondary antibody of FITC conjugated goat anti rabbit IgG (SantaCruz biotechnologies, Dallas, USA; sc-2012) (1:200) were used at RT for 1h (humidified chamber- in the dark) as the final probing layer. Counterstaining and wash were carried out simultaneously by addition of 10µg/ml Hoechst 33342 in PBS1X for 5min in the dark. The COCs were then mounted using 6µl Vectashield mounting media (Vector laboratories, Burlingame, CA, USA) and placed beneath

coverslips and visualized at 360nm and 470nm LED lamp under an Olympus BX60 fluorescence microscope, and photographs were obtained using tint filters (Blue for Hoechst, Green for FITC).

5.2.3. Statistical analysis:

All experiments were repeated at least 3 independent times. The proportional average of oocytes in different stages of meiotic resumption was calculated at the end of the culture period in comparison to the total number of cultured oocytes. Statistical analysis was carried out using PAWS statistics 18: Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA) using binary and ordinal logistic regressions via generalized linear model. Differences among treatment groups were considered significant if *P* values were < 0.05. Data are presented as mean ± SEM.

5.3. Experimental design

A total of 1457 COCs were used for these studies. COCs were cultured in groups of about 20 and specific time points were devised for each experiment. Summary of experiments and number of the COCs utilized are described in Table 5.1.

5.3.1. Insulin like growth factor 1 (IGF-1)

To investigate the influence of IGF-1 over *in vitro* maturation of canine oocytes an experiment was designed to put 226 oocyte in 3 repeats under treatment of IGF-1 at concentrations of 25, 50, 100ng/ml in the base maturation media (LH/FSH/E2/P4). Oocytes were cultured for 72h and then fixed and stained for nuclear maturation assessment.

5.3.2. Epithelial growth factor (EGF)

In order to study the effects of epithelial growth factors (EGF) on nuclear maturation of canine oocyte recombinant EGF (Sigma, Poole, UK) was supplemented in IVM culture media instead of LH in the base maturation media. COCs (n=212) were cultured in two comparison groups. As the EGF acts as

downstream molecular pathway of the LH induction in the treatment group LH 5µg/ml was replaced with EGF 10ng/ml in order to bypass the time consuming protein translation signal. Oocytes were cultured for 72h and then fixed and stained for nuclear maturation assessment.

5.3.3. Foetal bovine serum (FBS)

In order to study the effect of serum supplementation on nuclear maturation of oocytes a total number of 395 COCs were cultured in the presence of 10% FBS or 0.6% BSA. Experiment was repeated 4 times with the same batch of FBS and BSA reagents. Oocytes were cultured for 72h and then fixed and stained for nuclear maturation assessment.

5.3.4. Porcine growth hormone (PGH)

For investigation of previously reported (Chigioni *et al.* 2008) improving effect of PGH on canine oocyte maturation, COCs (n=269) were cultured in the increasing concentrations of PGH (10ng/ml, 100ng/ml and 1000ng/ml) and absence of this substance. Experiments were repeated 4 times. Oocytes were cultured for 72h and then fixed and stained for nuclear maturation assessment.

5.3.5. Melatonin

To study the improving effects of Melatonin on nuclear maturation of canine oocytes a total number of 295 COCs were culture in increasing concentrations (1nM, 100nM and 10µM) of Melatonin and the absence of it as the control group. Oocytes were cultured for 72h and then fixed and stained for nuclear maturation assessment.

5.3.6. Melatonin receptor (MTNR-A1) localization

In order to visualize the localization of MTNR-A1 in canine COCs a total number of 60 freshly collected (30 denuded oocytes + 30 COCs) samples were fixed on 6 slides for ICC.

Table 5.1. The summary of experiments in this chapter.

No. Exp.	No. COCs	Treatment	Measurements
5.3	**1457**	**Total COCs**	**Analysis**
5.3.1	**226**	**IGF-1**	**Staging**
5.3.2	**212**	**EGF**	**Staging**
5.3.3	**395**	**FBS**	**Staging**
5.3.4	**269**	**PGH**	**Staging**
5.3.5	**295**	**Melatonin**	**Staging**
5.3.6	**60**	**MTNR-A1**	**ICC**

5.4. Results

5.4.1. Effects of IGF-1 on nuclear maturation of canine oocytes

No significant difference was observed between control group and treatments in any stage of nuclear formation (P>0.05). Lowest degeneration rate (20.6% ± 12.1) and highest meiotic resumption (11.9% ± 4.4) were observed in 100ng/ml group. However the MII maturation rate in this group (1.57% ± 1.57) was still lower than the control group (2.8%±2.8) on average Table 5.2.

5.4.2. Effects of EGF on nuclear maturation of canine oocytes

Although there was a non-significant difference at MII maturation rates between LH (9.1% ± 0.5) and EGF (4.3% ± 2.2) groups, the degeneration rate was 35% on average in both groups. The number of oocytes at AI in LH group and TI at EGF group was significantly higher respectively (4.7%±2.6 vs. 0.0% ± 0.0; 7.5% ± 2.0 vs. 0.5% ± 0.5) Table 5.3.

5.4.3. Effects of FBS on nuclear maturation of canine oocytes

There was a significant decrease in percentage of oocytes in all nuclear stages except AI and TI in the 10% FBS treated group (Table 5.4; $P < 0.05$). Degeneration rate in the FBS supplemented group was significantly higher (74.6% ± 4.8) than the BSA control group (28.2% ± 3.2). Beside the drastically high degeneration in the FBS group, meiotic resumption was also significantly lower (10.2% ± 3.6) compared to the BSA control group (37.7% ± 3.7). Refer to Table 5.4 for detailed staging results.

*Table 5.2. Effect of IGF-1 on nuclear maturation of canine oocytes after 72 h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Mean ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Control	38.6±3.7	26.7±3.4	5.4±1.5	0.0±0.0	0.0±0.0	2.8±2.8	26.5±8.7	8.2±4.3	65
IGF1 25ng/ml	46.9±4.7	21.7±1.8	4.6±2.6	0.0±0.0	0.0±0.0	0.0±0.0	26.8±6.0	4.6±2.6	45
IGF1 50ng/ml	36.1±6.0	22.6±3.1	10.4±4.0	0.0±0.0	0.0±0.0	0.0±0.0	30.9±5.5	10.4±4.0	44
IGF1 100ng/ml	36.6±4.2	30.9±6.8	7.8±2.8	0.8±0.8	1.7±1.7	1.7±1.7	20.6±12.1	11.9±4.4	72
* *P*< 0.05 within the column compared to control , IGF-1 = Insulin like growth factor 1, Degen = degenerated									226

*Table 5.3. Effect of EGF on nuclear maturation of canine oocytes after 72 h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Mean ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	Total (n)
	GV (%)	GVBD (%)	MI (%)	AI (%)	TI (%)	MII (%)	Degen (%)		
LH 5µg/ml	21.5±4.3	22.3±3.9	6.6±3.3	4.7±2.6*	0.5±0.5	9.1±0.5	35.2±5.6	20.9±1.9	92
EGF 10ng/ml	27.6±3.0	15.7±3.0	9.1±2.4	0.0±0.0	7.5±2.0*	4.3±2.2	35.8±6.4	21.0±3.4	120
*** *P*< 0.05 within the column compared to control , EGF = Epithelial growth factor , Degen = degenerated**									212

*Table 5.4. Effect of FBS on nuclear maturation of canine oocytes after 72 h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Mean ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	Total (n)
	GV (%)	GVBD (%)	MI (%)	AI (%)	TI (%)	MII (%)	Degen (%)		
0.6% BSA	13.9±3.2*	20.1±3.4*	13.6±1.4*	3.2±1.2	2.2±1.4	18.7±4.0*	28.2±3.2	37.7±3.7*	205
10% FBS	4.4±1.9	10.7±2.9	2.7±1.8	2.5±0.5	0.0±0.0	5.0±2.4	74.6±4.8*	10.2±3.6	190
*** *P*< 0.05 within the column compared to control (0.6% BSA), Degen = degenerated**									395

5.4.4. Effects of PGH on nuclear maturation of canine oocytes

Meiotic resumption (MI-MII) was significantly higher in 100ng/ml (28.9% ± 10.0) and 1000 ng/ml (26.8% ± 3.8) compared to the control group (18.4% ± 4.3). Lowest percentage of oocyte paused at GV (11.4% ± 3.8) and lowest degeneration (27.3% ± 6.6) rate was in 100ng/ml and 1000ng/ml respectively. There was no significant different in MII maturation rate among different groups and except the 10ng/ml group (4.4% ± 4.4) the average MII rate was above 12%. Overall PGH 100ng/ml provided an optimal maturation profile regarding the meiotic resumption and MII maturation rate (Table 5.5).

5.4.5. Effects of Melatonin on nuclear maturation of canine oocytes

Melatonin at 100nM concentration has the optimal effect on the nuclear maturation profile of canine oocytes. Lowest percentage of oocytes remained at GV stage (6.7% ± 4.2), highest MII maturation rate (32.3% ± 6.4), minimum degeneration (20.5% ± 3.2) and maximal meiotic resumption (56.2% ± 8.6) were all resulted from 100nM supplementation of melatonin in the basic maturation medium (P <0.05). MII maturation rate of oocytes were also significantly higher in all melatonin treatments compared to the control group. Detailed staging results are summarized in Table 5.6.

*Table 5.5. Effect of PGH on nuclear maturation of canine oocytes after 72 h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Mean ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Control	21.8±6.4	21.6±5.9	3.7±3.7	5.5±3.2	0.0±0.0	12.7±1.6	34.6±2.2	18.4±4.3	59
PGH 10ng/ml	18.1±2.4	30.3±5.5	11.1±5.9*****	1.7±1.7	0.0±0.0	4.4±4.4	34.2±9.6	18.9±4.7	61
PGH 100ng/ml	11.4±3.8*****	27.5±9.4	9.6±4.3	4.7±2.6	4.7±2.6*****	14.6±4.5	27.5±8.5	28.9±10.0*****	69
PGH 1000ng/ml	18.3±0.8	25.7±2.9	10.7±0.5	2.3±2.3	2.0±2.0	13.8±2.2	27.3±6.6*****	26.8±3.8*****	80
* *P*< 0.05 within the column compared to control , PGH = Porcine growth hormone, Degen = degenerated									269

*Table 5.6. Effect of Melatonin on nuclear maturation of canine oocytes after 72 h. Table shows distribution of oocytes in different stages of meiotic division. Germinal Vesicle (GV); Germinal Vesicle Breakdown (GVBD); Metaphase 1 (MI); Anaphase 1 (AI); Telophase 1 (TI); Metaphase 2 (MII); Meiotic resumption of oocytes (total number of oocytes between MI and MII). * = P<0.05*

	Percentage of oocytes (Mean ± SEM) in different meiotic stages at 72h							Meiotic resumption (MI-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Control	15.5±4.2	27.6±8.4	8.5±4.8	0.0±0.0	6.6±4.6	11.8±2.6	29.9±7.4	27.0±3.2	70
MTN 1nM	10.4±5.5	20.1±2.5	15.7±8.8	0.0±0.0	4.6±2.3	21.1±6.3*****	28.0±6.1	41.4±12.8	68
MTN 100nM	6.7±4.2*****	16.6±1.3	11.9±1.2	3.2±1.7*****	8.7±2.8	32.3±6.4*****	20.5±3.2*****	56.2±8.6*****	80
MTN 10µM	9.1±4.9*****	23.3±3.4	8.1±4.4	0.7±0.7	4.0±2.0	26.0±4.8*****	28.7±6.1	38.8±6.4*****	77
* *P*< 0.05 within the column compared to control , MTN = Melatonin, Degen = degenerated									295

5.4.6. Immunolocalization of MTNR-A1 in canine COCs

MTNR-A1 was highly expressed in the oocytes and with lower intensity in the cumulus cells. The distribution of MTNR-A1 signal didn't follow a nuclear or peri-nuclear patterns and it was evenly scattered within the ooplasm. The very first layers of cumulus cells had also weak signal of the MTNR-A1 antibody (Fig. 5.1).

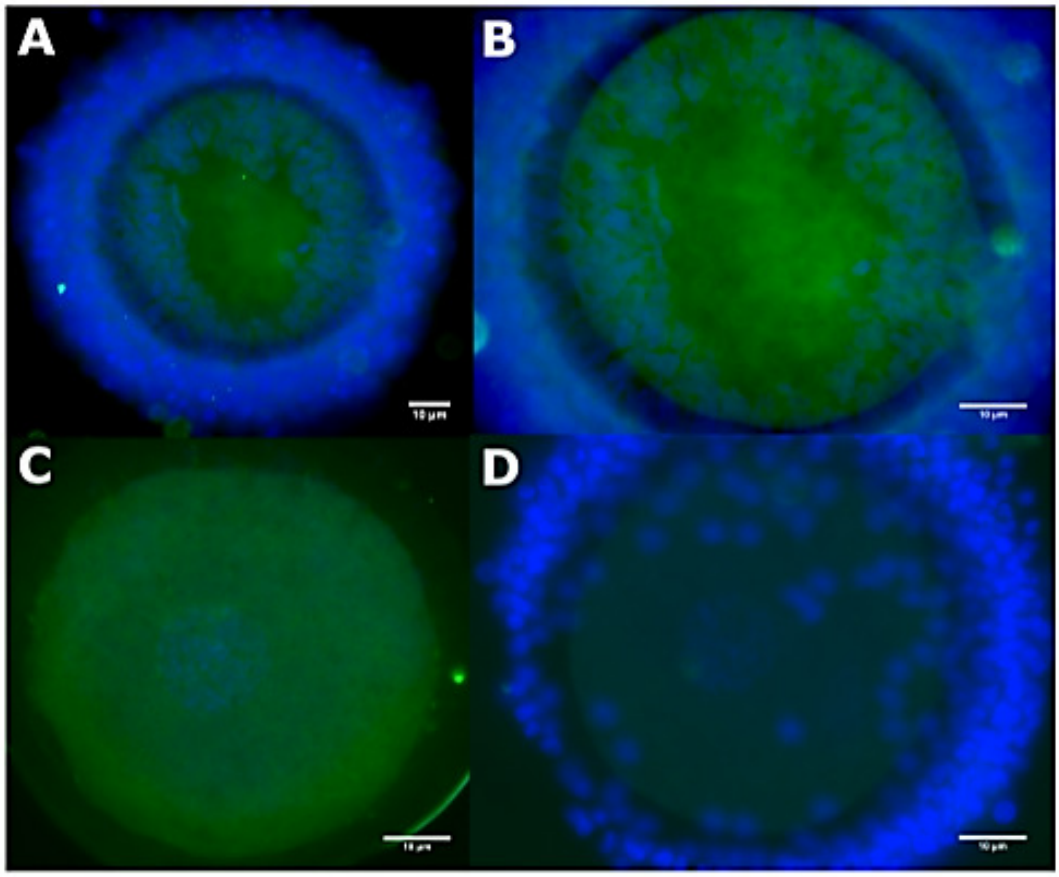


Figure 5.1. Fluorescent microscope images of canine COCs and denuded oocytes after ICC for MTNR-A1. Immunolocalization of MTNR-A1 within the oocyte and cumulus cells of canine COCs. A) COC 10x B) COC 20x C)Denuded Oocyte 20x D)Rabbit IgG -ve Control 20x. Scale bar = 10µm

The GV nuclear status of denuded oocyte was visualized using the Hoechst counter staining (Fig. 5.2).

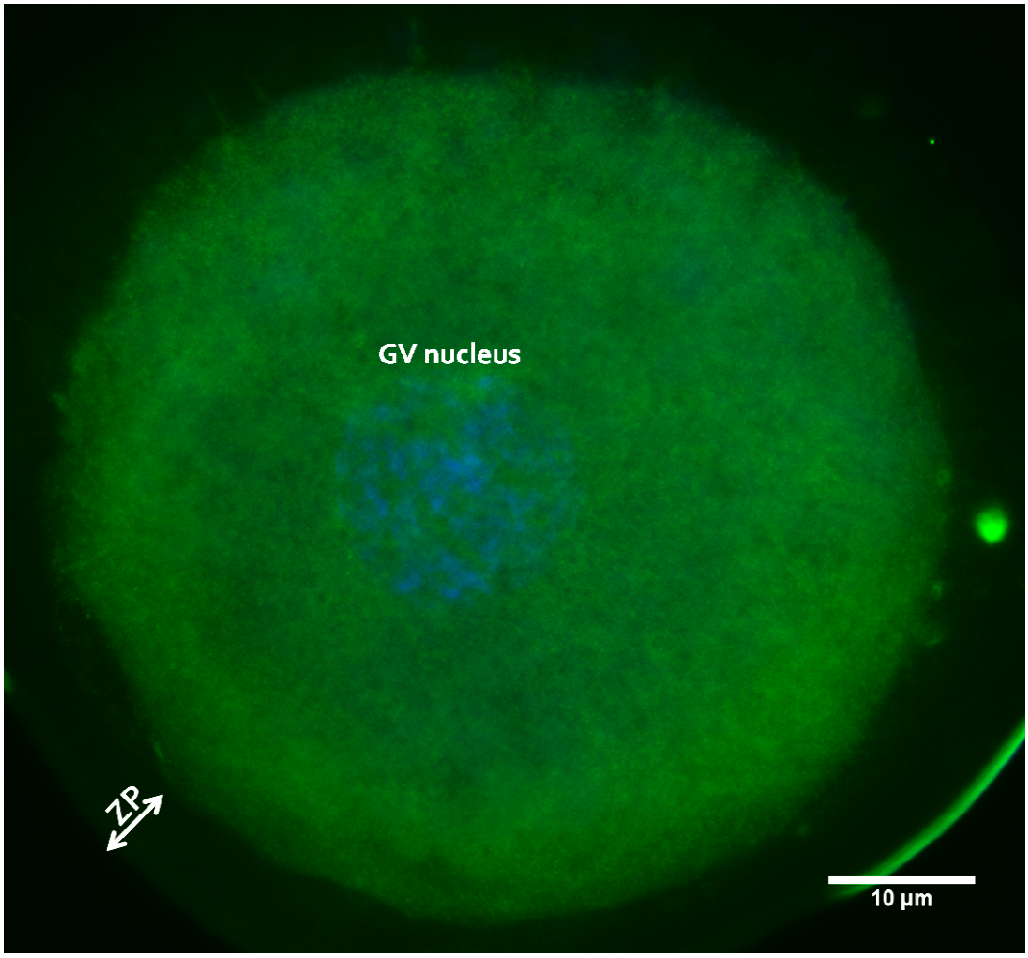


Figure 5.2. Fluorescent microscope images of canine denuded oocyte after ICC for MTNR-A1. Immunolocalization of MTNR-A1 within the oocyte is shown along with the GV stage nuclear and the thickness of Zona (20x). Scale bar = 10µm

5.5. Discussion

Studies conducted in this chapter illustrated a variety of results which should be interpreted separately at first. Results didn't show any improving effect over supplementation of IGF-1, EGF or FBS to maturation media, on meiotic progression of the oocyte nuclei toward MII stage. On the contrary the addition of PGH (100ng/ml) and melatonin (100nM) resulted in outstanding meiotic resumption and maturation rates.

5.5.1. Insulin like growth factor 1 (IGF-1)

In contrast with the expectations according to the key role of IGF-1 within the follicular fluid during the late folliculogenesis and previous reports, results of IGF-1 treatments in the maturation media were not satisfactory. Majority of oocytes treated with 25 and 50 ng/ml of the IGF-1 in the base maturation media were still arrested at the GV or GVBD stages. The MII oocytes in the 100ng/ml concentration group were even lower than the control group (Table 5.2). The addition of IGF-1 not only didn't promote nuclear maturation but also delayed the process. The high concentration of IGF-1 didn't seem to change the bioavailability of this hormone in terms of meiotic resumption. Degeneration rates were not affected under IGF-1 treatments but slightly reduced due to relatively higher GV and GVBD percentages.

5.5.2. Epithelial growth factor (EGF)

EGF was the only supplementation which was replacing LH in the maturation media with 10ng/ml concentration. Considering the fact that cumulus cells and the oocyte do not have LH receptors EGF family including AREG, EREG and BTC are the most important downstream molecules of LH induced oocyte maturation which function through G-couple receptors (Park *et al.* 2004). There are clear evidence for up-regulation of AREG, EREG and BTC just 3 h after the administration of LH analogues (Park *et al.* 2004). Phosphorylation of EGF receptors during the first 6h of LH treatment is gradually increasing and EREG constantly increases during the maturation after GVBD (Conti *et al.* 2006). To

accelerate the gonadotropin induction of meiotic resumption and cumulus expansion (EGF→ increased MAPK in cumulus cells (Song *et al.* 2010b)) it was hypothesized that replacement of LH in the base maturation media with EGF may lead into higher MII maturation rates. Although it's been reported by Song *et al.* in 2010 that EGF 10ng/ml treatment increased the MII maturation, our results showed otherwise. The EGF treated oocytes had lower MII maturation rate (4.3% ± 2.2) compared to the LH group (9.1% ± 0.5; *P* >0.05) Table 5.3. No significant difference was observed in the MII maturation rate, degeneration rate or meiotic resumption (MI-MII) between the two groups. Considering the results of this experiment, replacement of LH with its downstream messenger (EGF) doesn't have improving effects on meiotic resumption.

5.5.3. Foetal bovine serum (FBS)

In a challenge to compare 0.6% BSA and 10% FBS, the unknown components of serum seems to have more detrimental effects. According to the literature addition of serum (FBS) is not a good source of protein due to reduction of GSH level and increase of oxidative stress over oocyte maturation (Concannon *et al.* 2009, Rodrigues & Rodrigues 2010). The best replacement for FBS is either canine estrus serum (Concannon *et al.* 2009) or 0.3%-4% BSA (Hewitt & England 1999). Results of this experiment showed that presence of FBS reduces MII rate to 5.01% ± 2.39 compared to control group (18.7% ± 4.0; P<0.05). Degeneration rate in FBS treated group (74.6% ±4.8) was also significantly higher than the control group (28.2% ± 3.2; p<0.05). Meiotic resumption of the canine oocytes was also higher in the absence of the FBS (37.7% ± 3.7; *P* < 0.05).

5.5.4. Porcine growth hormone (PGH)

Canine and porcine growth hormones (cGH, pGH) have identical amino acid sequences and high level of biological similarity (Ascacio-Martinez & Barrera-Saldana 1994, Secchi *et al.* 2001). Supplementation of GH in canine oocyte maturation media improved meiotic resumption and reduced degeneration rates (Table 5.5). Porcine GH at, 100ng/ml significantly increased the percentage of

meiotic resumption from MI to MII (28.9% ±10.0; p <0.05). The lowest degeneration rate was observed in the 1000ng/ml group (27.26% ± 6.63; p <0.05). Results elucidated that pGH can improve meiotic resumption along with reducing degeneration rates in a very narrow window of concentration. This outcome could be due to vast influence of GH on IGF-1, EGF and cAMP molecular pathways which also requires further investigations.

5.5.5. Melatonin

Management of degenerative predisposition of canine oocytes due to their extended duration of culture for *in vitro* maturation is of essence. Melatonin according to the results showed to have significantly improving effects over MII maturation rates and reducing degeneration rate. Melatonin at, 100nM significantly increased the percentage of meiotic resumption to MII (32.34% ± 6.37; *p* <0.05). The lowest degeneration rate and GV arrested oocytes were also observed in the 100nM group (20.5% ± 3.21 and 6.71% ± 4.24 respectively; *p* <0.05). Presence of melatonin improved MII rate in all 3 concentrations compared to the control group (*p* <0.05). These results are in agreement with previous studies in goats (Rocha *et al.* 2012), pigs (Kang *et al.* 2009, Shi *et al.* 2009) and cattle (El-Raey *et al.* 2011). MTNRA1 was highly expressed in oocytes and with lower intensity in cumulus cells. Results showed that melatonin can improve nuclear maturation along with reducing degeneration rates. The improving effects of melatonin can originate from both antioxidative feature of this compound and its molecular influence through MTNRs. The highly expressed melatonin receptor within the COCs should be further investigated to clarify the origin of melatonin improving effects.

5.5.6. Conclusion

Although addition of IGF-1, FBS or substitution of LH with EGF had no improving effects on nuclear maturation of canine COCs, the improvements occurred under PGH and melatonin treatment were outstanding. PGH at 100 and 1000ng/ml and melatonin at 100nM concentration showed that via hormonal manipulation of canine oocyte culture media, higher maturation rates are achievable. Combination of PGH and melatonin treatments could be of great interest to be studied for illuminating the mechanism of this improvement.

Outcomes of this experimental chapter elucidated that:

- Addition of IGF-1 (25, 50 & 100ng/ml) to the maturation media did not improve the nuclear maturation rates of canine COCs.
- Replacement of LH (10ng/ml) with recombinant EGF in maturation media did not improve the MII maturation rates unprecedentedly.
- Supplementation of maturation media with 10% FBS not only did not improve the nuclear maturation rates compared to 0.6% BSA but also caused drastic increase in degeneration rate after 72h of IVM.
- Recombinant porcine growth hormone at 100ng/ml concentration had the most optimal influence on nuclear maturation of canine oocytes in the maturation media. PGH increased the MII maturation rates up to 14.6% ± 4.5 and significantly decreased degeneration rate.
- Melatonin supplementation of maturation media (100nM) increased MII maturation rate up to 32.3% ± 6.4 and meiotic resumption up to 56.2% ± 8.6 on average. The presence of MTNR-A1 was also confirmed in the oocyte and first layers of cumulus cells using immunocytochemistry.

6. Parthenogenetic activation and *in vitro* fertilization of *in vitro* matured canine oocytes

6.1. Introduction

In vitro embryo production in canine species is another unresolved issue in assisted reproductive techniques of mammals. In order to produce *in vitro* cultured embryos there are several approaches. The most frequently operated methods are as following:

1. *In vitro* fertilization (IVF) using fresh/chilled/frozen semen
2. Intracytoplasmic sperm injection (ICSI)
3. Subzonal insemination (SUZI)
4. Partial zonal dissection (PZD)
5. Somatic cell nuclear transfer (SCNT = cloning)
6. Parthenogenetic activation (PA)

Throughout the past decades attempts were made to produce canine embryos using IVF (Otoi *et al.* 2000b, De los Reyes *et al.* 2006, Saikhun *et al.* 2008), SCNT (Park *et al.* 2010) and PA (Lee *et al.* 2007c, Lee *et al.* 2009, Song *et al.* 2010a). The success rate of *in vivo* matured and picked up oocytes after IVF or SCNT have been higher than *in vitro* matured oocytes in general (Reynaud *et al.* 2005, Reynaud *et al.* 2006). So far, there have been very few reports of advanced embryonic development (morula and blastocyst) in canine (Luvoni *et al.* 2005, Chastant-Maillard *et al.* 2010). The traditional *in vitro* fertilization of canine oocytes has also been reported to have better results with fresh and chilled semen compared to frozen/thawed sperm (De los Reyes *et al.* 2009b). Although the sperm penetration (10-25%) (Rodrigues & Rodrigues 2006)and pronuclear formation (~30%) (Luvoni *et al.* 2006a, Saikhun *et al.* 2008) have happened within the *in vitro* matured oocyte, there seems to be an asynchrony between male and female counterparts as the zygotic genome activation is severely impaired (Rodrigues & Rodrigues 2006, Chastant-Maillard *et al.* 2010). Many

studies have reported the 8 cell block theory as the common feature in canine embryonic development of *in vitro* matured and fertilized oocytes (Lee *et al.* 2007b). Beside zygotic maternal transition, reports of polyspermia and untimely penetration of sperm (before reaching MII) have also been troubling this procedure (Reynaud *et al.* 2005, Reynaud *et al.* 2006, Chastant-Maillard *et al.* 2010). *In vitro* matured canine oocytes seem to have lower developmental potential and sperm interaction characteristics.

Parthenogenetic activation, as a sperm free fertilization method has been established in human (Brevini *et al.* 2009), porcine (Lee *et al.* 2004), canine (Lee *et al.* 2007c, Lee *et al.* 2009, Song *et al.* 2010a), ovine (Loi *et al.* 1998) and bovine (Campbell *et al.* 2000) studies. During the meiotic resumption the first polar body is extruded to subzonal region by the time the oocyte has reached the MII stage and waiting for sperm penetration (Brevini *et al.* 2008). A calcium influx occurs by sperm penetration and cortical reaction which drives the oocyte toward completion of second meiosis (Brevini *et al.* 2008). After sperm head fusion and insertion in to ooplasm the decondensation of sperm nucleus progresses the extrusion of the second polar body which will leave the haploid (n) female pronucleus ready for syngamy. The 2^{nd} polar body extrusion occurs for male and female genomic content to initiate the pronuclear formation procedure. In parthenogenetic activation the same calcium signalling is induced via substances such as Ionomycin, ethanol, dithiothreitol (DTT) or electric shock (Brevini *et al.* 2008). The underlying mechanism of these pulsatile calcium releases and initiation of cortical reaction is not fully understood. However; it's been proven that temporary exposure of oocytes to calcium ionophores such as Ionomycin (ION) induces the required calcium release to promote the zona hardening (cortical granules activation) and inactivation of protein kinases necessary for resumption of second meiosis (Rascado Tda *et al.* 2010). 6DMAP as a non specific kinase inhibitor promoted the inactivation of MPF (CDC2 kinase phosphorylation) and MAPK in order for the oocyte to enter the second meiotic division and extrude the second polar body (Vichera *et al.* 2010). Inactivation of MPF is

prerequisite for entrance to second meiosis which can be obtained via constant and prolonged incubation of oocyte with 6DMAP. This method have been established in human and porcine embryonic stem cell studies, in which pluripotent stem cells have been harvested from PA produced blastocysts (Brevini *et al.* 2009, Brevini *et al.* 2010).

The studies reported here have used parthenogenic activation protocol to analyse the developmental potentials of canine oocyte matured *in vitro* under several culture condition. In addition, attempts were made to produce presumptive zygotes through by conventional IVF using chilled semen.

6.2. Materials and Methods

Chemicals and reagents:

All chemicals were purchased from Sigma-Aldrich chemical Co. (Poole, UK) unless otherwise stated.

6.2.1 *In vitro* maturation

The IVM culture and oocyte staining/staging was carried out following the protocols described in the sections 2.2.-2.6. The basic maturation media in this chapter was devised according to previous chapter's results. Besides 5µg/ml LH, 5µg/ml FSH, 1µg/ml Estradiol and 1µg/ml Progesterone the SOF-0.6% BSA media was also supplemented with 100ng/ml PGH and 100nM Melatonin.

6.2.2. Parthenogenetic activation (PA)

Droplets (100µl) of PA buffers were placed in a 90 mm petri dish and warmed at 38.5^{o}C in 5%O_2, 5% CO_2 and 90%N_2 incubator. PA buffers were prepared as following:

Buffer	Volume
SOF *	7ml
SOF / ION$ 5mM	1ml
SOF / 6-DMAP£ 2mM	2ml

* Synthetic Oviductal fluid containing 0.6% BSA
$ Ionomycin prepared just before transferring the oocytes
£ 6-Dimethylaminopurine (6-DMAP)

Droplets were placed on the petri dish in the following order (Fig. 6.1) to avoid wasting time in transferring the oocytes:

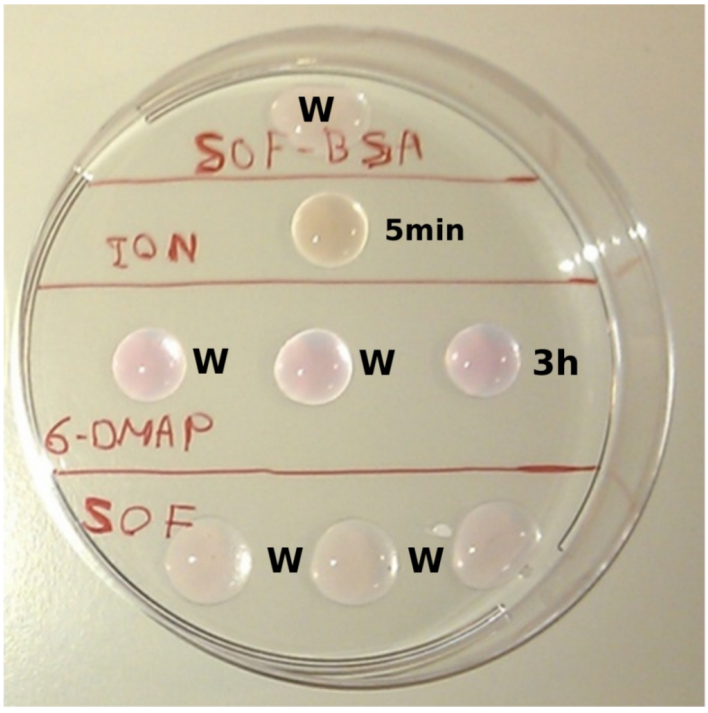


Figure 6.1. A parthenogenetic activation working petri dish. A 90mm petri dish containing droplets of PA buffers. Oocytes were washed before and after the PA in SOF-BSA0.6%. W = wash.

After IVM the COCs were denuded and oocytes were transferred to SOF media containing 0.6% BSA. The oocytes were incubated in the freshly prepared ION

droplet for 5min in the 38.5^{O}C incubator in the dark. Afterwards they were washed twice and transferred to 6-DMAP droplets and incubated for 3h in the same incubator. The oocytes were then washed 3 times in SOF-0.6%BSA droplets and transferred to a 4 well culture dish (VWR, UK). *In vitro* culture (IVC) of presumptive parthenotes was carried out in the same SOF media and at 38.5^{O}C and 5%O_2 5% CO_2 90%N_2 incubator. The cleavage rate was studied at 48h after PA and final assessment was carried out after 8 days of IVC.

6.2.3. *In vitro* fertilization and *in vitro* culture of embryo

Chilled sperm already diluted 1:4 (V/V) in INRA 96 extender (IMV-Technologies [INRA licensed], France) was provided as a generous gift from Professor Gary W.C. England (Nottingham University, UK) and kept in the fridge (4^{O}C) up to a week upon arrival. In order to wash away the extender and separate the motile sperm swim-up technique was performed. Calcium free sperm washing medium (Ca-free) was prepared according to appendix 3. The motility of sperm was assessed after swim up (capacitation) each time prior to IVF and only >70% motile batches were used. The motility of chilled semen was gradually decreasing till day 8 -10 when it was not suitable for IVF anymore. If the motility was lower than 70% swim up was repeated with more initial volume of extended semen. Three ml of fresh Ca-free medium was placed at the bottom of 15ml conical falcon tubes and equilibrated in 5%CO2 in air humidified incubator at 38.5^{O}C for 4 hours prior to swim. Fertilization media (appendix 4) was also prepared fresh and equilibrated/pre-warmed with Ca-free in separate tubes. In order to separate motile sperms 80-100µl of the extended semen was gently placed at the bottom of the 15ml tubes containing Ca-free media. Tubes where then settled in an oblique position (approx. 45^{o}) within the same incubator for 45min. Motile sperms was collected after the incubation period using sterile individually wrapped pastor pipettes (VWR, UK) and transferred to another pre-warmed 15ml conical tube and spun at 500g for 10min to pellet the sperms. Supernatant was carefully removed using a sterile pastor pipette (VWR, UK) not to disturb the precipitated sperms. The sperm pellet was then re-suspended in 1ml of pre-

warmed fertilization media. In order to count the total sperm number 10µl of sperm suspension was mixed with 90µl NaCl 3M in DDW (hyperosmolar solution immobilizes the sperms). The mixture was transferred to a Neubauer slide and the number of live spermatozoa was counted according to the following formula:

Total Sperm/ml = N x 10 x 10000

[n= sperms counted in the center box of the slide]

Sperm concentration was adjusted to $2x10^6$/ml and 500µl of the sperm solution was added to each well containing 20 COCs in a 4-well culture dish. After 16h IVF the presumptive zygotes were washed in pre-warmed SOF-0.6%BSA media to remove the remaining cumulus cells. The presumptive zygotes were then cultured for up to 8 days in 5%O_2, 5% CO_2 and 90% N_2 humidified incubator in a fresh SOF medium. SOF media was changed every 48h by transferring the presumptive zygote to new culture dishes. The cleavage rate was recorded at 48h post IVF under light microscope. Final assessment by 8^{th} day categorized cells in to: 2 cell, 4 cell, 8 cell, morula and blastocyst groups. Degenerated zygotes were defined with no cleavage or vacuolated cytoplasm.

6.2.4. Statistical analysis:

All experiments were repeated at least 3 independent times. The proportional average of oocytes in different stages of meiotic resumption or zygotes in development after fertilization was calculated at the end of the culture period in comparison to the total number of cultured oocytes. Statistical analysis was carried out using PAWS statistics 18: Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA) using binary and ordinal logistic regressions via generalized linear model. Differences among treatment groups were considered significant if *P* values were < 0.05. Data are presented as mean ± SEM.

6.3. Experimental design

A total of 1064 COCs were used for these studies. COCs were cultured in groups of about 20 and specific time points were devised for each experiment. Summary of experiments and number of the COCs utilized are described in Table 6.1.

6.3.1. Effect of PA on canine and bovine matured oocytes

Bovine COCs as the positive control for PA, were aspirated from 2-8 mm follicles . and matured in TCM199 containing 10% FBS, 5µg/ml LH & FSH and 1µg/ml Estradiol for 24h following the previously described protocol (Marei *et al.* 2009). A total number of 71 bovine and 129 canine COCs were matured and activated using the previous described method section 6.2.1.

6.3.2. Effect of Estrus Sheep Serum on IVM and IVF

A total number of 270 canine COCs were matured using the basic maturation medium, combined with 12h of 10mM Caffeine pretreatment (12h Caffeine + 60h SOF/LH/FSH/E2/P4/PGH/Melatonin) as previously described in chapter 4. IVF was carried out using fertilization media containing 2% estrus sheep serum (ESS) a generous gift from Professor Maria T. Paramio (University of Barcelona, Spain). Forty eight hours after IVF a proportion of the oocytes (n=110) were fixed and stained using Hoechst 33342 (10g/ml in PBS 1x) in order to visualize the fertilization status of presumptive zygotes. The remainder of the presumptive zygotes was cultured up to 8 days.

6.3.3. Pronase digestion of ZP prior to IVF

Due to low penetration rates in the latter experiment it was devised to digest the COCs using a generic protease (0.3% Pronase in SOF) for 3 min followed by deactivation in SOF-25%FBS and 3 separate washes (SOF) prior to IVF. For further optimization of IVF system the fertilization media was also replaced with SOF media containing 2.5 mM Caffeine and 2% ESS. A total number of 430 matured COCs (12h Caffeine pretreatment + 60h SOF/LH/FSH/E2/P4/PGH/Melatonin)

were digested and fertilized. Cleavage rate was recorded by 48h and final assessment was carried out by day 8 of IVC.

6.3.4. Effect of Post-Ovulation Estrus bitch serum on IVM/IVF/IVC

Post ovulatory estrus bitch serum (EBS) was provided as a generous gift from Prof. Gary W.C. England (University of Nottingham, UK). After 45 min 65^{o}C heat inactivation of the serum, it was utilized in IVM, IVF and IVC medium at 10, 2 and 5 % respectively to assess the effect of this supplement. A total number of 235 canine COCs were cultured following the same method previously described in section 6.3.3 with an additional 10% EBS in the maturation media. Following 3 min Pronase digestion, the oocytes were fertilized in SOF-2.5mM caffeine containing 2% EBS. After 48h post IVF 5% EBS was also added to the IVC medium.

Table 6.1. The summary of experiments in this chapter.

No. Exp.	No. COCs	Treatment	Measurements
6.3	**1064**	**Total COCs**	**IVM /IVF/ IVC**
6.3.1	**129**	**PA**	**IVM/IVC**
6.3.2	**270**	**ESS and IVF**	**IVF/IVC Staging**
6.3.3	**430**	**Pronase and ESS**	**IVC Staging**
6.3.4	**235**	**EBS and Pronase**	**IVC Staging**

6.4. Results

6.4.1. Effect of PA on canine and bovine matured oocytes

Bovine COCs were utilized in this experiment as a positive control for the parthenogenetic activation protocol. Bovine oocytes after 24h of maturation were fully expanded (95.7% ± 4.3) on average. Moreover; 48h after PA they achieved a cleavage rate of 33.4% ± 4.2 which was significantly higher than canine counterpart (2.7% ± 1.3). By the end of IVC (Day 8 after PA) bovine blastocysts were produced (11.0% ± 1.9; Table 6.2). Example images of the bovine parthenotes are presented in Fig 6.2.

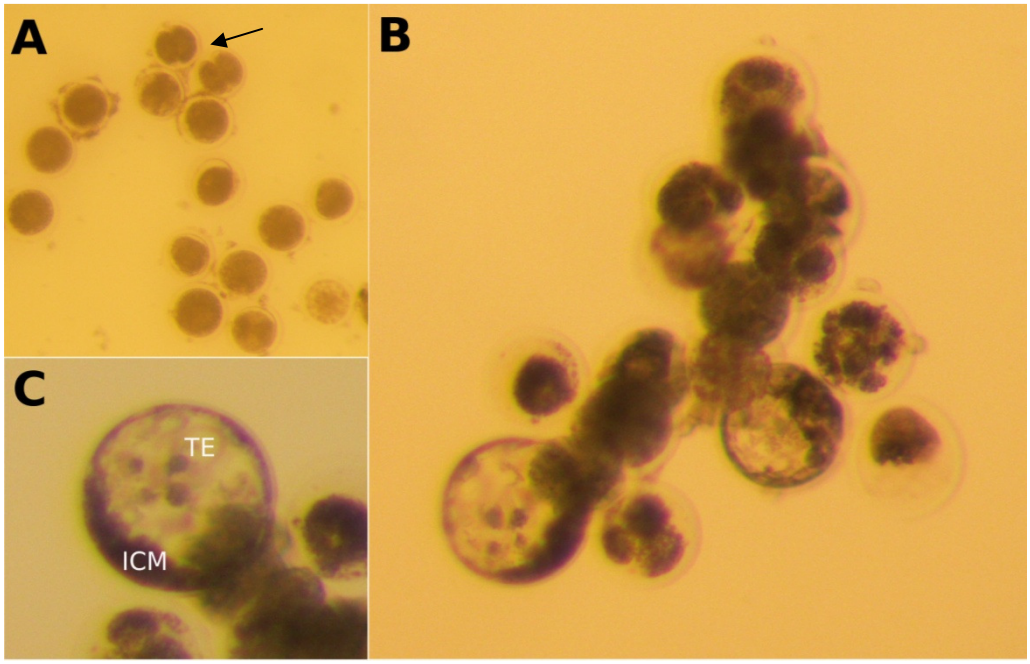


Figure 6.2. In vitro cultured (IVC) bovine parthenotes at different stages of embryonic development. A) Cleavage of bovine parthenotes 48h after PA (Black arrow shows 2cell embryos). B) Bovine parthenotes after 8 days of IVC. C) Bovine parthenote at expanded blastocyst stages with marked inner cell mass (ICM) and trophoectoderm (TE).

No blastocyst could have been produced using canine matured oocytes. Almost all of the presumptive parthenotes was degenerated by the end of IVC (97.3% ± 1.3)

*Table 6.2. Effect of PA on embryonic development of canine and bovine oocytes. Table shows distribution of presumptive zygotes in different stages of embryonic development. From 129 cultured canine oocytes in 3 repeats 49 oocytes were stained using Hoechst 33342 in order to clarify the MII maturation rate of the batch. Bovine oocyte maturation was only assessed by full expansion of the oocytes. Therefore all the bovine matured COCs (71) and only 80 canine COCs were activated in these experiments. * = P<0.05*

	Percentage of presumptive zygotes (Mean ± SEM) in different stages at day 8							Degenerated zygotes	(n)
	Matured[£]	Cleavage at 48h	2 cell	4 cell	8 cell	Morula	Blastocyst		
Bovine PA	95.7 ± 4.3	33.4 ± 4.2*	10.1 ± 2.1*	9.6 ± 3.0*	0.0 ± 0.0	20.8 ± 2.6*	11.0 ± 1.9*	44.2 ± 3.6	71
Canine PA	28.5 ± 1.3	2.7 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	97.3 ± 1.3*	80(129)
£ Full expansion for bovine COCs and MII matured oocytes in canine COCs									200

6.4.2. Effect of Estrus Sheep Serum on IVM and IVF

Addition of 2% ESS during IVF resulted in 14.8% ± 0.6 monospermic penetration (Fig. 6.3). However 9.9% ± 5.1 of oocytes were still at MII after 48h from the fertilization. In the stained presumptive zygotes high levels of degeneration was observed (75.3% ± 4.7) by 48h from IVF. There was no cleavage in the remainder of presumptive zygote and consequently no further development by day 8 of IVC (Table 6.3).

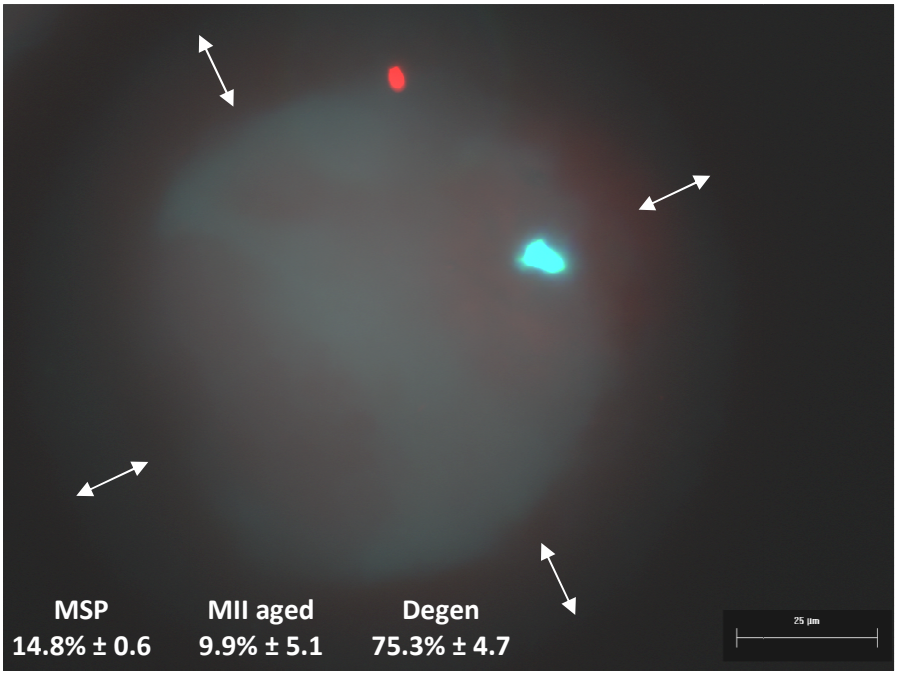


Figure 6.3. Monospermic penetration of canine sperm into sub zona region. Fertilized canine oocyte stained using Hoechst 33342 and the image is taken by a 40x object lens. Sperm nucleus (red) and oocyte genomic content (light blue) are tinted using GIMP2 software. The thickness of the ZP is apparent (white arrows) around the oocyte. (Scale bar 25µm; Monospermic Penetration= MSP)

6.4.3. Pronase digestion of ZP prior to IVF

Digestion of COCs for removal of cumulus cells and partial digestion of ZP led in to 6.4% ± 0.3 cleavage rate at 48h. By day 5 of the IVC in one of repeats a single 8 cell embryo was also produced (Fig. 6.4). No further development (morula or blastocyst) was observed (Table 6.3).

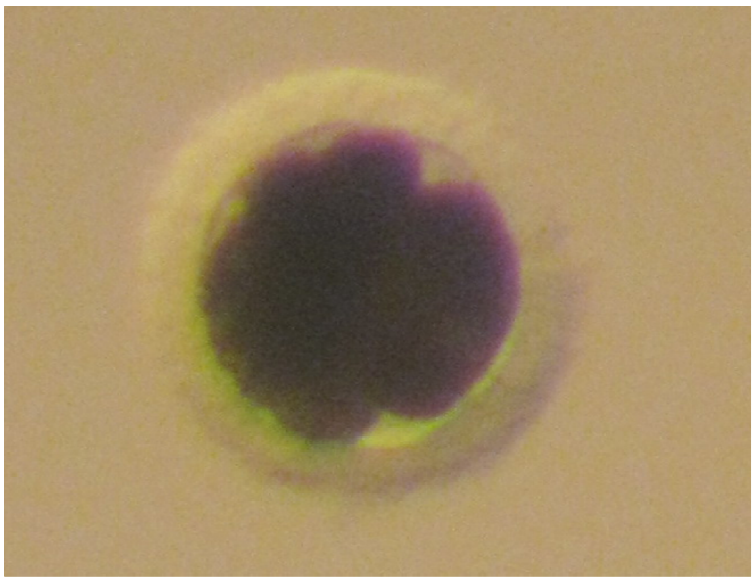


Figure 6.4. A photograph of an 8 cell canine embryo at day 5 of IVC.

6.4.4. Effect of Post-Ovulation Estrus bitch serum on IVM/IVF/IVC

Addition of EBS to IVM (10%)/IVF (2%) media resulted in 1.8% ± 0.5 cleavage rate at 48h and 98.7% ± 0.2 degeneration by day 8 of IVC. No further embryonic development was observed compared to previous attempts.

Table 6.3. Different fertilization protocols and its effect on embryo development of canine oocytes. Table shows distribution of presumptive zygotes in different stages of embryonic development. Estrus Sheep Serum = ESS; Estrus Bitch Serum = EBS; Degen= degenerated.

	Percentage of presumptive zygotes (Mean ± SEM) in different stages at day 8							Total (n)
	Cleaved at 48h	2 Cell	4 Cell	8 Cell	morula	blastocyst	Degen	
ESS 2% IVF	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	270
ESS 2% IVF + Pronase	6.4 ± 0.3	5.8 ± 1.0	4.1 ± 0.5	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	89.9 ± 1.6	430
EBS 10% IVM/ 2% IVF + Pronase	1.8 ± 0.5	0.9 ± 0.5	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	98.7 ± 0.2	235
								935

6.5. Discussion

Experiments devised in this chapter have assessed developmental potentials of canine oocytes after *in vitro* maturation. Attempts were made to produce *in vitro* cultured embryos via fertilization with sperm and oocyte activation (without sperm). There have been numerous reports of failed attempts to produce *in vitro* cultured canine blastocysts during the past years (Mahi & Yanagimachi 1976, Renton *et al.* 1991, Otoi *et al.* 2000b, De los Reyes *et al.* 2006, Hatoya *et al.* 2006, Luvoni *et al.* 2006a, Lee *et al.* 2007b, De los Reyes *et al.* 2009b, De los Reyes *et al.* 2012). Several obstacles have been hypothesized to prevent this achievement:

1. Low MII maturation rate of *in vitro* cultured oocytes (Luvoni *et al.* 2005)
2. Insufficient and unsynchronised cytoplasmic maturation of oocytes *in vitro* (Lee *et al.* 2007b)
3. High degeneration rates of canine oocytes due to high lipid content, susceptibility to oxidative stress and complex nutritional requirements (Salavati *et al.* 2012)
4. Sperm penetration and male nucleus decondensation (Reynaud *et al.* 2004, Reynaud *et al.* 2005, Reynaud *et al.* 2006)
5. High rate of polyspermia *in vitro* (Rodrigues Bde *et al.* 2004)
6. Prolonged culture conditions and environmental stressors (Alhaider & Watson 2009)

This chapter of studies was dedicated to assessment of developmental potentials of canine oocyte considering the improvements already made in previous chapters.

6.5.1. Parthenogenetic activation

Parthenogenetic activation of canine oocyte have been utilized during (Song *et al.* 2010a) and after IVM (Kim *et al.* 2010) in order to improve maturation rate and promote pronuclear formation respectively. In this study the PA was implicated after IVM as a sperm free fertilization method in order to produce canine embryos. PA in canine oocyte have been carried out via chemical or

electrical stimulation and there has been no report of blastocyst formation (Lee *et al.* 2007c, Brevini *et al.* 2008, Lee *et al.* 2009, Song *et al.* 2010a). Lee *et al.* In 2008 showed that 1.5kV/cm activation of canine oocytes results in 2.7% two cell embryo production but no further embryonic development was observed (Lee *et al.* 2009). In the experiments reported here, the combination of ION and 6-DMAP was used for activation of *in vitro* matured oocytes. Activation of bovine oocytes after 24h of maturation resulted in 33.4% ± 4.2 cleavage rate at 48h and 11.0% ± 1.9 blastocyst rate after 8 days of IVC. These results were in agreement with previous reports of bovine PA using ION and 6-DMAP (Vichera *et al.* 2010). However, canine matured oocytes after 72h of IVM only reached 2.7% ± 1.3 cleavage rate after 48h and were mostly degenerated by 8^{th} day of IVC ($P < 0.05$). The low cleavage rate were similar to previous reports of electrical or chemical activation of canine oocyte followed by no advanced embryonic development (Lee *et al.* 2007c, Lee *et al.* 2009, Kim *et al.* 2010, Song *et al.* 2010a). Although the bovine positive control parthenotes were cleaving at a satisfactory rate, the induction of calcium pulse is not investigated in canine oocyte. Further optimization of this activation method should be carried out to be tailored for canine oocytes.

6.5.2. Effects of estrus sheep serum on fertilization of dog oocytes

Sperm motility after swim up in Ca-free media in all the batches was above 70% and morphology of the sperm was satisfactory. The addition of estrus sheep serum to capacitation and fertilization media has been proven to promote cholesterol efflux, capacitation and acrosomal reaction of the sperm in ram semen (Huneau *et al.* 1994). Although there was no previous report of similar effects on canine sperm, in order to study the effect of ESS on fertilization and embryonic development of *in vitro* matured canine oocytes, ESS was added at 2% concentration to fertilization media. ESS presence resulted in 14.8% ± 0.6 monospermic penetrations. However, there was clear evidence of failure of sperm nucleus decondensation (Fig 6.3). This was concluded from single sperm

heads in the subzonal space without any sign of decondensation or further fusion with oolema.

6.5.3. Partial digestion of ZP

Since almost 1/3rd of matured oocytes (9.9% ± 5.1) were still at MII stage and the lack of sperm penetration was at doubt, Pronase was utilized for partial digestion of ZP and removal of possible physical preventive barriers in front of sperm. Replacement of fertilization media with SOF containing 2.5mM caffeine was also implicated in these experiments considering the literature mining (Hishinuma & Sekine 2004, Minelli & Bellezza 2011). Temporary digestion of oocyte prior to IVF using a generic protease such as Pronase resulted in 6.4 % ± 0.3 cleavage rate at 48 h. There was also further embryonic development in oocytes treated with Pronase prior to IVF (2cell = 5.8 % ± 1.0; 4cell = 4.1% ± 0.5; 8cell = 0.2 % ± 0.2). The unique structure of ZP in canine oocytes and deep invagination of cumulus cells within the glycoprotein matrix may result in this low penetration rate of sperm (Brewis *et al.* 2001, Blackmore *et al.* 2004, De los Reyes *et al.* 2009a). Changes in the ZP surface of canine oocytes occur during *in vivo* / *in vitro* maturation in order to facilitate the sperm orientation, penetration and acrosomal reaction (De los Reyes *et al.* 2009b). There is electron microscopic evidence of changes in pore size of ZP during the *in vitro* culture of canine oocytes (De los Reyes *et al.* 2009a). The pore size of ZP mesh in immature oocytes (0.69 ± 0.12 µm) significantly changed after 72h of IVM (1.56 ± 0.19 µm) and the extension of the culture (96h) has resulted in reduction of this pore size (De los Reyes *et al.* 2009a). Moreover; the capacitation of the sperm has been reported to happen during the penetration and recognition process which is highly affected by the maturation level of ZP proteins and surface structure (De Lesegno *et al.* 2008). The ZP thickness and tight inter cellular junctions block the penetration of sperm through simple physical interference. The partial digestion of zona before exposure of oocytes to sperm clearly increased the fertilization rate of canine oocytes (Table 6.3). However; the digestion of the zona might interfere with capacitation and physiological events required for ZP proteins

(ZP2, ZP3) recognition and sperm - oocyte communications. Further studies are required to elucidate the acrosomal ability of canine sperm for penetrating ZP and chronological order of zona hardening occurring simultaneously.

6.5.4. Effects of post ovulatory Estrus bitch serum on IVM/IVF/IVC

During the very last weeks of this PhD study, a reliable source of post ovulatory estrus bitch serum was kindly provided by Prof. Gary W.C. England (University of Nottingham, UK) which was immediately implicated to the IVF experiments of this chapter. Surprisingly the presence of EBS within maturation and fertilization media did not improve the cleavage rate or the embryonic development of canine oocytes. Its effects on maturation rates, and pronuclear formation should be further evaluated.

6.5.5. Conclusion

Parthenogenetic activation of canine oocyte in contrast with bovine +ve controls resulted in very poor developmental achievements. Further optimizations are required to narrow down the perfect activation protocol for canine oocytes. It's noteworthy to mention the ensemble of studies carried out within this chapter was production a single 8 cell embryo in the presence of 2% ESS in the fertilization media and ZP digestion prior to IVF. Since the *in vitro* fertilization of canine oocyte is a multifaceted phenomenon, further experimentations are necessary to shed light on low yield of this method. Timing of fertilization, the ingredients of capacitation/fertilization media and the specific embryonic supplementation are issues requiring further elucidation.

Summary of results in this chapter is as following:

- Parthenogenetic activation of canine *in vitro* matured oocytes (12h Caffeine pre-treatment + 60h LH/FSH/E2/P4/PGH/MTN), carried out using ION and 6DMAP chemical activation method, was unsuccessful. The cleavage rate below 5% at 48h was compared to 30% cleavage in bovine *in vitro* matured oocytes as the positive control in this sperm free fertilization method.
- *In vitro* fertilization of canine oocytes using chilled extended semen in the presence of 2% ESS in the fertilization media did not result in any embryo production.
- Partial digestion of the ZP prior to fertilization using Pronase in combination with 2% ESS in the IVF media resulted in 6.4% ± 0.3 cleavage at 48h. A single 8 cell embryo was produced from *in vitro* matured, fertilized and culture canine oocytes.
- Addition of EBS to IVM (10%), IVF (2%) and IVC (5%) culture media did not improve the cleavage rate even after PZD.
- Fertilization of canine oocytes *in vitro* requires further optimization in regards to sperm penetration, male nucleus decondensation and embryo development.

7. Canine follicular cells primary culture and its steroidogenic profile

7.1. Introduction

The source of cells cultured for this study was provided as a bi-product of COC extraction from canine ovarian tissues. Slicing the cortex of the ovaries throughout the studies presented in previous chapters had resulted in a cell suspension containing follicular and cortical ovarian stromal cells. There are limited studies concerning canine follicular culture (Bolamba *et al.* 1998, Skorska-Wyszynska *et al.* 2004, Abdel-Ghani *et al.* 2012) and there is no available data on establishment of canine granulosa and theca culture. Basic studies concerning ovarian and follicular primary culture enhance the understanding of folliculogenesis and ovarian cortex molecular dynamics. Physiological responses of canine ovarian cortical cells to hormones and pharmaceutical substances can provide quintessential information for oocyte maturation systems. The importance of further understanding these principles can also be of great value in animal modelling, conservation of endangered species and clinical application advancements as in ovarian cancer research. The cell lines of interest in follicular cultures are mainly theca interna and granulosa cells. Granulosa cells during the folliculogenesis and even immediately after ovulation participate in steroidogenic machinery of the follicle in close contact with theca cells (Scaramuzzi *et al.* 2011). In contrary with ruminant's ovulation model which is characterised by an oestrogen dominant proestrus and oestrous followed by ovulation and luteinisation of the dominant follicle (Scaramuzzi *et al.* 2011), ovulation in canid family differs (Concannon 2011). The P4 production temporal profile is unique and precedential in canine species (Concannon 2011). The pre-ovulatory luteinisation of canine follicles and above 2.5 ng/ml plasma P4 level in the presence of COCs within the follicular environment is the exceptional specifications of canine ovaries (Concannon 2011). The progesterone (P4) production of canine follicle is so reliable that has been considered as the clinical

marker of optimal mating time for dog breeders in veterinary medicine practice (Luvoni *et al.* 2001, Luvoni *et al.* 2005, de Gier *et al.* 2006b). The luteinisation time course and steroidogenic profile of canine granulosa and theca cells are expected to follow a different pattern from bovine (Allegrucci *et al.* 2003) or ovine (Campbell *et al.* 2010) counterparts. Each of these cell lineages has its temporal and spatial protein and steroid production profile. The steroidogenesis profile of theca and granulosa cells before ovulation is closely intercalated as theca cells provide androgens (mainly androstenedione and testosterone) (Zhang *et al.* 2009). The theca cells having luteinizing hormone (LH) receptors during the follicular phase utilize cholesterol to produce steroids. Follicle stimulating hormone (FSH) receptor orchestrates the steroidogenic transformations in granulosa cells during the same period (Jamnongjit & Hammes 2006, Zhang *et al.* 2009). The gonatropin (LH/FSH) receptors use cyclic adenosine mono phosphate (cAMP) as the secondary messenger and cAMP dependent protein kinase A (PKA) to regulate steroidogenesis which is initiated by transfer of cholesterol in to mitochondria via steroidogenic acute regulatory protein (StAR) and conversion to Pregnenolone via P450 Side chain cleavage protein (P450-SCC) (Orly & Stocco 1999). Pregnenolone will be further transformed to either progesterone (P4) via 3 beta-hydroxysteroid dehydrogenase (3βHSD) or dehydroepiandrosterone (DHEA) using Cytochrome P45017 (CYP17). Progesterone via CYP17 will be primarily converted to 17-hydroxy progesterone and then to androstenedione (AD). (Orisaka *et al.* 2006). DHEA can also be further transformed in to AD via 3βHSD and then testosterone (TT) through 17 beta-hydroxysteroid dehydrogenase (17βHSD) (Payne & Hales 2004, Jamnongjit & Hammes 2006). During the oestrogen dominant phase (follicular) these androgens (AD and TT) from theca cells leak into to granulosa counter parts through tight gap junction and basement membrane. Then AD will be metabolized through Cytochrome P45019 (CYP19; Aromatase) to oestrone and finally via 17βHSD in to estradiol (E2) (Payne & Hales 2004). TT will also be transformed in to E2 via CYP19 (Payne & Hales 2004). This collaborative steroidogenic machinery is in place during the

selection of follicles, after which during the dominance the granulosa cells start to express LH receptors and produce their own supply of androgens and produce large amounts of E2 (Jamnongjit & Hammes 2006). After the ovulation phase both theca and granulosa cells differentiate into small and large luteal (P4 producing) cells respectively.

The present studies were conducted to characterize steroidogenic activity of canine ovarian cortex cultured *in vitro*. Morphological changes in the cells attachment and aggregation were recorded. Functional analysis of the cells were carried out by these cells response to physiological (LH, FSH, AD) and pharmaceutical substances (25 hydroxy cholesterol [25OHC], dibutyryl cAMP [dbcAMP]). P4 and E2 production profile of these cells were measured by radioimmunoassay (RIA) and the mRNA expression profile of LH receptor, FSH receptor, CYP19, StAR and 3βHSD was analysed via relative qPCR.

7.2. Materials and Methods

All the chemicals were supplied via Sigma (Poole, UK) unless stated otherwise.

7.2.1. Dissection of Ovaries and Separation of Follicular cells

Collection and culture media were prepared at least 3 hours prior to initiation of culture and equilibrated inside the 38.5°C (5% Co_2 in air) humidified incubator. The ovaries were dissected separately from the ovarian bursa using a scalpel blade in warm PBS 1x, and then transferred to a new 90mm Petri dish containing pre-warmed collection media (TCM199 supplemented with 20mM HEPES, 10% FBS, Penicillin 100IU/ml, Streptomycin 100 µg/ml and Amphotericin B 2.5 µg/ml). A contrivance of multiple blades (Fisher Scientific, Loughborough, UK) as described previously (Salavati *et al.* 2012) was used to scratch the surface (cortex) of the ovaries to release the follicular cells (FC) . Cumulous oocyte complexes (COC's) were removed under a dissecting microscope and utilized elsewhere.

The tissue extract was passed through a sterile cell strainer (BD falcon, 100 µm) into a 50ml conical tube to remove large size debris, including any COCs which were not removed via selection. The cells were then pelleted in 3-5ml of collection media using 900g for 10min centrifugation. The supernatant was discarded in order to access the pellet. In case of presence of RBC (red layer on top of the cell pellet), 1ml of filtered, sterile water was added, and the suspension was shaken for 2 minutes, followed by the addition of 1ml filtered PBS 1x sterile buffer to the tube and shaking for a further 1 minute. Finally, 2ml of culture media (McCoy's 5A supplemented with 20mM HEPES, 10% FBS, Penicillin 100IU/ml, Streptomycin 100 µg/ml and Amphotericin B 2.5 µg/ml) was added immediately to re-equilibrate the solution for cells to optimal osmolarity and pH range. The cells were once again pelleted at 900g for 10 min. The supernatant was discarded. The cells were then re-suspended in 1ml of culture media; ready to be counted. Ten µl of the cell suspension was mixed with 90µl of trypan blue, and the cells were counted and estimated according to the Neubauer slide universal instructions. The live cells were cultured in 100,000/well manner in 50µl volume of a 96 well Nunc-Nalgene culture plate (NUNC, VWR International, Milan, Italy). The cells were distributed among treatments in quadruplicates. Once the cells were settled, the well was topped-up with 200µl of culture media (total 250µl). The culture was continued for 96h and the culture media were changed every 24h.

7.2.2. Immunocytochemistry for CYP19

The cells were cultured on sterile glass coverslips laid at the bottom of the 24well culture dish (NUNC, VWR International, Milan, Italy) and fed every 48h with control media (McCoy's 5A, 10% FBS) up to 6 days. After retrieval of the coverslips from the bottom of the well, they were fixed in cold acetone 99% for 10min at -20^{o}C and glued on a glass slide. Slides were rehydrated in PBS for 5min, before blocking nonspecific binding with 8% normal horse serum in PBS for 1h 30m at room temperature in a humidified chamber. The slides were probed overnight at 4^{o}C using mouse anti human cytochrome P450 (CYP19) aromatase

(MCA2077S, AbD serotec, Oxford, UK) at 1:100 dilution. After a wash in PBS cells were visualized using sheep anti rabbit IgG-FITC conjugated secondary antibody (1:2000). Hoechst 33342 at 10µg/ml in PBS1X was used as the counter stain. Slides were mounted using Vectashield mounding media (Vector Laboratories, Burlingame, CA, USA) and examined under Olympus BX60 fluorescence microscope (Olympus, UK).

7.2.3. Assessment of Gene Expression from Canine Follicular Cells at 96 hours

7.2.3.1. Primer Design and Setup

The primers for LHR, StAR, 3βHSD and GAPDH were designed using the web-based software Primer3 from the sequence information found in NCBI's 'Gene' database. The FSHR and Cyp19 primers were a generous gift from Dr. Waleed Marei (University of Cairo, Egypt), which was originally designed in cow but had high BLAST score with canine FSHR and CYP19 genes. Sequences of the primers were as follows:

Table 7.1. Sequence of the designed primers for LHR, FSHR, StAR, 3ϐHSD, Cyp19 and GAPDH. B = bovine, C = canine

Genes Name	Reference Sequence	Oligos(5'→ 3')	Tm(°C)	bp
LHR	ENSCAFT00000036195	F:acacataaccaccataccagga R:gctccattgtgcatcttctcta	61	174
FSHR	B: NP_776486.1 C: XM_538488.4	F:tgcagtcgaactgaggtttg R:gaaggttctggaaggcatca	59	218
StAR	ENSCAFT00000009932	F:agctctctgcttggttctcg R:acatctgggaccaccttactga	59	182
3βHSD	ENSCAFT00000015969	F:agcaagaccaagctgaccat R:acaggagctgagtacccttcag	59	172
Cyp19	B: NM_174305.1 C: NM_001008715.1	F:gccaagaatgttccttacaggt R:atcttctcaacacaccgacctt	59	200
GAPDH	NM_001003142.1	F:gtgatgctggtgctgagtatgt R:atggatgactttggctagagga	59	233

7.2.3.2 RNA extraction from ovarian tissue

A sample of canine ovarian tissue was processed for RNA extraction using TRI Reagent® protocol (AB Applied Biosystems, UK; AM9738). This was used as an alternative source to test all the primers prior to realtime-PCR.

7.2.3.3. RNA extraction from cultured cells

QIAGEN RNeasy Mini Kit was used to isolate RNA from the samples, and the manufacture's protocol was followed. After 96 hours of culture, the culture media was removed and the wells were treated with RLT (350µl) RNA Lysis Buffer. A 1ml insulin syringe with a 23G needle was used to lyse the cells before transfer of the lysates in to RNeasy spin columns. RNA extraction and reverse transcription were carried out using the method previously described in section 3.2.3.2-3. Real time PCR and analysis was carried out according to the methods described in section 3.2.3.4-6.

7.2.4. Progesterone and Oestradiol Hormone assay

Media stored at -20^{o}C from each time point, were analysed using previously established method of charcoal-dextran coated RIA (Robinson *et al.* 2002) for progesterone and estradiol concentration. Standard range was 0.8ng/ml-10ng/ml for progesterone and 0.01ng/ml-2.5 ng/ml for estradiol. Briefly the progesterone and estradiol antiserum (generous gift from Dr. Zhangrui Cheng, Royal Veterinary College, UK) were mixed with standard and samples accordingly followed by addition of the tritiated tracers (in separate tests): [2,4,7,16,17-3H]-Estradiol (Amersham,UK; TRK587) and [1,2,6,7,16,17-^{3}H]-Progesterone (Amersham,UK; TRK641). Tubes were incubated at 4^{o}C overnight. The suspension of 0.4% dextran (T-70; Amersham Pharmacia Biotech) and 2% neutralized charcoal was added to all tubes except the total count. After 10min 4^{o}C incubation followed by centrifugation at 2000 g for 10 min, the supernatant was transferred into 6 ml scintillation vials and topped up with 4 ml scintillant (Ultima gold; Packard Bioscience BV, Pangbourne, Berks, UK). All the tubes were counted

for 2min. The intra-assay coefficient of variation for P4 and E2 were 4.6% / R^2 = 0.97 and 9.6% / R^2 = 0.93, respectively.

7.2.5. Statistical analysis

All the experiments were repeated at least three times. The quantitative data were evaluated by comparison among the different treatment groups within the 24 hour time intervals, and furthermore, the time intervals were assessed per treatment group, through the use of a Linear Mixed Model (SPSS, Inc., Chicago, IL, USA. The results were expressed as mean $\pm$ SEM. All differences were considered significant at a confidence level of $p<0.05$.

7.3. Experimental Design

7.3.1. Origins of cultured cell population

The first experiment was designed to determine identity of the isolated cells based on morphological features and presence of granulosa cell marker CYP19. In the first experiment cells were cultured on the surface of a sterile circular cover slip placed at the bottom of a 24 well culture dish. Photographs were taken in order to illustrate the culture proliferative behaviour and cellular morphology. Expression of CYP19 was assessed by immunocytochemistry.

7.3.2. Steroidogenic machinery of follicular cells

To study the steroidogenic profile of this primary culture, experiments were devised (Non-Androstenedione [Non-AD] and Androstenedione [AD]) and repeated 4 times. During these experiments culture media were collected to be assessed for progesterone and estradiol production of these cells and its temporal profile. Photographs were also taken from the cells cultured for Non-AD at 24, 48, 72, 96h after culture in order to observe the attachment and intercellular behaviour of the follicular cells under hormonal and chemical treatments. In Non-AD experiments follicular cells were isolated from canine ovaries and plated in 96 well culture dishes (100,000/well). The cells were fed with 150 µl of fresh culture treatment media every 24 hours (up to 96 hours).

The collected media was stored at -20°C for later inspections. There were 5 separate treatment groups: 1. LH (100ng/ml); 2. FSH (100ng/ml); 3. 25OHC (5 mM); 4. dbcAMP (5 mM); 5. Ctrl (no treatments). At 96 hr the culture was terminated and the cells were retrieved for RNA extraction and cDNA construction to study the expression level of genes involved in steroidogenesis. The changes in mRNA expression of LHR, FSHR, StAR, 3βHSD and CYP19 were analysed using realtime qPCR.

In the AD experiment the same principle of culture and treatment was conducted with complete removal of the media at 24h and replacing it with treatments and control media containing 100nM AD. The media was collected for RIA and culture was terminated at 96h for RNA extraction.

Table 7.2. The summary of experiments in this chapter.

No. Exp.	Treatment	Measurements
7.3.1	**Culture origin and morphology**	**Imaging / ICC**
7.3.2	**Steroidogenic machinery**	**Imaging/RIA/qPCR**

7.4. Results

7.4.1. Follicular/Stromal morphology

The attachment of the total cell population after 24h was stable during the study. Aggregation/clumping pattern of the cells after 96h culture in the control group has been shown in Fig. 7.1:

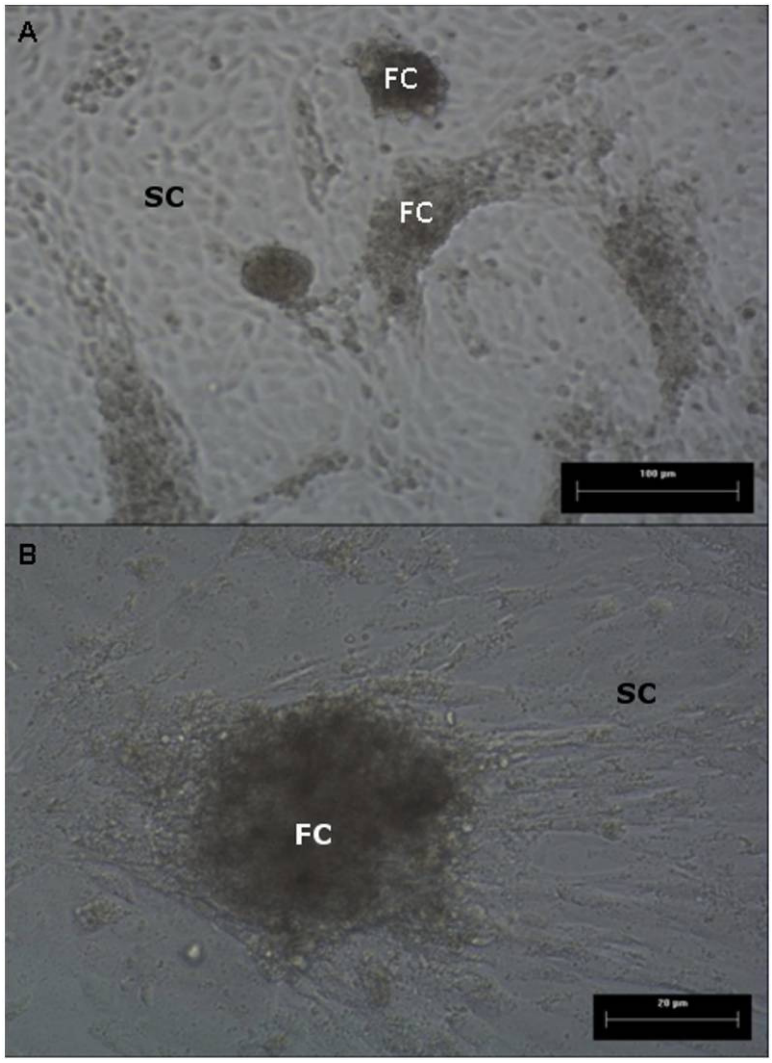


Figure 7.1. Photographs of the Non-AD control group after 96h of culture. Photographs were obtained using a digital camera installed on a light microscope at 10x (A) and 20x (B) magnification. SC =presumptive somatic cells, FC= potential follicular cells (e.g. granulosa/theca/luteal)

Three distinctive cell populations were observed by the end of 96h culture duration. Two morphologically different cellular clumps were surrounded by a pavement of somatic cells (Fig 7.1 A&B). The aggregated clumps were hypothesised to be of follicular origin as the pattern of their connections was changed under hormonal treatments such as LH (Fig. 7.2):

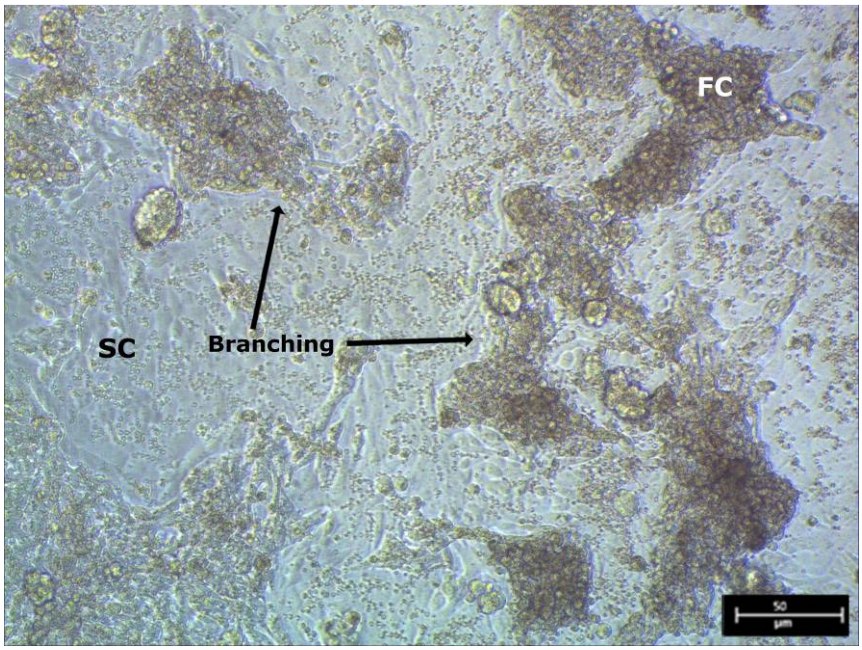


Figure 7.2. Photographs of the Non-AD LH treated group after 96h of culture. Photograph obtained at 10x, depicts possible follicular cell clumps (FC) branching after 96 hours from, with presumptive stromal cells (SC) in the background.

7.4.2. Cell type characterization

The origin of the culture cells was investigated using monoclonal antibody raised against CYP19 (P450 aromatase) which is found in granulosa cells producing estradiol from androgens. The cells cultured on the coverslip were probed for the presence of CYP19 enzyme using immunocytochemistry. The circular FC clumps (Fig. 7.3B) and some individual cells (Fig. 7.3A) among the stromal pavement proved to have Cyp19 expression (+ve). The patchy FC clumps (-ve) and SC pavement didn't show any fluorescent signal of CYP19 (Fig. 7.3D&E).

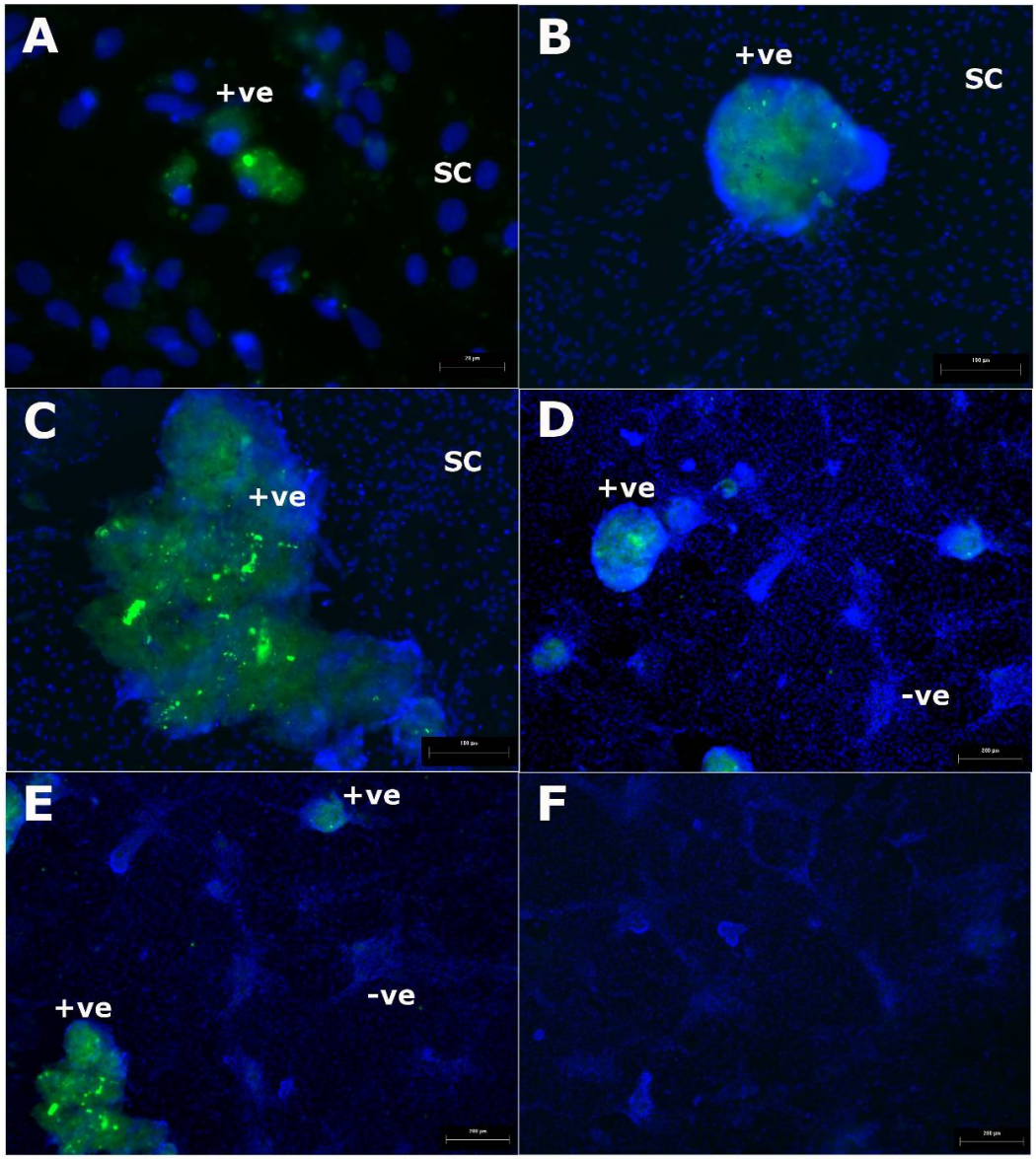


Figure 7.3. Immunocytochemistry of Cyp19 (p450 Aromatase) from cells cultured for 6 days. Illustration shows the images taken using fluorescent microscope (FITC conjugate) from follicular cells cultured for 6 days and probed with Cyp19 monoAb in immunocytochemistry. A) Cyp19 +ve FC cells at 40x magnification (20µm scale bar). B&C) Cyp19 +ve Clumps at 10x magnification (100µm scale bar). D&E) Cyp19 +ve and -ve FC clumps at 4x magnification (200µm scale bar). F) Mouse IgG control at 4x magnification (200µm scale bar).

7.4.3. Culture morphology

Images of cell attachment and inter cellular connection were captured after daily feeding in Non-AD treatments (Fig. 7.4). The images show a visible increase in the size of cell clumps, as well as, an increase in their density over time. There is also evident extending and branching of these cell clumps after 96 hours of culture. The LH and FSH groups notably represent these changes and are in resemblance to the control group. (Fig. 7.4)

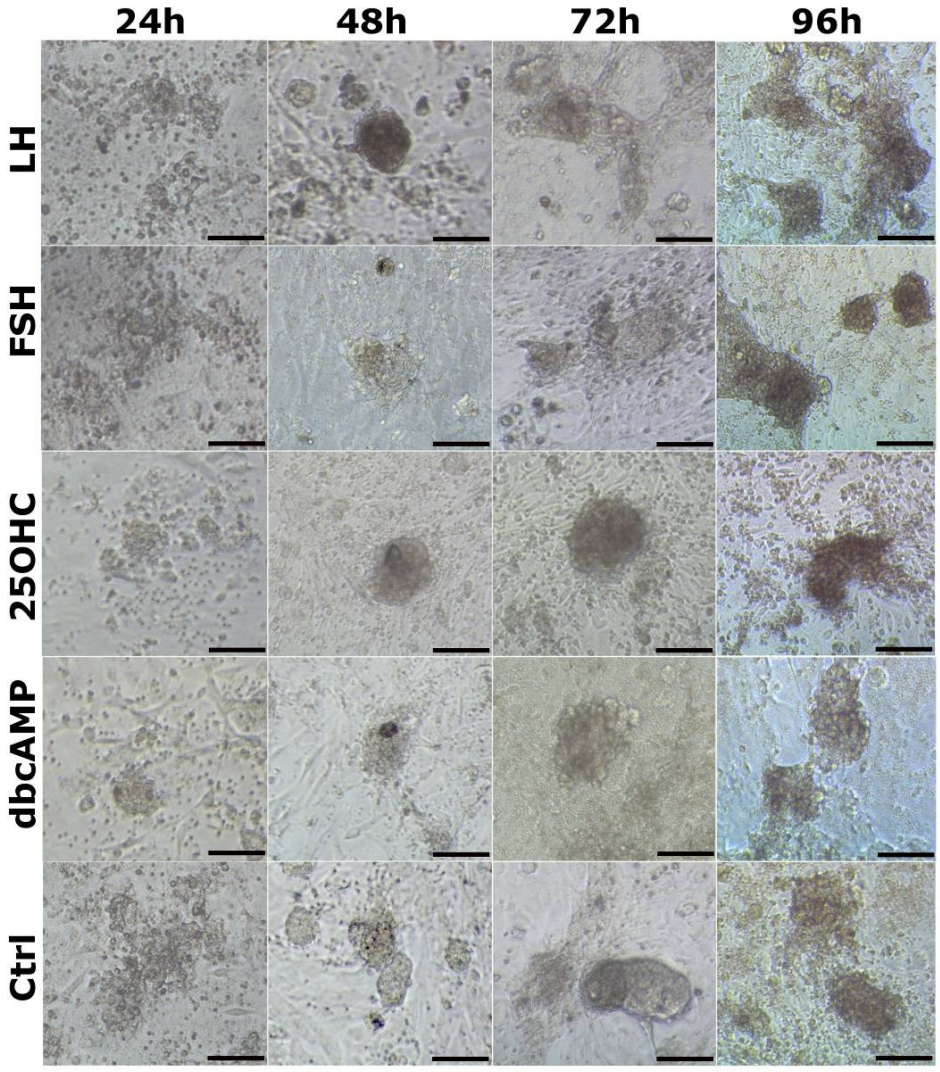


Figure 7.4. Photographs of cell cultured under LH, FSH, 25OHC and dbcAMP treatments at 24, 48, 72 and 96h. The panel shows the morphological status of cells cultured in Non-AD experiments during 96h of culture period. Scale is shown at 20µm.

7.4.4. Non-AD Progesterone RIA

Progesterone concentration in the culture media was analysed using RIA in Non-AD supplementation. Results are presented as cumulative concentration mean ± SEM (ng/ml) (Fig. 7.5). Progesterone concentration in LH, FSH and 25OHC showed no significant increase compared to the control group, however; there was significant increase in P4 production over time at 72h and 96h compared to 24 hours for all groups (*P*<0.05). Estradiol concentration was undetectable in cultures without androstenedione.

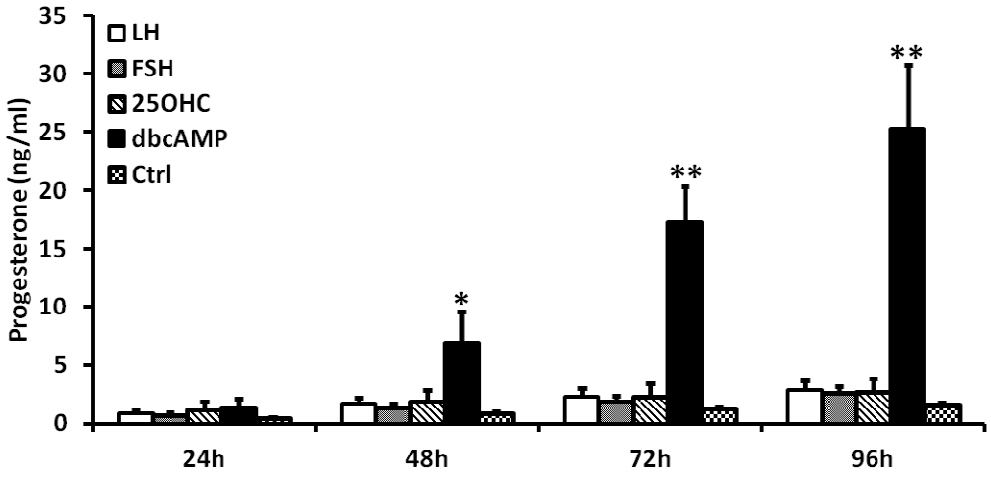


*Figure 7.5. Time course changes in progesterone production by in vitro cultured canine follicular cells. The graph shows the cumulative concentration of progesterone (ng/ml) over 96h of culture in LH, FSH, 25OHC, and dbcAMP and control group in Non-AD experiment. * = P <0.05; ** = P<0.0001.*

7.4.5. Non-AD Steroidogenic pathway mRNA expression

Primers were first tested in conventional PCR in order to confirm the specificity of the reaction (Fig. 7.6). The cDNA utilized was from ovarian cortical tissue mRNA extracted as the +ve tissue for designated genes.

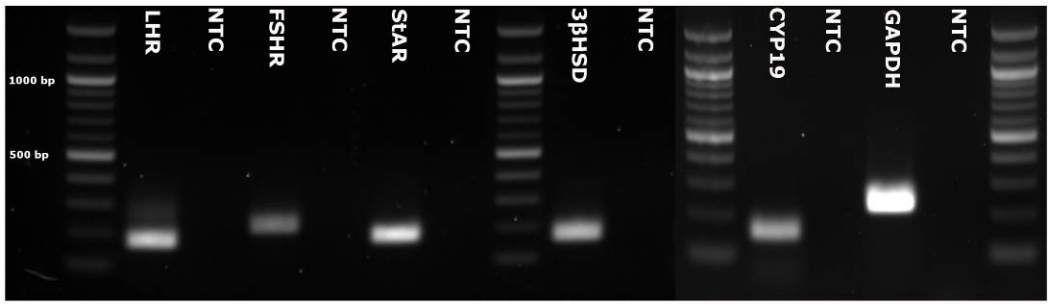


Figure 7.6. Conventional PCR products of canine primers setup. PCR products from the reaction of LHR, FSHR, StAR, 3βHSD, CYP19 and GAPDH primers with canine ovarian cortex sample as the +ve control, visualized on the 1.5% agarose gel. DNA ladder contained two bright bands marked as 1000 and 500bp. NTC= non template control for each primer was loaded on the lane to the right.

Relative real-time qPCR was carried out using canine GAPDH as the housekeeping gene, which had a stable expression among repeats and different groups (Levene's test, P = 0.2; one-way ANOVA P = 0.058). Normalized expression ratio of target genes in treatment groups were compared to the control as the calibrator. This analysis was carried out as an end point mRNA expression ratio after 96h of culture. After 96 hours of culture, there was no significant difference in the expression level of LHR, FSHR, CYP19 or 3βHSD. However, the treatment of dbcAMP increased the StAR expression significantly compared to LH, FSH and 25OHC (Fig. 7.7).

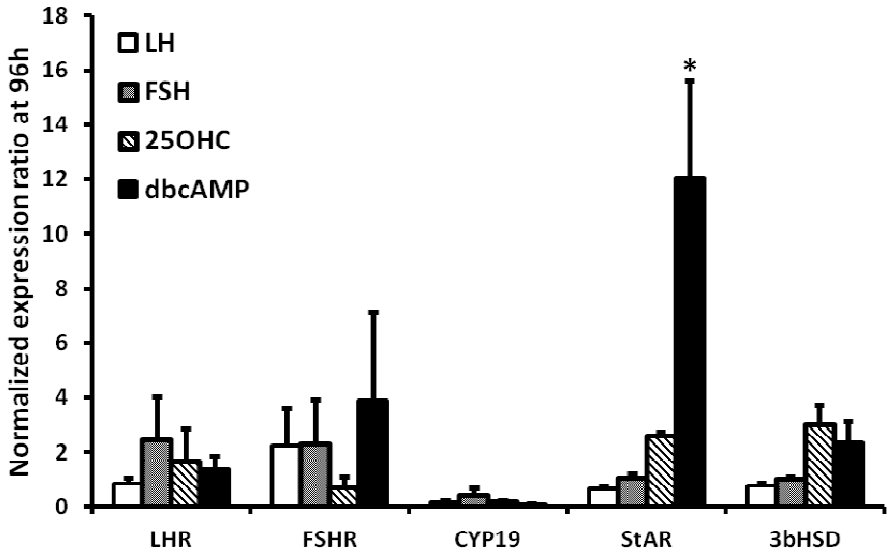


Figure 7.7. Expression of steroidogenic genes in canine follicular cells cultured in vitro. The graph shows the normalized expression ratio of LHR, FSHR, CYP19, StAR and 3ϐHSD in Non-AD experiment (LH, FSH, 25OHC and dbcAMP) against the house keeping gene (GAPDH) after 96h of culture. = P <0.05*

7.4.6. AD Progesterone and Estradiol RIA

Progesterone and Estradiol concentration in the culture media was analysed using RIA in cultures with androstenedione supplementation after 24h. The dbcAMP induced significant progesterone production in AD cultures from 72h onwards higher than other groups (Fig. 7.8). There was no significant difference in progesterone production between treatment groups at 24 and 48h. Results are presented as cumulative concentration mean ± SEM (ng/ml). (Fig. 7.8)

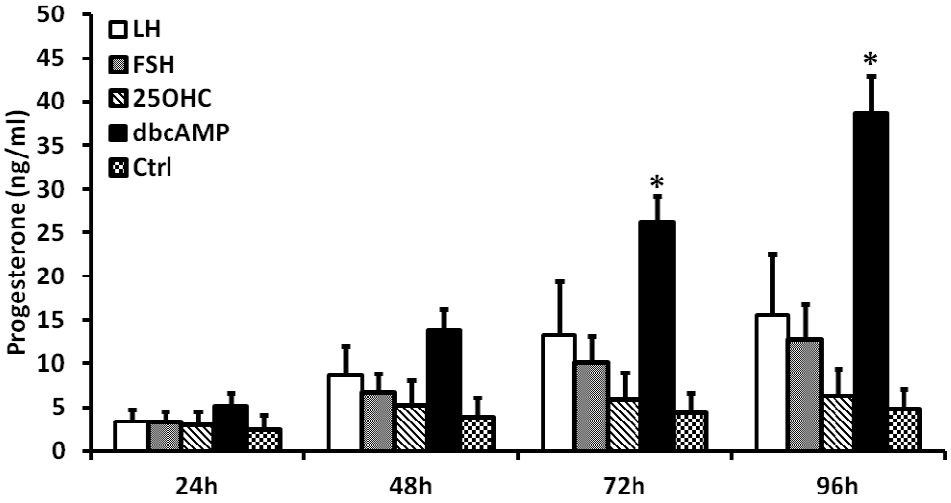


Figure 7.8. Time course changes in progesterone production by in vitro cultured canine follicular cells. The graph shows the cumulative concentration of progesterone (ng/ml) over 96h of culture in LH, FSH, 25OHC, and dbcAMP and control group in AD experiment. (= P <0.05)*

There was no detectable level of estradiol at 24h of culture (before the AD supplementation). After replacement of media with treatments containing 100nM AD estradiol concentration reached detectable levels at 48h onwards. There was a significantly higher estradiol production in the FSH+AD group compared to others at 72h onwards (Fig. 7.9).

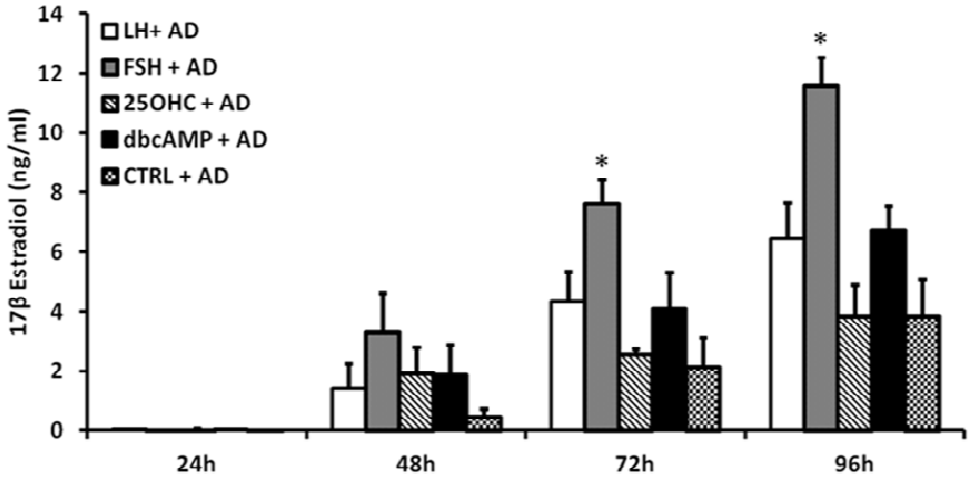


Figure 7.9. Time course changes in estradiol production by in vitro cultured canine follicular cells. The graph shows the cumulative concentration of estradiol (ng/ml) over 96h of culture in LH, FSH, 25OHC, and dbcAMP and control group in AD experiment. (= P <0.05).*

7.4.7. AD Steroidogenic mRNA expression

After 96 hours of culture, there was no significant difference in the expression level of LHR, FSHR, CYP19, StAR or 3βHSD. However, the expression of StAR gene was elevated in the presence of AD compared to other genes (*P* >0.05) (Fig. 7.10).

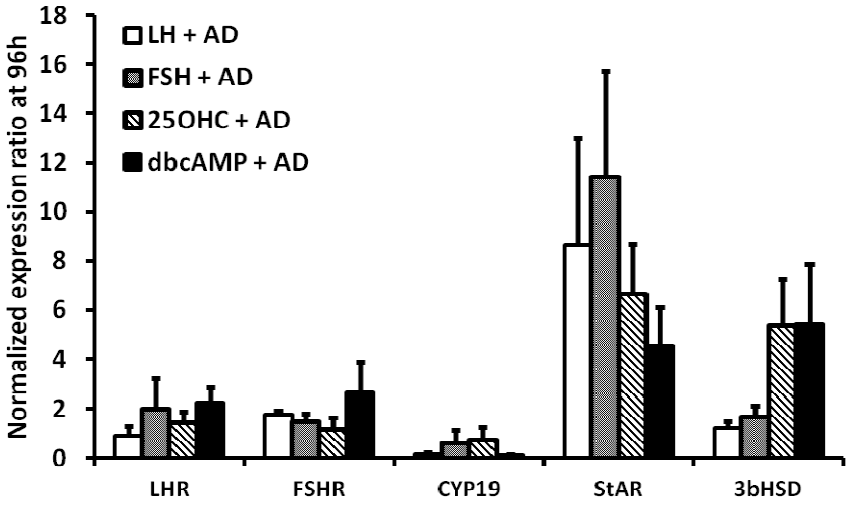


Figure 7.10. Expression of steroidogenic genes in canine follicular cells cultured in vitro. The graph shows the normalized expression ratio of LHR, FSHR, CYP19, StAR and 3ϐHSD in AD experiment (LH, FSH, 25OHC and dbcAMP) against the house keeping gene (GAPDH) after 96h of culture.

7.5. Discussion

7.5.1. Morphological characteristic changes of canine follicular cells during *in vitro* cell culture

This study showed successful extraction and culture of potential canine follicular cells as evidenced by morphological assessment and analyses of functional steroidogenic activity and presence of CYP19 aromatase. The increase in size and density of cell clumps from 24 to 96 hours indicated proliferation which may be comparable to other studies (Meidan *et al.* 1990, Pescador *et al.* 1999). Another morphological feature displayed in these cells is the characteristic branching and extension of projections from their centre. Other studies have reported these features in bovine granulosa cells during *in vitro* culture, particularly, increased frequency of these membrane projections and increase cell convexity (Kotsuji *et al.* 1990). The common view is that the granulosa cells become large luteal cells which demonstrate these membrane projections during differentiation (Kotsuji *et al.* 1990). The outgrowth behaviour of granulosa cells during luteinisation may play an important role in blood vessel recruitment (Redmer & Reynolds 1996,

Picton *et al.* 1998), paracrine signalling, transfer of nutrients and other important events needed for follicular maturation and steroidogenesis (Picton *et al.* 1998). The evident extending and branching of the presumptive cell clumps after 96 hours in this study may suggest such events, as well as, cell to cell interaction and inter-exchangeable cellular matrix. However, there is evidence in some models that granulosa cells retain their capacity to proliferate *in vitro* (Peluso *et al.* 1995), even after luteinisation (Pescador *et al.* 1999), suggesting that these events are not mutually exclusive. The nature of the proliferative process in differentiated luteal cells is unclear, however continuation of growth is dependent on FSH (Scaramuzzi *et al.* 2011).

The cell culture images reflect the importance of FSH; as this group, along with the LH treatment group, showed noticeable increases in cell clumps and branching compared to the other treatment groups (25OHC & dbcAMP) (Fig. 7.4). It has been shown *in vitro*, that supplementation of FSH exerts a positive influence on canine follicle growth and steroidogenesis (Serafim *et al.* 2010, Songsasen *et al.* 2011).

7.5.2. Steroidogenic Characterization along with Gene Expression

One of the key findings in this study revealed that at 72 hours the production of P4 increases significantly regardless of the presence/or absence of AD. This may suggest the point at which luteinisation begins to occur *in vitro* for canine follicular cells. It is known that plasma P4 increases through oestrus from a baseline of 2-4ng/ml at the time of LH surge, to 4-10ng/ml, and finally reaching 15-90ng/ml 15-30 days after the LH surge (Concannon 2011). This study has demonstrated values well within the post-LH surge range from potential follicular cells cultured *in vitro*. With the treatment of dbcAMP, these levels were achieved, with significance, with and without AD supplementation by 72 hours (Fig. 7.5 & 7.8). This is further supported by the significant expression of StAR by this group compared to its expression of CYP19 coupled with low estradiol production in the non-AD experiments. The significant difference between the

expressions of StAR and CYP19 in the dbcAMP treatment of non-AD experiment, as well as, the increased levels of P4 by 72 hours and undetectable E2 production, possibly demonstrates the shift from E2 production to P4 dominated production; imitating the possible similar events which occur during the luteinisation.

The significant increases in P4 and StAR from the primary cell culture of canine follicular cells show responsiveness to physiological and pharmaceutical stimuli. From the treatment of dbcAMP, it is evident that this group behaves differently. During steroidogenesis, cAMP acts directly on PKA, which initiates transcription of enzymatic genes. Thus by supplying this direct link to the activation of PKA, with the treatment of dbcAMP, the cultured cells promoted the transcription of StAR, which drove the production of progesterone over estradiol.

When the cells further treated with AD, which may have imitated potential thecal androgen paracrine signalling, E2 production reached detectable levels, particularly from the FSH and LH groups. The addition of AD to the cultures may indirectly suggest the granulosa cells were isolated with minimal theca cell contamination. As there was undetectable E2 production without the addition of AD; therefore, there are presumably no remarkable endogenous AD supply in non-AD cultures. The E2 production in the FSH treated cells was also significantly higher than the rest from 72h (Fig. 7.9). The compilation of these findings hints at a sequential temporal luteinisation profile, but may be further supported by the evidence shown with the addition of AD. While the production of P4 is retained with the addition of AD, the production of E2 became relevant. This may suggest that the cells' machinery could be utilized for both P4 and E2 and external AD could effectively interfere with the process of luteinisation. To further support this, characterization of the cells for thecal/granulosa/luteal specific markers is required.

This study has shown that E2 production from these cultured cells retain this ability for up to 96h, thus CYP19 activity is maintained. It is most notable in

groups FSH and LH at 72 hours (Fig. 7.9). It has been shown, that the capacity for androgen to sensitize granulosa cell response to FSH declines with follicular maturity (Hillier & Tetsuka 1997). However, in the early stages of follicular growth, there is evidence that androgens directly stimulate this growth (Weil *et al.* 1999) and increase granulosa responsiveness to FSH (Hillier & Tetsuka 1997, Walters *et al.* 2008). This study showed an increase in response with the treatment FSH supplemented with AD over time. From this, perhaps a case can be made for the importance of thecal cell paracrine signalling with regards to follicular conditioning. The causality chain of this achievement in canine follicles remains unclear.

7.5.3. Conclusion

This study has demonstrated an establishment of presumptive follicular primary cell culture from canine ovaries with steroidogenic machinery that is responsive to both physiological and pharmacological manipulations. Further clarification of potential follicular/stromal cell populations and characterization of their secretory profile require future studies in pure canine granulosa and theca primary culture. The pure population separation and characterization of canine granulosa and theca cells is of importance due to several reasons. Firstly the modelling of their stable culture or luteinized model could be very helpful in the field of ovarian cancer research. The preovulatory lutenization is commonly shared feature of canine granulosa cells with human and rodents counter parts (Chastant-Maillard *et al.* 2011). Shared hormonal profile enables this animal model to be utilized in medical reproductive research. Secondly the thoroughly investigated secretory profile of these cells could be utilized in improvement of oocyte co-culture systems in gaining knowledge about their folliculogenesis and oogenesis. The communication between COCs and follicular cells and its impact on oocyte competency (final oocyte programming prior to ovulation) could also be of great scientific value. The corner stone of hormonal treatments in maturation media used for canine oocyte maturation could be based on the response of their follicular cells to physiological and pharmaceutical compounds.

Thirdly more light could be shed on unique physiological characteristics of canine reproduction by analysis of RNA transcriptome and proteomics of pure isolated cell population of canine ovarian cortex.

Results presented in this section postulated:

- The culture of canine presumptive follicular (granulosa, theca) cells is possible through mechanical extraction and bedding in 96 well plates.
- The morphology of FCs in culture takes effect from hormonal and synthetic substances.
- Under certain circumstances (72h culture without AD for progesterone and from 24h culture with AD for Estradiol) the steroidogenic machinery of this primary cell culture in functional at physiological levels and could be manipulated to sustain significant production of E2 & P4.

8. Summary and General Discussion

Since 1960's assisted reproductive technologies in canine species have been under improvement to reach applicapable perfection (Bartlett 1962, Guraya 1965, Wales & White 1965).These early studies pointed out the outstanding differences between canine gametes and other domesticated animals such as cattle, sheep and pigs (Holst & Phemister 1971, Mahi & Yanagimachi 1976, Archbald *et al.* 1980, Tesoriero 1981, Tesoriero 1982). Its note worthy that in cattle, sheep and pigs the process of oocyte collection from hundreds of ovaries provides us with the elite oocytes (follicular size and competency) from each animal resulting in high maturation and embryonic development rates. Logistical obstacles in canine studies such as this one, limit oocyte population to the ones collected from 1 bitch at a time (single pair of ovaries). To normalize and compare the success rate of these IVM systems the number of maturated oocytes should be divided by the number of animals (pairs of ovaries) utilized in order to obtain the efficiency ratio. As an example a batch of COCs collected from bovine ovaries come from aspiration of most fit (140 COCs; 2-8mm follicle size) follicles from 100 ovaries (50 cows) in each abattoir batch. These collected COCs are the one with the highest chance of resuming their meiosis up to MII from each animal. Considering 85% MII maturation rate of these oocytes after 24h of maturation (119 COCs / 140 total) the MII matured oocytes from each cow is (119/50 =2.38 [~2.5] MII oocyte per cow). In this study from an average of 70 COCs obtained from a single bitch, 30% of oocytes resumed meiosis up to MII (~20 MII oocytes per bitch).

Within the course of these studies attempts were made in order to establish an optimized *in vitro* maturation system for canine oocytes during the extended culture period. These endeavours resulted in an IVM method characterized by a chemically/hormonally defined maturation media, optimized oxygen concentration of culture incubator and tuned duration of culture respectful of nuclear maturation status. Besides an effective and accurate oocyte nuclear staining method was designed during the setup phase of this PhD which provides clear chromatin visualization for nuclear assessment of the oocytes.

Effects of Caffeine as a non specific PDE inhibitor on nuclear and cytoplasmic maturation of canine COCs were also studied. Moreover; several hormonal treatments (e.g. IGF-1, EGF, pGH, Melatonin and Foetal bovine serum) were utilized in order to improve the

nuclear maturation rate of the oocytes in the previously defined time frame. For assessing the developmental potential of oocytes, parthenogenetic activation and *in vitro* fertilization with chilled semen were conducted. Studying the canine ovarian cortex primary cell culture, the steroidogenic machinery of extracted cells was briefly investigated to elucidate the preovulatory lutenization and steroidogenic profiles.

During the studies presented within this PhD very first elements required for *in vitro* maturation of canine oocytes was attended and investigated. Staining and nuclear staging of canine oocytes were optimized to maximum efficiency using fluorescent microscopy. Low oxygen culture conditions have proven to be the optimal choice for canine oocyte maturation (72h). The improving effect of caffeine pre-treatment on oocytes was confirmed by nuclear and cytoplasmic maturation markers. Presence of growth hormone and melatonin within the maturation media beside LH, FSH, E2 and P4 showed tremendous improvement over MII maturation rates and reduced degeneration. However; parthenogenetic activation and IVF of *in vitro* matured canine oocytes did not result in production of blastocysts, thus require further elucidations. The primary culture of canine presumptive follicular cells and their maintenance up to 96h were carried out successfully *in vitro*. Steroidogenic profile of latter cells was functional and also responsive to hormonal/chemical stimuli.

8.1. Summary of results

Experiments conducted in chapters 3-7 and their outcomes are summarized in the Table 8.1.

Table 8.1. Summary of all experiments carried out during this PhD

No. Exp.	No. COCs	Treatment	Outcome
3.3.1	5753	Effect of age and weight	Age ↓ = COC↑
3.3.2	1488	Cumulus expansion 52h IVM 24h vs. 52h	No expansion
3.3.3	118	52h Oxygen LO vs. HO	LO = ↑MR
3.3.4	460	52 vs. 72 vs. 84 Low oxygen	72h optimal
3.3.5	130	ROS (H2O2) Oxygen Comparison	HO = ↑ROS
3.3.6	340	ROS repairing enzymes	HO = ↑oxidative stress
3.3.7	201	Vit. E vs. Oxygen comparison	Still ↑ Degen
4.3.1	192	Caffeine (12+60) 72h Biphasic IVM	↑MII
4.3.2	234	Caffeine (12+72) 84h Biphasic IVM	Not improved
4.3.3	720	Cytoplasmic maturation markers Biphasic IVM (72h)	AKT no change - MAPK1&3 ↑
4.3.4	308	MPF kinase activity Biphasic IVM (72h)	MPF ↑
4.3.5	168	BCB screening	↑immature oocytes (BCB-)
5.3.1	226	IGF-1 effects on IVM	Not improved
5.3.2	212	EGF effects on IVM (replacing LH)	↓MII
5.3.3	395	FBS enrichment of maturation media	↓MII + ↑ Degen
5.3.4	269	Porcine GH effects on IVM	100ng/ml = ↑MII
5.3.5	295	Melatonin effects on IVM	100nM = ↑↑MII
5.3.6	60	MTNR-A1 expression (ICC)	↑Oocyte and CC
6.3.1	129	Parthenogenetic activation	↓cleavage
6.3.2	270	ESS 2% in IVF	Not improved
6.3.3	430	Pronase digestion + ESS 2%	↑cleavage
6.3.4	235	EBS 10% IVM - 2% IVF + Pronase	Not improved
7.3.1	-	Ovarian cortical culture morphology	Cyp19 +ve clumps
7.3.2	-	Steroidogenic profile	+E2/+P4 ; ↑72h
Total COCs	>9896		

8.2. Canine oocytes optimized culture conditions (IVM setup)

The *in vitro* maturation process for canine oocyte should retrospectively compensate for two main stages of change:

1. The proestrus and pre-ovulatory stage hormonal changes (9 days on average)

2. Early estrus hormonal changes and oviductal transition changes (5 - 9 days on average)

Hormonal supplementations, media components, gas composition and protective reagents during the extended culture time required for maturation of canine oocytes are of great importance (Luvoni *et al.* 2005). Studying the nuclear maturation stage of the canine oocyte using a quick and efficient staining system is a challenging step. Unorthodoxically high lipid content of ooplasm in canine oocytes and highly integrated ZP / cumulus cell complex are main obstacles in nuclear visualization of canine oocytes. Limited number of oocytes collected from each pair of ovaries (60 - 80 COCs) and extended duration of culture (72h) were the main logistical obstacles throughout the studies presented in this thesis. Discriminative nuclear staging of canine oocyte have been a difficult task to undertake according to previous studies (Reynaud *et al.* 2006). The staining method established in chapter 3 resulted in less than hour, efficient and distinctive nuclear content visualization method.

Low oxygen (5%O_2, 5%CO_2, and 90% N_2) gas composition provides optimal culture condition in combination with serum free maturation media for the extended culture of canine COCs up to 72h. Meiotic progression of oocytes (GVBD- MII) is significantly higher in low oxygen environment. Moreover; the H_2O_2 level (ROS) produced during the IVM culture was reduced in low oxygen culture oocytes compared with high oxygen (in air) cultures. The higher oxidative stress in oocyte can result in meiotic retardation of canine oocyte specifically accompanied by up-regulation of ROS repairing enzymes such as SOD1&2, GPX1,

GSR and CAT. This oxidative pressure had effects on nuclear maturation of oocytes, which was not compensated even in the presence of vit. E as an antioxidative compound. However; 100 µM vitamin E in high oxygen concentration reduced the degeneration rate significantly compared to the control counterpart.

8.3 Effects of caffeine in biphasic in vitro maturation of canine oocytes

Considering the fact that meiotic resumption is a phenomenon triggered around and close to the pre ovulatory LH surge (Mehlmann 2005, Rodrigues *et al.* 2009b, Tripathi *et al.* 2010), a biphasic IVM system was hypothesized in the 4th chapter. Changes in the level of cAMP within the COC complex and downstream molecular changes following this event was the corner stone of this design. Biphasic *in vitro* maturation systems are relatively new methods described in murine and bovine ART (Albuz *et al.* 2010). The sharp increase in the cAMP concentration within the oocyte and cumulus cells as a consequence of gonadotropic induction has been mimicked using cAMP modulators (Fig. 8.1). In this study caffeine as a non selective PDE inhibitor which has proven to reversibly disrupt the PDEs activity and maintain cAMP concentration, was applied. Results showed that 12h pre-treatment of COCs with 10mM caffeine has significant improving effects on MII maturation rates of canine oocytes by the end of the culture period. This temporal and relative increase of cAMP in the caffeinated oocytes also resulted in better cytoplasmic maturation profile compared to the control group. MPF catalytic subunit, P^{34}CDC2 kinase with the help of its regulatory subunit, Cyclin B, regulates the cell cycle in eukaryotes (Tripathi *et al.* 2010). Meiotic progression in mammalian oocyte is specifically regulated between GVBD and first metaphysial plate formation and at MII stage via MPF kinase activity (Campbell *et al.* 1996). Activation of MPF (dephosphorylation of P^{34}CDC2 and Cyclin B) is also promoted via phosphorylation (activation) of MAPK1&3 directly (within the oocyte) and indirectly (through cumulus cells and

connexin tight junctions [CX]) (Luciano *et al.* 2004, Luvoni *et al.* 2006b). Compared to previous reports (Suzukamo *et al.* 2009) the MPF kinase activity and MAPKs phosphorylation was increased through 72h of maturation with time point differences. Caffeine pre-treated oocyte showed significant increase in MPF activity at 12h (just after caffeine wash; high phosphorylation of MAPK1) and 48h (with high MAPK3 phosphorylation). There was no meaning full change in the phosphorylation pattern of AKT during 72h of culture.

The success of biphasic maturation system with caffeine pre-treatment of the oocytes was elucidated through significantly higher MII percentage compared to the control group. The biphasic maturation system considering the delayed meiotic progression of canine oocyte could be the perfect match for an optimized IVM protocol. As described in Fig. 8.1, the ensemble of factors and hormones programming the canine oocytes prior to ovulation differ from oviductal elements. Physiology of microenvironment surrounding the COCs and biological peculiarities, changes by ovulation. Therefore COCs are exposed to different hormonal and chemical compounds during the oviductal transition compared to the pre-ovulatory follicle. Canine oocytes are either pre-programmed to resume meiosis within 3-5 days of oviductal transition or there are specific mechanisms capable of tweaking the speed of these nuclear changes (Rodrigues & Rodrigues 2010). A clear time course of meiotic progression is of great value in order to narrow down the molecular pathways and biological agents responsible for this seemingly retarded resumption (compared to other species).

8.4 Effects of hormonal supplementations on nuclear maturation of canine oocytes

Apart from biphasic maturation protocol, several hormonal supplementations were introduced to the maturation media in the experiments of chapter 5. Following the literature mining, Insulin like growth factor (IGF-1), epithelial growth factor (EGF), recombinant porcine growth hormone (PGH) and melatonin

(MTN) were added to the maturation media one at a time. The basic maturation media contained LH, FSH, E2 and P4 considering the plasma concentration at peri ovulation and previous studies. Addition of IGF-1 and replacement of LH with EGF didn't improve the nuclear maturation rates compared to the control group. Supplementation of maturation media with 10% FBS had even significant detrimental effects on MII and degeneration rates. However, additions of PGH at 100-1000ng/ml improved the nuclear maturation rate and significantly increased MII percentage. These results were in agreement with previous reports (Chigioni *et al.* 2008). Moreover; the supplementation of maturation media with increasing concentration of MTN led in to drastic increase in the number of MII matured oocytes. MTN at 100nM concentration promoted nuclear maturation of canine oocyte up to 32.3 % ± 6.4 on average. To the best of my knowledge there is no previous report of MTN effects on canine oocyte maturation. The Immunolocalization of MTNR-A1 within the COCs was confirmed using specific monoclonal antibody against this receptor. The improving effects of MTN could also originate from potent antioxidative properties of this compound. Considering the previously reported predisposition of canine oocytes to oxidative stress due to lack of sufficient protecting mechanisms such as glutathione (GSH *in vitro* (Kim *et al.* 2007) and high lipid content, (Wesselowski 2008, Silva *et al.* 2009, Lopes *et al.* 2011, Salavati *et al.* 2012) controlled and adequate presence of an antioxidant could have improving effects on nuclear and cytoplasmic maturation of canine oocytes. The underlying molecular pathway and chemical events of this improving effect of melatonin on canine oocytes is to be investigated further.

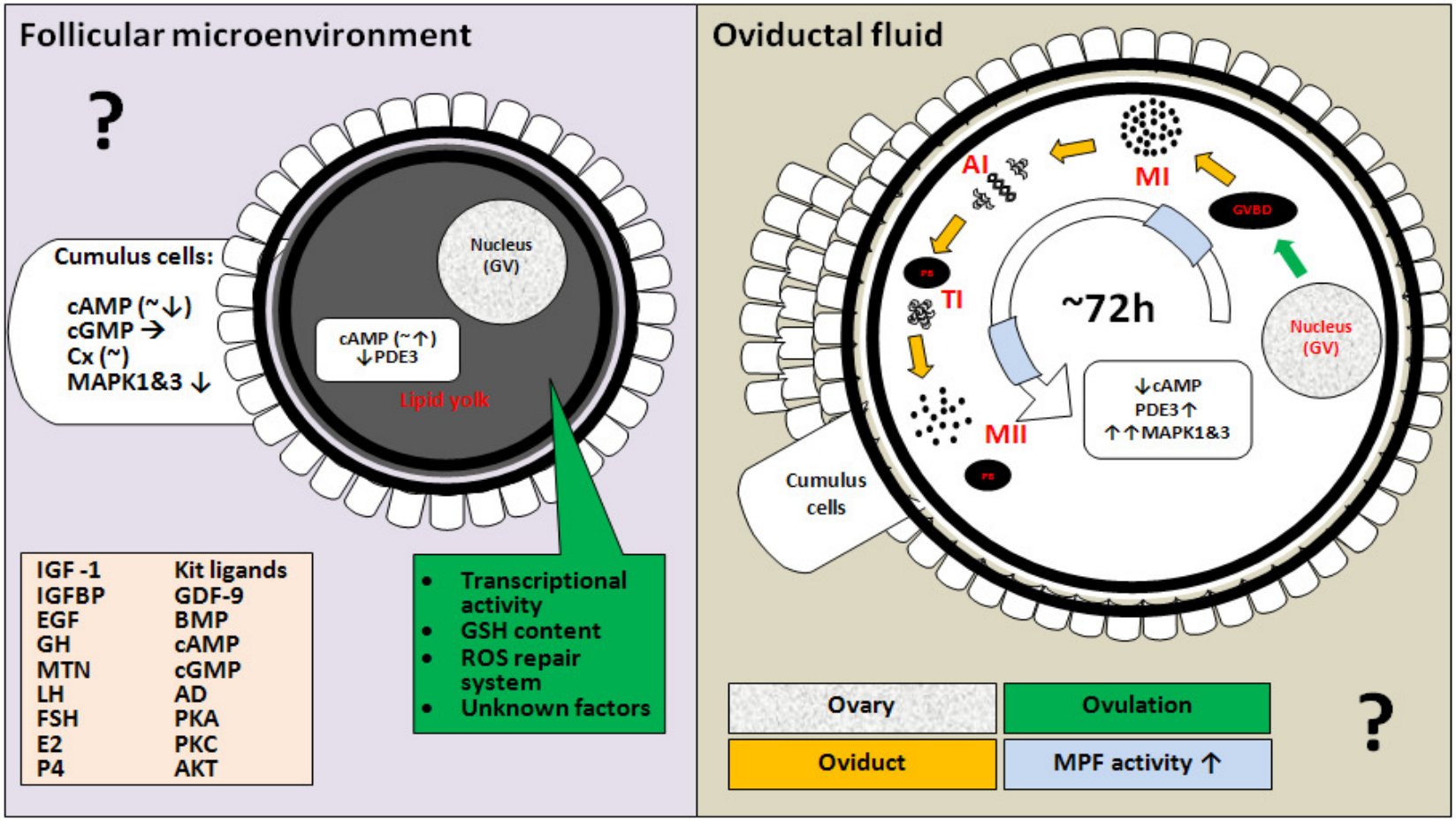


Figure 8.1 Schematic illustration of pre and post ovulation microenvironment in which canine COCs maturate.

The figure shows the two separate microenvironments that canine COCs experience before and after ovulation. ↑ = increase; ↓ = decrease; → = secret from CCs to oocyte; ~ = maintained; IGF-1 = Insulin like growth factor; IGFBP = IGF binding proteins; EGF = Epithelial growth factor; GH = Growth hormone; MTN = Melatonin; LH = Luteinizing hormone; FSH =Follicle stimulating hormone; E2 = Estradiol; P4 = Progesterone; GDP-9 = Growth differentiation factor 9; BMP = Bone morphogenetic protein; cAMP = cyclic adenosine mono phosphate; cGMP = cyclic guanidine mono phosphate; AD = adenylate cyclase; PKA = Protein kinase A; PKC = protein kinase C; AKT = Protein kinase B (PKB[PI3K/AKT]); MPF = maturation promoting factor (P^{34}CDC2 + CyclinB); MAPK1&3 = Mitogen activated protein kinase 1 & 3[ERK2&1]; GV = germinal vesicle [prophase I]; GVBD = germinal vesicle break down; MI = metaphase I; AI = anaphase I; TI = telophase I; MII = metaphase II; PB = polar body; PDE3 = phospho diesterase 3.

8.5 Parthenogenetic activation and in vitro fertilization of canine oocytes

Developmental potentials of *in vitro* matured canine oocytes compared to other species such as mouse (Albuz *et al.* 2010), pigs (Sirard *et al.* 1993), cattle (Marei *et al.* 2009) and sheep (Loi *et al.* 1998), is very low (Luvoni *et al.* 2005, Luvoni *et al.* 2006a). Parthenogenetic activation of second polar body in canine oocytes have been previously carried out using electrical (Lee *et al.* 2009) and chemical (Song *et al.* 2010a) stimulation. Pronuclear formation and cleavage of canine *in vitro* matured oocytes were very low in both approaches. In our study the activation method was evaluated with a bovine positive control with resulted in cleavage of 1/3 of matured oocytes on average (11.0% ± 1.9 blastocyst formation). Canine oocytes differed in cleavage and embryonic development which required further investigations. The induction of Ca^{2+} influx and subsequent molecular events leading to activation of the second polar body should be exclusively tailored for canine oocytes.

The usage of chilled dog semen in the swim up process and utilization of motile sperm ($2x10^6$/ml conc.; 16h of co-incubation) for IVF was also studied in this thesis. The fertilization led to detectable, yet impaired embryonic development

only in the presence of 2% ESS and partial digestion of ZP prior to IVF. Although cleavage rate in IVF exceeded the PA outcomes, it was still below 10% of the matured population. The reduction of ZP thickness and its effect on the penetration rate (cleavage rate consequently) using partial digestion, elucidates one of the obstacles within the fertilization process of the canine oocytes. It appears that fertilization, sperm penetration and fusion of sperm head to the oocyte require thorough further optimizations.

8.6 Primary cell culture of canine follicular cells and their steroidogenic profile

Culture of canine follicular cells as evidenced by morphological assessment and analyses of functional steroidogenic activity was investigated in this study. The extraction and primary culture of these cell types were addressed due to lack of available literature and the indispensible role of granulosa/theca cells in pro-estrus programming of the oocyte (Abdel-Ghani *et al.* 2012). The knowledge of morphology and steroidogenic profile of these cells could provide basic information about canine ovarian cortex and its possible influence on oocytes during folliculogenesis (Fig. 8.2). These cultures contained follicular and somatic cells colonies of which specific CYP19 (aromatase) positive clumps were identified using Immunocytochemistry. Latter clumps were considered to be the source of estradiol production. Production of P4 in these cultures showed a significant increase at 72h regardless of the presence/or absence of AD. Responsiveness to physiological and pharmaceutical stimuli was observed through increase in P4 production and expression of specific genes (StAR). Further treatment of the cells with AD also promoted E2 production to detectable levels by RIA. Result of control groups, suggested that canine follicular cells retain steroidogenic machinery necessary for production of both P4 and E2 up to 96h. These results could be the corner stone for establishment of canine follicular cells *in vitro* lutenization model and its contribution to pre-ovulation programming of the oocytes.

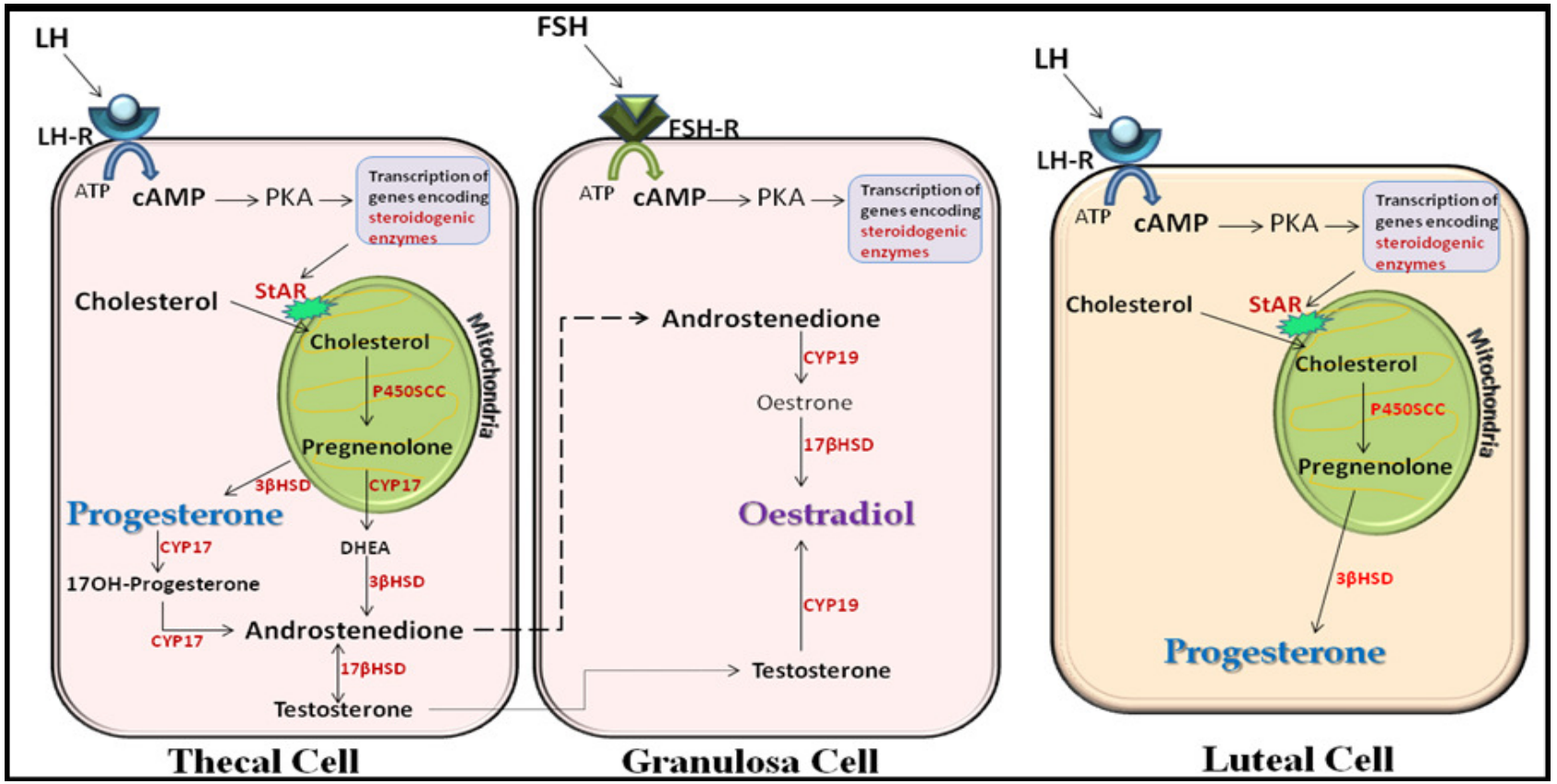


Figure 8.2 Steroidogenesis in Theca, Granulosa & Luteal Cells. The graph illustrates a schematic flowchart of steroidogenic pathways between granulosa/theca cells as a combined unit and luteal cells separately. LH = Luteinising hormone; LH-R = LH receptor; FSH = Follicle stimulating hormone; FSH-R = FSH receptor; ATP = adenosine tri-phosphate; cAMP= cyclic adenosine mono-phosphate; PKA= protein kinase A; StAR = Steroidogenic acute regulatory protein; P450SCC = cytochrome oxidase P450 side chain cleavage protein; CYP17 = Cytochrome P45017; 3ϐHSD = 3 beta-hydroxysteroid dehydrogenase; 17ϐHSD = 17 beta-hydroxysteroid dehydrogenase; CYP19 = Cytochrome P45019.

8.7. Future work

Multiple enterprises should be addressed for the further studies in order to improve the IVM/IVF/IVC techniques for canine oocytes:

1. Super ovulation programmes are not successful in breeder dogs due to unknown hormonal interactions during transition from diestrus to anestrus and also from anestrus to estrus (Luvoni *et al.* 2005, Luvoni *et al.* 2006a). Regarding the fact that *in vivo* in each oestrus cycle 6-12 oocytes are ovulated to the bitch's oviduct and not all of them result in conception, realistic expectations should be drawn (Reynaud *et al.* 2009). Average COCs collection of 60 - 70 from each bitch and approximately 30% MII maturation rate obtained in the studies presented in this thesis could be the extreme optimization for nuclear development of canine oocytes. Attempts for *in vitro* production of canine embryos have been strongly affected by the quality of matured oocytes. However; limitation of developmental potentials in canine oocytes should not only be solely penalized on poor cytoplasmic and nuclear maturation of these cell types. Comparison of IVF/PA outputs in these species with other mammals such as cows, sheep and pigs must be based on the efficiency of IVM systems per animal and unique considerations required for optimized canine oocyte/sperm interactions.

2. The unique histological/physiological specifications of canine ovaries and follicles are still unexplained and could be of great value in ART establishments. The evolutionary strategies preserved in these species should be thoroughly studied such as:

- Pre-ovulatory lutenization of granulosa cells accompanied by high levels of progesterone in follicular fluid and plasma even prior to ovulation,
- Ovulation of oocytes at prophase of first meiosis instead of metaphase II,
- Meiotic resumption of oocytes much slower than ruminants/rodents/human within the oviductal environment instead of the follicle,

- Superior high lipid content within the ooplasm compared to other mammals,
- Embryonic uterine translocation and delayed implantation tailored to season or circadian cycles,
- And existence of multiovular follicles and the mechanism of dominance/selection within those follicles.

3. The genomic and proteomic analysis of canine follicular fluid and follicular cells beside characterization of secretory profile of the oviductal segments (infundibulum, ampulla and isthmus) could narrow down key role molecules involved in *in vivo* maturation and fertilization process. Above all, the epigenetic investigations of COCs and their cellular communication network within the follicle and later in contact with oviductal cells could shed light on the conundrum of delayed meiotic resumption and *in vivo* fertilization window of these oocytes. RNA interference (e.g. miRNA, regulatory RNA [rRNAs]) and biological secretory profile of the oviduct (e.g. Heat shock proteins [HSPs], Osteopontin, Hyaluronic acid and etc.) might have indispensible influence on oocyte maturation or oocyte-sperm interaction.

4. The ensemble of biological changes during the 9 days of pro-estrus and 5-7 days of oviductal transition of canine oocytes (during estrus) is an utterly difficult concision to fit in to 72-96h of *in vitro* culture. The biphasic approach in canine oocyte maturation using caffeine as a modulator of cAMP pathway seems to be an efficient model to be further characterized. Addition of PDE inhibitors to the washing media and collection buffers can further improve the preservation of nuclear stage *in vitro* by preventing the drop in cAMP concentration early enough. Profound biochemical analysis of cAMP concentration within the COCs and oocyte can shed more light to the causality chain of this phenomenon.

5. Drastic improvement of Melatonin and Growth hormone treatments should be further analysed through cellular and molecular studies. Hormonal cascade leading into resumption of meiosis in canine oocyte trajects involvement of

GH/GHR (JAK2-cAMP), IGF1 bioavailability and EGF (down-stream gonadotropic induction of meiotic resumption) molecules. In studies concerning PGH during this PhD the effect of this hormone was only analyzed through its end point impact over nuclear maturation rates. The activation of GHR through exposure of oocytes to PGH in canine oocytes should be studied in combination with its effects on secretion of IGF-1 from cumulus cells. The promoting effect of GH on the oocyte nucleus could be transduced through IGF-1 expression in collaboration with FSH/LH activation of AREG, EREG and BTC (EGFs) (Izadyar *et al.* 1996, Bevers & Izadyar 2002). This molecular pathway leads to activation of MAPK1&3 and progress of cell cycle toward completion of first meiosis and maintenance of the oocyte at MII via high levels of MPF activity. Besides the interactive collaboration between GH and IGF1 via the cAMP activated/dependant kinases, the bioavailability of IGF-1 should be studied via characterization of IGFBP 2,4,5 secretory profiles within the follicle and their involvement in maintenance of meiotic arrest adjacent to high concentrations of cAMP within the oocyte itself.

Melatonin beside hormonal interaction can improve culture of canine oocytes through its receptor via antioxidative influence. The changes in the oxidative stress levels within the COCs under MTN treatments could be a confirming indicator of this fact. Antiapoptotic (BCL2, BCLw up-regulation or down regulation of BAD, BAK, BAX, IAP, Caspase family and Oxo-dG [OGG1]) and ROS repairing marker (SOD1&2, GPX1, GSR, CAT and GCS) mRNA expression levels could also be studied and analyzed under MTN treatments. The MTN receptors (MTNRA1 and B1) and their involvement in regulation of progesterone production in follicular cells should be further investigated. The latter clarification better be carried out, aiming MTN steroid (P4) regulatory function in relationship with preovulatory lutenization of canine granulosa cells.

6. Natural fertilization window of canine oocytes in terms of sperm penetration, sperm head fusion and male pronucleus decondensation/formation must be

studied. The changes required in the surface of ZP in order to facilitate sperm recognition and penetration in canine oocytes is still unknown. The zona maturation alongside changes in the cellular network of COCs should be investigated. Basic knowledge concerning sperm recognition by ZP proteins (ZP2, ZP3 and connexin family) and the chemical changes in the matrix of ZP such as pore size, biological conformation and protein profile would help improvement of the IVF systems. The sperm capacitation and the suitable media required for this process should be further studied in order to have optimal *in vitro* penetration rates. The oocyte cortical granules and their compartmentalization/rearrangement during the fertilization window could also be of great importance. The oocyte sperm interaction should be thoroughly studied *in vitro* to clarify conditions required for optimal IVF methodology. The further elucidation of this fertilization window provides discriminative criteria for separation of competent MII oocytes from aged (unsuitable for fertilization) or developmentally incompetent oocytes.

7. The induction of Calcium wave after chemical PA should be validated and confirmed. The activation protocol should be further optimized regarding the duration of calcium influx induction and protein kinase inhibition periods. The cortical reaction, ZP hardening, prevention of 2^{nd} polar body extrusion and pronuclear formation would be the main concerns.

8. Sperm nucleus decondensation and GSH content of the cultured oocytes can be improved through usage of reducing agents (e.g. Dithiothreitol [DTT]) to accelerate the reduction of sperm chromatin di-sulphide bonds and male pronuclear formation.

9. Tight junctions between cumulus cells and oocyte such as connexin family should be further studied to understand the communicative behaviour of these cells. Investigation of this matter beside the expression profile of hyaluronic acid and extra cellular matrix can elucidate limited expansion and mucification of canine COCs.

10. Separate primary culture of granulosa and theca cells via Flowcytometric cell sorting can lead in to pure primary population of canine follicular cells for establishment of lutenization models.

11. In case of successful *in vitro* blastocyst production in canine species using PA or IVF, retrieval of embryonic stem cells from inner cells mass would be of great value in animal and human disease modelling. Reprogramming of embryonic stem cells in to engineered tissues or stable differentiated cell lines is of empirical medical importance in biomedical research.

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Appendices

Appendix 1:

THE ROYAL VETERINARY COLLEGE
UNIVERSITY OF LONDON

Information sheet for owner and separate consent form

In vitro maturation of canine oocytes

Dr. Ali Fouladi, DVM, MSc, PhD
Mr. Mazdak Salavati (PhD student)

Reproduction Research Group, The Royal Veterinary College, Hawkshead Campus, Hawkshead Road, AL9 7TA. Email: afouladi@rvc.ac.uk

Background
We are undertaking a research project to study oocyte (egg) maturation in dog. Immature eggs are present in all ovaries (whether from mature or prepubertal young bitches) and these can be obtained from ovaries after they have been removed at routine sterilisation (spaying). The ovaries will be retained for oocyte recovery rather than be disposed of immediately.
Maturation of egg is essential for its development after fertilisation and production of offspring. Currently the rate of oocyte maturation and embryo development in the laboratory is very low. Therefore, research into development of methods for improvement of oocyte maturation is necessary.

The project
The aim is to develop in vitro methods to increase the breeding potential of stud animals to supply dogs with a range of abilities such as guide dogs for blind people.

Oocytes will be recovered from the ovaries of dogs. Many eggs can be recovered from a single ovary. The eggs are then placed into culture media to provoke development. Experiments will be conducted altering the culture conditions for development to discover optimal procedures.

Ethical Issues
Ovaries will be collected following routine spaying. Therefore, there is no additional treatment of your dog for this project. There are no additional risks to the patient.

Your participation
Your participation in this project is entirely voluntary and unwillingness to participate will not affect your pet's care. If you are happy to assist us with this study, please sign the attached authorisation.

THE ROYAL VETERINARY COLLEGE
UNIVERSITY OF LONDON

In vitro maturation of canine oocytes

OWNER'S CONSENT FORM

I give permission for my dog to participate in this study as described. I have read and understood the Owner Information Sheet and have been given the opportunity to ask questions and receive answers to my satisfaction.

PLEASE COMPLETE IN BLOCK LETTERS UNLESS OTHERWISE STATED:

Owner's Name: _____

Animal's Name: _____

Case No: _____

Owner's Signature: _____

Date: _____

If, during the course of the study, you have any concerns about the welfare of your dog please contact Dr. Ali Fouladi.

Two copies of this Consent Form should be completed – one to be retained by the Owner and the other to be retained by the investigators for a minimum period of five years.

Reproduction Research Group, The Royal Veterinary College, Hawkshead Campus, Hawkshead Lane, AL9 7TA. Tel: 01707 - 666662

Appendix 2:

Synthetic oviductal fluid (SOFaaci) was the base of maturation media which has 14 components. SOF media devised from Holm *et al.* 1999:

1. Stock C (33mM Pyruvate)

Sodium Pyruvate **18mg in 5ml** Embryo Transfer (ET) water.

Filter and store at 4°C for up to **one week**.

2. Stock GLN (10mM glutamine)

L-Glutamine **1ml of 200mM aliquots in 19ml** of ET water

Filter and store at 4°C for up to **one month.**

3. Stock B (250mM $NaHCO_3$)

Phenol red solution **20mg in 20ml** of ET water (Store up to **3months**)

NaHCO3 **0.5025g in 22.5ml** ET water and add 2.5ml of 1mg/ml phenol red solution. Filter and store at 4°C for up to **two weeks**.

4. Stock T

Tri-Sodium Citrate **50mg in 10ml** ET water.

Filter and store at 4°C for up to **two weeks**.

5. Stock L (330mM Na Lactate)

Na Lactate (60% syrup) in the fridge **705µl in 15ml** ET water.

Filter, store 4°C for up to **one month**.

6. Stock S3

NaCl **3.145g**

KCL **0.265g**

KH_2PO_4 **0.081g**

Add the amount to 50 ml ET water, filter and store in fridge for up to **3 months.**

7. Stock D (171mM $CaCl_2.2H_2O$)

$CaCl_2.2H_2O$ **1.260g in 50ml** ET water.

Filter and store in fridge for up to **3 months**.

8. Stock M (49mM $MgCL_2.6H_2O$)

$MgCl_2.6H_2O$ **0.5g in 50ml** ET water.

Filter and store in fridge for up to **3 months**.

9. Stock G (60mM Glucose)

D-Glucose **0.54g in 50m** ET water.

Filter and store in fridge for up to **3 months**.

10. Stock I

Myo-Inositol **499mg in 20ml** ET water.

Filter and store for up to **3 months.**

To prepare SOF media with 0.6% BSA follow the combination table:

100ml	**50ml**	**25ml**	
Stock S3	10.0ml	5.0ml	**2.5ml**
Stock B	10.0ml	5.0ml	**2.5ml**
Stock GLN	10.0ml	5.0ml	**2.5ml**
Stock C	3.0ml	1.5ml	**750µl**
Stock L	3.0ml	1.5ml	**750µl**
Stock G	2.5ml	1.25ml	**625µl**
Stock T	2.0ml	1.0ml	**0.5ml**
Stock I	2.0ml	1.0ml	**0.5ml**
Stock D	1.0ml	0.5ml	**250µl**
Stock M	1.0ml	0.5ml	**250µl**
BME	2.250ml	1.125ml	**562µl**
MEM	500ul	250ul	**125µl**
BSA	600mg	300mg	**150mg**
Water	52.75ml	26.375ml	**13.185ml**

Adjust the pH to 7.4; Osmolarity should be between 270-280 mOsmol. Filter and store in fridge for up to two weeks.

Appendix 3:

Stock solutions for Calcium free and fertilization media: (shelf life 3 months)

CaCl2.2H2O	78mg/ml	Add 1.560g to 20ml sterile water
MgCL2.6H2O	20mg/ml	Add 400mg to 20ml sterile water
Na2HPO4	9.4mg/ml	Add 188mg to 20ml sterile water
Heparin	10mg/ml	Add 200mg to 20ml sterile water
Epinephrine	1mg/ml	Add 20mg to 20ml sterile water
Hypotaurine	1mg/ml	Add 20mg to 20ml sterile water
Phenol red	1mg/ml	Add 20mg to 20ml sterile water

Filter and store in fridge up to 3 months

Calcium Free Medium

Component	**Concentration**	**100ml**
NaCl	5.3 g/l	530mg
KCl	0.23 g/l	22mg
Na2HPO4 2H2O	**0.040 g/l**	**425µl of stock**
MgCl2 6H2O	**0.31 g/l**	**1.5ml of stock**
NaHCO3	2.1 g/l	200mg
Caffeine	0.1 g/l	10mg
Phenol red solution	**0.002 g/l**	**200ul of 1mg/ml**
Kanamycin monosulphate	0.075 g/l	7.5mg
Pyruvic acid	1.0 g/l	100mg
BSA	6.0 g/l	600mg
Na Lactate (60% syrup)	**3.7 ml/l**	**370ul**

pH: 7.7 Osmolarity: 280-290 mOsmol Storage: up to one month at 4°C

Appendix 4:

Fertilisation Medium

Component	**Concentration**		**100ml**
NaCl	5.44	g/l	544mg
KCl	0.23	g/l	23mg
Na2HPO4 2H2O	**0.040**	**g/l**	**0.5ml of stock**
MgCl2 6H2O	**0.10**	**g/l**	**0.5ml of stock**
NaHCO3	2.1	g/l	220mg
Caffeine	0.27	g/l	27mg
Heparin	**0.001**	**g/l**	**100ul of stock**
Hypotaurine	**0.00005**	**g/l**	**50ul of stock**
Epinephrine	**0.00005**	**g/l**	**50ul of stock**
Kanamycin monosulphate	0.075	g/l	7.5mg
Pyruvic acid	1.0	g/l	100mg
BSA	6.0	g/l	600mg
Na Lactate (60% syrup)	**1.86**	**ml/l**	**186ul**
CaCl2 2H2O	**0.78**	**g/l**	**1ml of stock**

pH: 7.7 Osmolarity: 278 mOsmol Storage: up to one month at 4°C

Publications and presentations

REPRODUCTION
RESEARCH

Effects of oxygen concentration on *in vitro* maturation of canine oocytes in a chemically defined serum-free medium

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Abstract

Canine oocytes require an extended period of culture (72 h) *in vitro* for nuclear maturation to the metaphase II stage, which also results in high degeneration. Canine cumulus oocyte complexes were isolated by slicing from ovaries collected after ovariohysterectomy and cultured in serum-free synthetic oviductal fluid incubated at low (5%) or high (20%) oxygen levels. Changes in oocyte nuclear maturation rates, H_2O_2 levels within the oocytes and mRNAs of reactive oxygen species inhibitory genes superoxide dismutase 1 and 2 (*SOD1* and *2*), glutathione reductase (*GSR*), glutathione peroxidase (*GPX1*), and catalase (*CAT*) were quantified. Higher meiotic resumption from germinal vesicle breakdown up to MII was observed in low O_2 (41.8±13.1%) compared to high O_2 (15.8±8.2%) ($P=0.014$) after 52 h of culture ($n=112$). Extension of the culture period up to 84 h at low O_2 ($n=457$ oocytes) produced the highest meiotic resumption at 72 h (64.1±6.0%; $P=0.008$), compared with 52 h. Oocytes ($n=110$) cultured in high O_2 contained higher levels of peroxidase measured using the 2′,7′-dichlorodihydrofluorescein diacetate fluorescence assay after 72 h of culture compared with low O_2 ($P=0.004$). High O_2-cultured oocytes also showed higher amounts of *SOD1*, *SOD2*, *GSR*, *GPX1*, and *CAT* mRNA. Vitamin E in high oxygen level was able to decrease degeneration ($P=0.008$) but had no improving effect on percentage of oocytes in MII. These results for the first time showed that low oxygen gas composition improves nuclear maturation rates and alleviates the oxidative stress for canine oocytes during *in vitro* maturation.

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Introduction

Dogs have been the most popular companion animals in the history of mankind and have always been part of the social and personal activities of modern human life. Despite all efforts, due to its unique features, a successful system for *in vitro* maturation (IVM) and IVF could not be established for canid family (Songsasen & Wildt 2007, Rodrigues & Rodrigues 2010). In contrast to the majority of mammals, canine oocytes are ovulated at the prophase of the first meiotic division (germinal vesicle, GV) and complete the maturation process to metaphase II stage (MII) 48–72 h after ovulation in the oviduct (Hewitt & England 1999, De los Reyes *et al.* 2011). High degeneration (>50%) and very low MII maturation rates (16.2±4.2%) (Luvoni *et al.* 2005, Rodrigues & Rodrigues 2010) are the main features of current IVM in the dog.

Dog oocytes contain abundant lipid droplets that occupy 80–90% of the visible ooplasm surface (Guraya 1965, Tesoriero 1982, Songsasen *et al.* 2009). This may reflect the importance of energy supply during the prolonged period of oviductal travel and the maternal zygotic transition period (Guraya 1965, Luvoni *et al.* 2005, Lopes *et al.* 2010). However, presence of these abundant lipid droplets in ooplasm has an impeccable influence on predisposition of cumulus oocyte complexes (COCs) to oxidative stress by reactive oxygen species (ROS; Wakefield *et al.* 2008, Whitaker & Knight 2008, Tao *et al.* 2010). Oxygen concentration during the IVM culture period can contribute to the extent and velocity of this oxidative stress of which oocyte nuclear and cytoplasmic maturation and development pattern may perturb (Kim *et al.* 2007, Whitaker & Knight 2008).

Glutathione (GSH) is the main non-enzymatic cellular defense mechanisms against ROS and other free radicals (Guerin *et al.* 2001, Menezo *et al.* 2010). GSH becomes a substrate of glutathione peroxidase (GPX) in alliance with catalase (CAT), which degrades hydrogen peroxide (H_2O_2) to water and oxygen (Whitaker & Knight 2008). H_2O_2 itself is the resulting product in neutralization of ROS by superoxide dismutases (SOD) (cytosolic SOD=Cu-Zn SOD1, mitochondrial SOD=Mn-SOD2) (Guerin *et al.* 2001). In order to recover GSH, oocytes use

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another enzyme, glutathione reductase (GSR), to reduce the GS-SG (oxidized form of GSH) back to GSH (Guerin *et al.* 2001). Because of lower levels of GSH within *in vitro* matured canine oocytes (<8 pMol/oocyte) in comparison with *in vivo* (19.2 pMol/oocyte) (Kim *et al.* 2007), high levels of H_2O_2 and ROS within COCs during IVM can severely impair maturation and increase degeneration rates (Whitaker & Knight 2008). These studies showed that the H_2O_2 level in oocytes is a good indication of oxidative stress.

High oxygen level has detrimental effects on cumulus cell survival (Silva *et al.* 2009), which influences expansion during oocyte maturation *in vitro*. In the majority of IVM techniques in domestic animals, cumulus cell expansion and nuclear maturation are concomitant phenomena during the culture period. In other words, expansion of cumulus cells could be a good indicator of the right conditions for nuclear maturation of oocytes within the IVM process (Chen *et al.* 1990, Qian *et al.* 2003). In canine oocytes, the relationship between cumulus expansion and nuclear maturation is quite controversial (Otoi *et al.* 2007, Chebrout *et al.* 2009, Reynaud *et al.* 2009), and there have been counter-intuitive interpretations for cumulus expansion in canine IVM (Reynaud *et al.* 2005, 2006, Lee *et al.* 2007*a*, Chastant-Maillard *et al.* 2010). *In vivo*, expansion of cumulus cells may not concomitant with meiotic resumption as the maturation occurs after ovulation while the expansion initiation time is not clear (Reynaud *et al.* 2009). *In vitro*, extensive mucification of cumulus cells occurred only in the presence of canine serum in the maturation media (Lopes *et al.* 2011). The credibility of cumulus expansion as a reliable indicator of oocyte maturation is to be further elucidated. In addition, due to entrapment of villi of the two or three most inner layers of cumulus cells inside the zona pellucida (ZP; Blackmore *et al.* 2004, De los Reyes *et al.* 2009), it is difficult to denude the oocytes for assessment of nuclear maturation or manipulation for other assisted reproduction techniques.

Vitamin E (α-tocopherol) as a lipid-soluble antioxidant has been proven to have beneficial effects on oocyte maturation and embryo development in pigs (Tao *et al.* 2010) and alleviates the degeneration rates of bovine and ovine oocytes (Dalvit *et al.* 2005, Natarajan *et al.* 2010). This study investigated the impact of oxidative stress on oocyte nuclear maturation and degeneration. Changes in H_2O_2 content of oocytes cultured in two oxygen levels were analyzed, and the protective role of an antioxidant (vitamin E) was investigated in pursuit of oxidative stress alleviation and improvement of cumulus cell expansion and oocyte nuclear maturation rates.

Results

Cumulus cell expansion and oxygen concentration

Expansion of cumulus cells was assessed in COCs cultured under low- or high-oxygen conditions. No full mucification was observed throughout IVM in either group (absence of extracellular matrix cloud) at 52 h (0.0% full expansion in both groups; not expanded oocytes 97.1±1.6% in low O_2, and 95.0±2.7% in high O_2 ($P>0.05$)). However, after 52 h, limited numbers of cumulus cells were disintegrated from the COCs, with higher disintegration in COCs cultured in the high-oxygen incubator (5.0±2.7% in high O_2 vs 2.9±1.6% in low O_2; ($P>0.05$)). Moreover, in high-oxygen culture, disintegrated cells were less attached to the culture dish (Fig. 1).

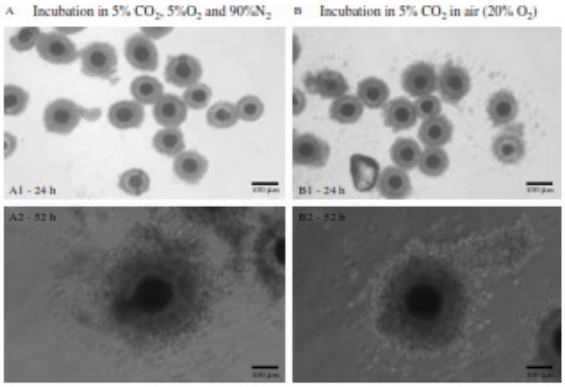

Figure 1 Effect of oxygen level during oocyte culture on cumulus expansion of canine oocytes at 24 and 52 h. Canine oocytes cultured in low (A) or high (B) levels of oxygen (5 vs 20%). Photographs were taken with light field optics to visualize cumulus expansion pattern (A1 and B1 = oocytes at 24 h of culture (4× objective)), (A2 and B2 = oocytes at 52 h of culture (10× objective)).

Table 1 Effect of oxygen concentration during oocyte culture on nuclear maturation of canine oocytes. Table shows distribution of oocytes in different stages of meiotic division. Meiotic resumption of oocytes (total number of oocytes between GVBD and MII) was significantly higher in low O_2 condition.

	Percentage of oocytes (average ± S.E.M.) in 52-h oxygen comparison							Meiotic resumption	
	GV	GVBD	MI	AI	TI	MII	Degen	(GVBD-MII)	(*n*)
Low O_2	35.0±13.4	27.1±12.1	4.0±4.0	4.8±2.5	3.8±2.4	2.0±2.0	23.2±6.6	41.8±13.1*	66
High O_2	52.3±9.9	12.7±6.4	3.0±3.0	0.0±0.0	0.0±0.0	0.0±0.0	31.9±2.2	15.8±8.2	52

*P value <0.05, high O_2 compared with low O_2. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.

Nuclear maturation

A total of 118 oocytes from three different repeats were cultured in low- or high-O_2 conditions. After 52 h of culture (Table 1), there was no significant difference in degeneration rates of the oocytes cultured under two gas compositions ($P>0.05$). However, a higher percentage of oocytes resumed meiosis after GV breakdown (GVBD) toward MII stage in low O_2 (41.8±13.1%) compared with the oocytes cultured in high-O_2 incubator (15.8 ±8.1%) (P value−0.014; Table 1).

Extended IVM

A total number of 460 oocytes (three repeats) were cultured in base maturation media for 52 h (n−66), 72 h (n−231), and 84 h (n−163). The number of oocytes that remained at GV stage in the 52 h group was higher ($P<0.05$) than the other two and the lowest MII stage percentage occurred within this group. Highest meiotic resumption (GVBD-MII) was in the 72-h cultured group (64.1±6.0%), which was higher than 52 h ($P<0.05$; Table 2). On the other hand, the 84-h group resulted in the highest rate of degeneration with 43.2±4.5% compared with 52 h ($P<0.05$; Table 2). Although there were no significant differences in anaphase I (AI) or telophase I (TI) percentages among the three groups, the highest MII maturated oocytes (7.5±4.0%) occurred at 84 h ($P<0.05$; Table 2).

ROS and oxygen level

To analyze the effects of oxygen level during IVM, oocytes (n−130) were stained for H_2O_2 levels using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) after 72 h culture. Densitometry using ImageJ software showed that the overall intensity of fluorescence was greater for high O_2 oocytes higher than low-O_2 oocytes (P−0.004; Fig. 2).

ROS repair enzymes mRNA expression

Analyses of the fold induction of target genes (*GPX1*, *SOD1*, *SOD2*, *GSR*, and *CAT*) at 0 and 72 h in culture showed significant end point differences between COCs cultured in low- and high-oxygen incubators after 72 h ($P<0.05$). The expression of mitochondrial *Mn-SOD2* among other enzymes involved in GSH metabolism was more prominent in the high-oxygen group (Fig. 3).

Vitamin E and oxidative stress

COCs cultured in low- and high-oxygen incubators were supplemented with vitamin E during 72 h of IVM. Vitamin E at 100 μM reduced degeneration in the high-oxygen group compared with its control (41.7±7.6% down to 30.4±2.3%; P−0.008; Table 3). Meiotic resumption in control (P−0.0001) and vitamin E (P−0.007)-treated oocytes in the low-oxygen group was higher than their high-oxygen counterparts (Fig. 4). Also vitamin E has no significant effect on the percentage of MII-matured oocytes in both control groups (low−13.1±3.1% and high−4.6±2.5%; P−0.072; Table 3). However, in the presence of vitamin E, the MII maturation rate in the low-oxygen group was higher than in high-oxygen group (P−0.008; Fig. 4).

Table 2 Effect of the duration of culture period from 52 to 84 h on nuclear maturation of canine oocytes (5% O_2, 5% CO_2, and 90% N_2). Table shows distribution of oocytes in different stages of meiotic division.

	Percentage of oocytes (average ± S.E.M.) in 52 vs 72 vs 84 h and low oxygen							Meiotic resumption	
	GV	GVBD	MI	AI	TI	MII	Degen	(GVBD-MII)	(*n*)
52 h	35.0±13.4	27.1±12.0	4.0±4.0	4.8±2.4	3.8±2.4	2.0±2.0	23.2±6.6	41.8±13.1	66
72 h	4.0±1.1*	37.2±8.4*	16.5±2.5	2.9±1.8	3.7±0.8	3.8±0.8	31.9±5.4*	64.1±5.9*	231
84 h	0.7±0.7*	22.5±4.2	20.6±4.8*	2.8±1.4	2.5±1.3	7.5±3.9*	43.2±4.4*	56.0±5.1	163

*P value <0.05, within the column compared with 52 h. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.

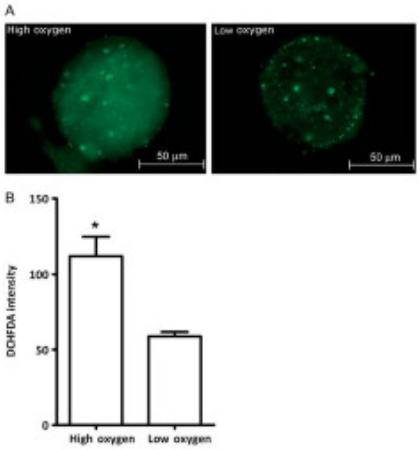

Figure 2 Effect of oxygen concentration on H_2O_2 (ROS) levels of canine oocytes after 72 h of culture. (A) Oocytes were stained with DCHFDA for 30 min at 38.5 °C in the dark and then mounted on superfrost slides using Vectashield mounting media. Slides were visualized using fluorescence (470 nm wavelength LED lamp) and photographs taken immediately. (B) The total intensity of DCHFDA fluorescence signal of oocytes cultured in low (5%) and high (20%) oxygen levels in the base maturation media after 72 h. DCHFDA intensity is a direct indicator of H_2O_2 level. *P value <0.05.

Discussion

Establishment of an efficient IVM system for canine oocytes, which is complicated by unique and complex canine reproductive physiology, is still unsolved. During this study, attempts were made to establish a chemically defined and time course optimally modified IVM protocol for canine oocytes. The majority of IVM protocols are based on serum-enriched culture media (Otoi *et al.* 1999, Luvoni *et al.* 2005, Oh *et al.* 2005) in which the exact beneficial effects of serum are unknown and in most cases unpredictable (Bolamba *et al.* 2002, Lee *et al.* 2007*b*). In addition, results from our preliminary experiments (unpublished data) indicated that presence of fetal bovine serum tends to have detrimental effects on nuclear maturation of canine oocytes and significantly increases degeneration rates. Thus, in this study, all the experiments were carried out in a chemically defined medium and without serum supplementation.

Staining and staging

Nuclear staining and staging of canine oocytes after IVM is one of the most critical issues in canine-assisted reproductive techniques. Therefore, it is worth mentioning that we have optimized a protocol that allows precise assessment of oocyte nuclear stage during meiotic maturation. It includes a procedure for complete denuding of cumulus cells and fixation followed by staining of oocytes and visualization of oocyte chromatin under a fluorescence microscope. Denuding canine oocytes due to their highly inter-digitated cumulus, ZP attachment (Blackmore *et al.* 2004, De los Reyes *et al.* 2009) after culture, is challenging (Hewitt *et al.* 1998). In addition, the lipid droplets inside the oocyte make it very difficult to visualize the chromatin content by simple aceto-orcein staining unless oocytes are fixed for 5–7 days (Song *et al.* 2010). After trying several denuding buffers with different timetables of incubation and vortexing (Reynaud *et al.* 2004), incubation in sodium tri-citrate 1% (Hewitt & England 1999) and denuding using an oocyte holding needle with gauge of 135 m (Yellow EZ-Strip Research Instruments Limited, Cornwall, UK) resulted in complete removal of the cumulus cells. For nuclear staining, the combination of 10-min fixation with acetone at −20 °C followed by 5 min staining in 10 μg/ml Bisbenzimide (Hoechst 33342) solution provided a quick and reliable solution. Using this method, we were able to provide a panel of images that can be used as guidance for investigators (Fig. 5).

Oxygen level and IVM

Expansion of cumulus cells did not differ after 52 h of culture of COCs in low- (5%) or high (20%)-oxygen

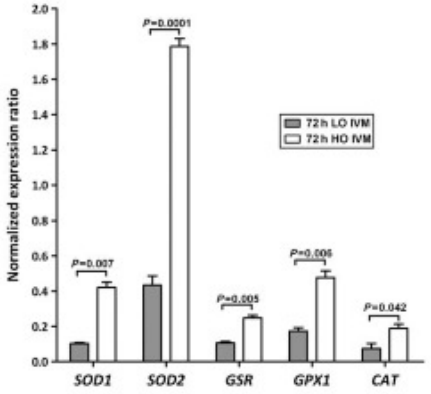

Figure 3 Effect of oxygen concentration on mRNA expression profile of ROS repairing enzymes in canine oocytes. Graph shows the fold induction of ROS repairing enzymes after 72 h of IVM compared with 0 h and normalized to the GAPDH housekeeping gene. SOD1, cytosolic superoxide dismutase; SOD2, mitochondrial superoxide dismutase; GSR, glutathione reductase; GPX1, glutathione peroxidase; CAT, catalase; LO, low oxygen; HO, high oxygen.

Table 3 Effect of vitamin E on the nuclear maturation of canine oocytes in low and high oxygen. Table shows distribution of oocytes in different stages of meiotic division.

	Percentage of oocytes (average±S.E.M.) in vitamin E experiment (72 h)							Meiotic resumption	
	GV	GVBD	MI	AI	TI	MII	Degen	(GVBD-MII)	(*n*)
Ctrl low	14.9±4.1	31.5±5.4†	7.6±2.6	2.3±2.3	2.8±2.8	13.1±3.1	27.9±4.2	57.2±5.5†	39
Vitamin E low	16.9±2.9	24.9±4.7‡	8.0±4.8	0.0±0.0	3.6±2.2	10.5±2.5‡	35.9±2.3	47.2±2.7†	51
Ctrl high	27.2±2.2†	16.2±3.1	8.6±3.7*	2.1±2.1	0.0±0.0	4.6±2.5	41.6±7.5*	31.2±7.3	45
Vitamin E high	34.7±5.7‡	16.5±3.6	7.6±1.5	1.3±1.3	4.6±2.9	4.8±1.6	30.4±2.3	34.8±7.3	66

*P value <0.05 within the column in low or high oxygen compared with their controls; †,‡P value <0.05 within the column between control groups (†), between vitamin E groups (‡). GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.

incubators; nevertheless, the disintegration of cumulus cells differed in high oxygen (Fig. 1). The oviductal oxygen level is almost one-forth to one-third of the normal air oxygen level (e.g. 5 vs 20%) (Rodrigues & Rodrigues 2010). Despite the presence of anti-apoptotic intracellular mechanisms acting via GSH against ROS (Silva *et al.* 2009), having high lipid content in canine COCs can reduce GSH level and predispose them to oxidative damage and induce apoptosis in the cumulus cells (Silva *et al.* 2009). This can contribute to lowered oocyte maturation rates (Kim *et al.* 2007, Rodrigues & Rodrigues 2010). Expansion of cumulus cells *in vivo* or during culture of COCs *in vitro* occurs through production of hyaluronan by cumulus cells under the influence of LH. Cumulus cells must be viable and express hyaluronan synthase II in the cell membrane (Marei *et al.* 2012). Induction of apoptosis in COCs through oxidative stress during the extended culture period is expected to hamper production of hyaluronan and cumulus cells expansion. Albeit that cumulus cell expansion is not a convincing marker of canine oocyte maturation (Reynaud *et al.* 2005, Otoi *et al.* 2007, Rodrigues & Rodrigues 2010), there was significantly higher number of maturing oocytes (GVBD-MII) after 52 h culture in the low-oxygen (41.8±13.1%) group compared with the high-oxygen group (15.8±8.2%). This was accompanied by lower degeneration rate in the low-oxygen group (23.2±6.6%) compared with the high-oxygen group (31.9±2.2%) ($P<0.05$), confirming the detrimental effects of high oxygen tension on dog oocytes.

Duration of culture period for IVM of canine oocytes ranges from 48 to 96 h in different studies (Luvoni *et al.* 2005, Rodrigues & Rodrigues 2010). *In vivo*, oocytes need 2–4 days for completion of this stage (Concannon 2011). Therefore, it was decided to extend the culture period up to 84 h in order to find the optimal culture period for canine oocytes, which result in highest maturation and lowest degeneration rates in the low-oxygen atmosphere. Extension of culture period to 72 h resulted in increased meiotic resumption to 64.1±5.9% (Table 2). However, further extension of the culture period to 84 h resulted in elevation (7.4%) in the percentage of MII oocytes (Table 2) but increased degeneration rate. Similar findings were reported by other research groups (Otoi *et al.* 2002, Rodrigues Bde *et al.* 2004). Therefore, for the remainder of experiments presented here including H_2O_2 comparison, ROS, and vitamin E experiments, 72-h incubation period was used to avoid high degeneration but achieve highest meiotic resumption.

Oxidative stress and IVM

In the H_2O_2 staining (DCHFDA) experiment, a comparison was done between low and high oxygen levels and the total amount of H_2O_2 produced inside ooplasm of canine oocytes after 72 h of culture. Oocytes stained with DCHFDA (Fig. 2A) produced significantly higher levels of H_2O_2 ($P=0.004$) in the high-oxygen group (Fig. 2B). It is noteworthy that H_2O_2 is a byproduct of the ROS protection system in which SOD enzymes transform damaging oxygen-free radicals into less aggressive H_2O_2 molecules (Whitaker & Knight 2008). As oxidative stress is introduced to oocytes immediately after mechanical extraction till the end of the culture period via various sources of free radicals, light and physical trauma (Guerin *et al.* 2001, Menezo *et al.* 2010), it is necessary to provide these cells with least amount of

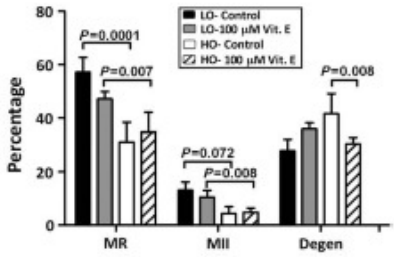

Figure 4 Effect of vitamin E on the nuclear maturation of canine oocytes in low and high oxygen. Graph shows the percentages of meiotic resumption (MR; GVBD-MII), metaphase II (MII), and degenerated (Degen) oocytes cultured in the presence or absence of vitamin E. LO, low oxygen; HO, high oxygen.

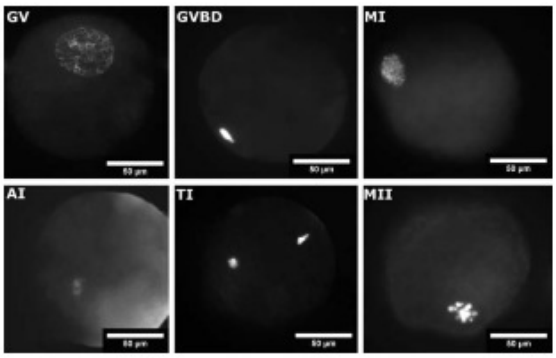

Figure 5 Canine oocytes at different stages of meiotic division: oocytes were stained with Hoechst 33342 (10 μg/ml) and visualized by fluorescence microscope with 40× objective. Prophase I (germinal vesicle, GV), germinal vesicle breakdown, GVBD accompanied by chromatin condensation, metaphase I (MI), anaphase I (AI), telophase I (TI), metaphase II (MII).

stressors. Reduction of oxidative stress can profoundly contribute to improved nuclear and cytoplasmic maturation.

Results of the real-time qPCR showed that mRNA of ROS repair enzymes are significantly higher in oocytes cultured in high oxygen (Fig. 3), which is concomitant with high H_2O_2 level, lower meiotic resumption, and higher degeneration rates. SOD, present in mitochondria (*SOD2*), showed remarkable increase in mRNA expression in the high-oxygen group, which is in agreement with previous reports (De los Reyes *et al.* 2011). Involvement of mitochondrial mobilization during cytoplasmic maturation of canine oocytes in terms of perturbation due to oxidative pressure could be the main retarding factor in canine IVM. Moreover, oocytes cultured in high oxygen showed increased *GPX1* and *GSR* expression in response to high oxygen tension (Fig. 3), indicating that the mitochondrial oxidative profile requires further investigation.

Supplementation of vitamin E in the maturation medium did not prevent the detrimental effects of high oxygen tension on degeneration rates of canine oocytes during 72-h culture. Meiotic resumption of oocytes cultured in low oxygen tension was higher in both control and 100 μM vitamin E-treated oocytes than in the high-oxygen group. Vitamin E in the presence of high oxygen was able to decrease degeneration ($P=0.008$) but did not improve the percentage of oocytes in MII. The beneficial effect of vitamin E on oocyte maturation and embryo development was previously reported in animal species regardless of lipid content, including porcine cells (Tao *et al.* 2010), which contain high lipid, or in ovine oocytes (Natarajan *et al.* 2010), which contain relatively low lipid contents.

The studies presented here concerned culture of canine oocytes in a serum-free and chemically defined maturation media. Culture period of 72 h in low oxygen level (5%) was the optimal condition for canine COCs. Oxidative stress and the level of ROS in canine ooplasm affected maturation efficiency, particularly involving mitochondrial activity. To the best of authors' knowledge, this is the first study that demonstrated the importance of oxygen tension during canine IVM and its possible contributions to maturation and degeneration.

Materials and Methods

Chemicals and reagents

All the materials and chemical reagents were purchased from Sigma–Aldrich Chemical Co. unless otherwise stated.

Collection of ovaries

The sample collection procedure was carried out after routine ovariectomy of bitches by approval of the ethics committee of the Royal Veterinary College from a small animal hospital under supervision and consent of dog owners. Ethical approval was also verified by the University of Bedfordshire Ethical Scrutiny Committee. Due to the nature of this study and also previously published reports (Otoi *et al.* 2000, 2001, 2002, Songsasen & Wildt 2005), sample collection was blind to reproductive stage of the animal, breed, age, weight, or size.

Collection of COCs

Ovaries were collected immediately after ovariectomy and placed in a 60 ml container (VWR International, Westchester, PA, USA) half full of warm (37 °C) sterile PBS and transferred to the laboratory <2 h after surgery. The container was immersed in a thermos flask containing warm PBS. Before dissection of follicle and oocytes, the ovaries were washed with warm PBS and trimmed of the ovarian bursa and other debris with a scalpel blade. The ovaries were washed with a sterile filtered (0.2 μm microbial filter (Anachem, Bedfordshire, UK)) washing media (TCM199+20 mM HEPES buffer +10% FBS (PAA

Laboratories, Dartmouth, MA, USA)) and then sliced gently using a set of multiple blades (Fisher Scientific, Loughborough, UK) as described previously (Alhaider & Watson 2009). COCs with an oocyte diameter over 100 μm (surrounded by at least three layers of cumulus cells) having dense and homogenous lipid yolk in the ooplasm were selected and washed twice before culture.

COCs selection criteria for canine IVM protocols used to be quite controversial (Songsasen & Wildt 2005, Concannon *et al.* 2009). Recently, it has been accepted that age (older than 6 months), breed, weight, and the stage of estrous cycle are less likely to affect the maturation rate of oocytes to MII stage or their degeneration rate (Concannon *et al.* 2009). Instead cellular criteria have been introduced for oocyte quality including condensed and homogenous lipid yolk, oocyte diameter above 100 μm excluding the ZP, and being surrounded with at least three layers of cumulus cells (Otoi *et al.* 2000, 2001, 2002). Oocyte diameter above 100 μm is one of cellular criteria during selection that is highly recommended by many reports (Songsasen & Wildt 2005). Nevertheless, large size of the oocyte is not necessarily a guarantee of either meiotic competency or cumulus expansion (Rodrigues & Rodrigues 2010). During all experiments of this study, oocytes were collected from ovaries according to the above cellular criteria to maintain the unity.

In vitro *maturation*

Oocytes were cultured in 25 oocyte maximum groups according to Hewitt & England (1999) with minor modifications. Selected oocytes were cultured in four-well culture dishes (NUNC, VWR International, Milan, Italy). The base maturation media was modified synthetic oviductal fluid (mSOF; Holm *et al.* 1999, Marei *et al.* 2009) supplemented with LH 5 μg/ml (Leutropin; Bioniche Animal Health, Belleville, ON, Canada), FSH 5 μg/ml (Follitropin; Bioniche Animal Health), 17β-estradiol 1 μg/ml, progesterone 1 μg/ml, 50 μg/ml gentamicin sulfate, and 6 mg/ml BSA.

Assessment of cumulus cell expansion

Cumulus expansion (mucification) was described by the partial or complete loosening of cumulus cells into extracellular matrix surrounding the oocyte (Lee *et al.* 2007*a*, Marei *et al.* 2009, Silva *et al.* 2009). COCs were assessed for expansion of cumulus cells at 24 h after the beginning of the culture and by the end of 52 h under a stereomicroscope.

Denuding oocytes and assessment of nuclear maturation

Oocytes were denuded by incubation in 1% (W/V) Tri-Sodium Citrate in PBS buffer for 3–5 min and vortexed for 2 min at maximum agitation inside a 15 ml conical centrifuge tube (VWR International). Remaining cumulus cells were denuded using an oocyte holding needle with gauge of 135 m (Yellow EZ-Strip Research Instruments Limited). The oocytes were placed in a drop on superfrost slides (VWR International) and air dried in room temperature for 20 min. Afterward, the oocytes were fixed in cold (−20 °C) 99% Acetone (Merck) for 10 min. Hoechst 33342 fluorescent DNA dye (excitation/emission = 350/461 nm) was prepared in PBS at 10 μg/ml for simultaneous staining and rehydration of slides for 5 min at 4 °C. Hoechst 33342 and PBS were dried with a stripped filter paper as much as possible before mounting the oocytes. Oocytes were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) beneath a coverslip fixed with four paraffin/vaseline drops (1/40; w/w) at the corners of the coverslip. Nuclear stage of the oocytes was assessed with an Olympus BX60 fluorescence microscope (Olympus, UK).

During maturation, oocytes go through different stages between prophase I and MII. By the time they had reached the end of culture period, they were distributed among seven groups according to nuclear stage. The number of oocytes at each stage was recorded (Hewitt *et al.* 1998). Degenerate oocytes with undetermined, disappeared, or morphologically abnormal nuclear material were categorized as a separate group (Degen) in all experiments. A panel of stained oocytes at different meiotic stages is shown in Fig. 5, which was used as a guide for all experiments.

ROS staining using DCHFDA

A total number of 130 oocytes from high- and low-O_2 groups in three replicates were denuded after 72 h of culture and stained using DCHFDA. This substance hydrolyzes via intracellular esterase to produce $2^{\prime},7^{\prime}$-dichlorodihydrofluorescein (DCHF), and the latter metabolite will be oxidized via H_2O_2 to $2^{\prime},7^{\prime}$-dichlorofluorescein (DCF; Nasr-Esfahani *et al.* 1990, Wakefield *et al.* 2008). With DCF excitation at 470 nm wavelength, the emission at 522–530 nm could be captured by fluorescence microscopy. Thus, the oocytes were washed twice in 0.04% PVP in PBS and then incubated for 30 min at 38.5 °C in the dark in the 0.04% PVP-PBS buffer containing 10 μM DCHFDA. Afterward, oocytes were washed two times in the same buffer and mounted on slides using Vectashield mounting media (VectorLabs, Peterborough, UK) under a coverslip. Oocytes were visualized by 470 nm LED lamp Olympus BX60 fluorescence microscope, and photographs were obtained from ten oocytes of the two groups in each repeat. The intensity of fluorescent signal was quantified using ImageJ software particle analysis plug-in (Abràmoff *et al.* 2004).

Real-time PCR and expression profile of ROS repair enzymes

Mechanically dissected COCs (n = 340) cultured in high and low oxygen were snap frozen after 72 h of culture in PBS-PVP 0.4% (polyvinylpyrrolidone) using liquid nitrogen and kept at −20 °C until analysis. RNA extraction was done using a QIAGEN RNAeasy kit (Qiagen). Briefly, 20 COCs were lysed in 350 μl lysis buffer (RLT), mixed with equal amount of 70% ethanol, and transferred to the RNAeasy spin columns. Washes and centrifugation were carried out according to the manufacturer's instruction, and RNAs were finally eluted in 30 μl nuclease-free water and the concentration was measured by a TECAN plate reader (TECAN, Switzerland). RNA concentration was normalized among samples by dilution to 50 ng in 8 μl

Table 4 Sequence of the designed primers and accession numbers of the ROS repairing enzyme genes.

Canine genes	Accession number	Oligos (5′→3′)	Product size (bp)
GPX1	NM_001115119.1	F: GACACCACTGCGCTAATGAC R: AGGGAAAGGAGGGTTGCCTA	215
SOD1	NM_001003035.1	F: ACCATTACAGGGCTGACTGAAG R: TGGACAGAGGATTAAAGTGAGGA	115
SOD2	XM_533463.3	F: AGAAGGGTGACATTACAGCTCA R: AATCACGTTTGATGGCTTCC	153
CAT	NM_001002984.1	F: CCCATTGCAGTTCGATTCTC R: CTATGGATAAAGGACGGAAACA	179
GSR	XM_532813.3	F: CTACGTGAGCCGCCTAAATAC R: CTGTGGCAATCAGGATGTGAG	155
GAPDH	NM_001003142.1	F: GTGATGCTGGTGCTGAGTATGT R: ATGGATGACTTTGGCTAGAGGA	233

volume. DNA digestion was carried out using RQ1 RNAase-free DNAse kit (Promega; M6101). RT of RNA samples was conducted by Promega RT kit (Promega) in a equal amount of 50 ng RNA (total 20 μl) and the PCRs were done in 12.5 ng cDNA per reaction in duplicate tubes and four sets of repeats. Conventional PCR (38 cycles) was conducted on every batch of cDNA including the negative control for RT for every primer before temperature gradient optimization. A temperature gradient real-time PCR was done in the range of 50–60 °C and except *CAT* (57 °C), the rest of primers had an optimal melting temperature at 59 °C. Quantitative PCR was done using KAPA SYBR FAST qPCR Kits (Kapa Biosystems, Bedfordshire, UK) and 20 μM primers in 20 μl volume and 38 cycles via Bio-Rad CFX96 real-time machine (Bio-Rad) using CFX manager 1.7 software (Bio-Rad). Genomic sequences were obtained from PubMed, and primers were designed using Primer3 (Rozen & Skaletsky 2000) web-based software (Table 4).

Relative real-time qPCR was carried out using canine *GAPDH* as the housekeeping gene, which had a stable expression among three groups (control (0 h) and the two treatments) in four replicates (Levene's test, $P=0.086$; one-way ANOVA (equal variances assumed, LSD post-HOC); P value $=0.227$). The fold induction (expression) of target genes was analyzed using Livak method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen 2001).

Experimental design

Experiments were repeated at least three times. A total of 1249 oocytes presenting homogenous and condensed lipid contents in the cytoplasm surrounded by at least three layers of cumulus cells were used. During the first experiment, 118 COCs were cultured in humidified incubators under two different gas compositions for 52 h at 38.5 °C: high O_2 ((5% CO_2 in air (~20% O_2)) or low O_2 (5% CO_2, 5% O_2, and 90% N_2) in order to study the pattern of cumulus expansion. COCs were photographed twice during the 52 h of culture (at 24 and 52 h) using a digital camera and an inverted microscope to record any pattern of cumulus expansion within different oxygen levels. At the end of culture (52 h), COCs were denuded fixed and stained to assess nuclear maturation of the oocytes. Each stage of meiotic resumption was reported as a percentage of total number of the oocytes cultured from one animal/replicate.

From results of the nuclear maturation rates, a second experiment was designed to compare nuclear maturation rates of canine oocytes in longer incubation periods. A total number of 460 COCs were cultured in the low oxygen for 72 and 84 h time periods; oocyte maturation and degeneration rates were analyzed after staining.

The third experiment was designed to assess the role of oxidative stress as a detrimental factor, which may contribute to the high degeneration and low maturation rates in canine IVM oocytes. A total number of 130 oocytes were cultured for 72 h in the low or high oxygen gas atmospheres. The H_2O_2 concentration in oocytes as the product of ROS was quantified after DCHFDA fluorescent staining. Photographs of stained oocytes were analyzed via ImageJ software to quantify the spot density of fluorescence, which is directly correlated with the amount of H_2O_2 produced inside each oocyte (Nasr-Esfahani *et al.* 1990, Wakefield *et al.* 2008). An average of total intensity was compared between the low- and high-oxygen groups.

For investigating the mRNA expression profile of ROS-defensive enzymes, 340 oocytes in four repeats were cultured in low- and high-oxygen incubators (groups of 20). At the beginning of the experiment, 20 COCs were snap frozen as the 0-h control group. At the end of the 72-h culture period, oocytes were snap frozen as low- and high-oxygen treatment groups. PCR reactions were carried out in duplicates and four repeats. In Livak analysis, cycles of threshold (Ct) of the target genes (TG) were first deduced by the Ct of housekeeping gene (HKG; *GAPDH*): ΔCt = average C(t) (TG) − average C(t) (HKG). Resulting values for treatment groups (72 h) were normalized against the 0-h control using the following equation: ΔΔCt (TG) = ΔCt treatment 72 h (TG) − ΔCt Ctrl 0 h (TG); normalized expression ratio $=2^{-\Delta\Delta Ct\ (TG)}$.

Experiment 4 concerned protective effects of vitamin E over oxidative stress. A total of 201 oocytes were cultured in low- and high-oxygen incubators in two groups in absence (control) and presence of 100 μM vitamin E. Nuclear maturation rates and meiotic resumption of oocytes were analyzed after 72 h of culture using fluorescent staining.

Statistical analysis

All experiments in this study were repeated at least three times. The proportional average of oocytes in different stages of meiotic resumption was calculated at the end of the culture

period in comparison to the total number. The oocytes were categorized in to seven groups of GV, GVBD, metaphase I, AI, TI, MII, and degenerates (Degen). Statistical analysis was carried out in PAWS statistics 18: Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, USA) using binary and ordinal logistic regressions via generalized linear model. Analysis of qPCR data was carried out using CFX manager software (Bio-Rad) and one-way ANOVA (LSD *PostHoc* multiple comparisons) in PAWS statistics 18: Statistical Package for Social Sciences (SPSS, Inc.).

Declaration of interest

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

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Effects of Caffeine on *In vitro* Maturation of Dog Oocytes

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Caffeine has competitive non-selective inhibitory effects on Phosphodiesterases (PDE) which potentially influence molecular changes affecting nuclear development of oocytes during *in vitro* culture. Maintenance of the oocyte in the meiotic arrest is believed to be orchestrated via the high cAMP concentration within the oocyte which prevents the activation of pathways responsible for meiotic resumption. Moreover high levels of cAMP in the cumulus cells improve oocyte maturation rates in a range of mammalian species. In the present study the effects of caffeine as a phosphodiesterase inhibitor which prevents the degradation of cAMP and thus enables the maintenance of high levels of cAMP were investigated throughout *in vitro* maturation process. Dog ovaries were collected after routine ovariohisterectomy. Cumulus oocyte complexes (COCs) were isolated by mechanical separation and cultured in a serum free SOF media (Synthetic oviductal fluid) up to 72hrs. The culture was carried out in a humidified atmosphere of 5% O2, 5% CO2 and 90% N2. COCs were cultured in absence (Control) or presence of increasing concentrations of caffeine (5, 10, 20mM). In the first experiment, oocytes (n=314) were cultured for varying periods of time up to 72hrs in the presence of caffeine. The results of this experiment indicated that the pre-treatment of canine oocytes with 10mM caffeine for 6-12 hrs in the maturation media increases the percentage of MII oocytes (6 and 12hrs; 7.6% and 13.3% respectively). However longer periods of caffeine treatments (24 and 72hrs) had detrimental effects on maturation rates. In the subsequent experiment, treatment of canine oocytes (n=192) in 10mM caffeine for 12hrs also improved maturation rate to M-II by up to 21.43% (on average 16.7% ± 2.4 $P<0.05$) relative to control, 5mM and 20mM groups. Results of this investigation elucidated that short time treatment of oocyte at the beginning of *in vitro* culture process will improve maturation rates of canine oocytes.

Keywords: *caffeine – cAMP – Canine oocytes – In vitro maturation*

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Contrasting effects of porcine growth hormone and foetal bovine serum on *in vitro* maturation of dog oocytes

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Introduction: Managing the degenerative predisposition of canine oocytes due to their extended duration of culture for *in vitro* maturation is of essence. Canine and porcine growth hormones (cGH,pGH) have identical amino acid sequences and high level of similarity. Supplementation of growth hormones (GH) in canine oocyte maturation media containing bovine serum albumin (BSA) improved meiotic resumption and reduced degeneration rates. On the other hand Foetal bovine serum (FBS) as a classic supplement due to its unknown hormonal and chemical composition has had contrasting effects over maturation and degeneration rates of canine oocytes

Methods: The effects of pGH or FBS as maturation promoting factors were compared during dogs oocytes maturation in a serum free culture system. Dog ovaries were collected after routine ovariohysterectomy. Cumulus oocyte complexes (COCs) were isolated by mechanical separation and cultured in SOF media (synthetic oviductal fluid) up to 72hrs in a humidified atmosphere of 5% O2, 5% CO2 and 90% N2. Cumulus oocytes complexes were cultured in the absence (control) or presence of increasing concentrations (10, 100 and 1000ng/ml) of pGH (n=269) or 10% FBS (n=395). Oocytes nuclear maturation (resumption of meiosis; GVBD to metaphase II stages) and degeneration rate was assessed.

Results and discussion: Porcine GH at, 100ng/ml significantly increased the percentage of meiotic resumption from GVBD to MII (61.02% ± 8.91; $p < 0.05$). The lowest degeneration rate was observed in the 1000ng/ml group (27.26% ± 6.63; $p < 0.05$). Presence of FBS reduced MII rate to 5.01% ±2.39 compared to control group (18.72%±3.97; $P<0.05$). Degeneration rate in FBS treated group (74.59% ±4.76) was significantly higher than control group (28.25%±3.25; $p<0.05$). Results elucidated that pGH can improve meiotic resumption along with reducing degeneration rates in contrast with FBS that has outstanding negative effects on canine oocytes maturation.

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Effects of Oxygen Concentration on *In vitro* Maturation of Canine Oocytes

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Canine oocytes require extended period of culture up to 72 h *in vitro* for nuclear maturation to metaphase II stage which also results in high degeneration rates when cultured in high oxygen gas atmosphere. High levels of fat deposit in these oocytes and incompetent repair mechanisms are expected to predispose these oocytes to oxidative stress and may contribute to high degeneration and reduced nuclear maturation rates. Canine cumulus oocyte complexes (COCs) were isolated by mechanical separation from ovaries collected after routine ovariohisterectomy and cultured in serum free SOF media (synthetic oviductal fluid) incubated at low (5% O_2 + 5 % CO_2 + 90 % N_2) or high (5% CO_2 in air) oxygen concentrations. Changes in oocyte nuclear maturation rates, H_2O_2 levels within the oocytes and expression of reactive oxygen species inhibitory genes; superoxide dismutase 1 &2 (SOD1&2), glutathione reductase (GSR), glutathione peroxidase (PGX1) and catalase (CAT) were analysed. Higher meiotic resumption from germinal vesicle break down up to metaphase II was observed in low O_2 (41.75% ± 13.1) compared to high O_2 (15.76% ± 8.15) (P = 0.014) after 52h of culture (n=112). Extension of the culture period up to 84h at Low O_2 produced the highest meiotic resumption at 72h (64.10%±5.98; P=0.008), (n=457) compared to 52h or 84h. Oocytes (n=110) cultured in High O_2 contained higher levels of peroxidase measured using 2',7' –dichlorodihydrofluorescein diacetate (DCHFDA) fluorescent assay after 72h of culture (P= 0.004). High O_2 cultured oocytes (n=300) also showed higher fold induction of SOD1, SOD2, GSR, PGX1 and CAT at mRNA level using relative qPCR analysis. These results for the first time that Low oxygen gas composition improves nuclear maturation rates and alleviates the oxidative stress for canine oocytes during *in vitro* maturation.

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Improving effects of melatonin on *in vitro* maturation of canine oocyte

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Introduction: Melatonin is an indolamine hormone secreted from pineal gland influencing the reproductive cycle and hormonal changes in uterus and ovaries though its dedicated G- protein coupled receptors (MTNR-A1&B1). MTNRA1 mRNA expression has been reported in both oocyte and cumulus cells; however MTNRB1 is only present in cumulus cells. Besides regulating estradiol and progesterone production within the follicle, melatonin has proven to have improving effects on oocyte maturation rate and protecting the cumulus oocyte complexes (COCs) from oxidative stress.

Methods: After obtained approval from ethics committee and given consent of the owners, dog ovaries were collected after routine ovariohysterectomy. COCs and denuded oocytes were isolated by mechanical separation and stained for MTNRA1 using immunocytochemistry. COCs (n=295) were cultured in SOF media (synthetic oviductal fluid) up to 72hrs in a humidified atmosphere of 5% O2, 5% CO2 and 90% N2. Culture was carried out in the absence (control) or presence of increasing concentrations (1 nM, 100nM and 10µM) of melatonin. Oocytes nuclear maturation (resumption of meiosis; GVBD to metaphase II stages) and degeneration rate were assessed.

Results & Discussion: MTNRA1 was highly expressed in oocytes and with lower intensity in cumulus cells. Melatonin at, 100nM significantly increased the percentage of meiotic resumption to MII (32.34% ± 6.37; p <0.05). The lowest degeneration rate and GV arrested oocytes were also observed in the 100nM group (20.5% ± 3.21 and 6.71% ± 4.24 respectively; p <0.05). Presence of melatonin improved MII rate in all 3 concentrations compared to the control group (p <0.05). Results showed that melatonin can improve nuclear maturation along with reducing degeneration rates. The improving effects of melatonin can originate from both antioxidative feature of this compound and its molecular influence through MTNRs.

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