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In vitro maturation of canine oocytes: a unique conundrum

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Abstract

Oocyte maturation depends on multiple variables. In fact, in dogs, the requirements for oocyte development *in vitro* are so complex that achievement of female gamete maturation has been viewed as a difficult problem. The conundrum, thus far unanswered, of achieving dog oocyte maturation *in vitro*, is nowadays the major puzzle of the *in vitro* program in the canine species. In *in vitro* studies of dog oocytes, researchers are dealing with an entire spectrum of reproductive functions, from oocyte maturation to development, which are not entirely understood for the canine species. There have been many trials focusing on animal selection, oocyte quality identification, establishment of environmental conditions, and medium composition. Different systems were tried but have not produced satisfactory results. Differences in metabolic requirements are considered to be some of the major causes underlying the specific sensitivity of the canine oocyte to *in vitro* development. The main aim of this review is to provide the reproductive biologist with information on the novel trials being performed in the field of *in vitro* assisted reproductive technology (ART) for canines. The article focuses on *in vitro* oocyte maturation.

Keywords: dog, oocyte, *in vitro* maturation, conundrum.

Introduction

Although somatic cell nuclear transfer (cloning) is currently undoubtedly at the forefront of biotechnological research, both basic and applied reproductive studies are crucial to any further progress supporting *in vitro* techniques in canine species. The knowledge of factors involved in canine oocyte *in vitro* maturation is largely based on experiments performed in other mammals. Despite the fact that some information can be interchanged across species, a major challenge compromising the efficiency of canine oocyte *in vitro* maturation (IVM) is the highly variable and vulnerable behavior of the oocyte itself, whether at the level of gene expression or metabolism, which interferes with its own developmental competence. Thus far, no *in vitro* system has produced the maturation rates verified *in vivo*, a shortcoming that has been attributed to specific and highly complex requirements of *in vitro* canine oocyte maturation (Chastan-Maillard *et al.*, 2006). Once research results improve, the reproductive techniques of *in vitro* maturation, *in vitro* fertilization, and embryo transfers promise to be useful tools for animal breeding, endangered species conservation, and clinical application progresses. To learn more about the controlling mechanisms of *in vitro* oocyte maturation, events linked to natural steps of *in vivo* gamete maturation and fertilization are of particular importance. Furthering our understanding of the principles that govern these processes will help to shed light on suitable conditions to attain *in vitro* oocyte developmental competence and effective methods for manipulating the complex behavior of canine oocytes. This way, the ultimate goal of the *in vitro* program in dogs, which is embryo transfers, may progress reasonably well.

In this paper we review current approaches and limitations of assisted *in vitro* canine reproduction technologies, mainly about approaches to *in vitro* maturation from various studies, and focus on published data in this exciting field of research.

Primary factors influencing *in vitro* oocyte maturation

Oocyte maturation is linked to the adequate regulation of many molecular pathways, which are possibly those associated with genes that are only expressed by the maternal transcriptome (Fair *et al.*, 2007). Expression of pro- and anti-apoptotic genes may shift the oocyte´s developmental potential towards either cell death or cell survival (Van Soom *et al.,* 2007). From distinguishable ovarian populations, only fully-grown oocytes are able to resume meiosis and progress to maturation. MessengerRNA (mRNA) transcription is first down regulated in the fully-grown immature oocyte and ceases after germinal vesicle breakdown (GVBD; Fair *et al.*, 2007). Furthermore, certain genes are specifically up or down regulated in the metaphase II (MII)-stage oocytes relative to germinal vesicle (GV)-stage (Fair *et al.*, 2007). Location of transcripts to specific regions of the cell ensures that adequate concentrations of the encoded proteins are available where needed (Brevini *et al.*, 2007), and therefore available to specific signaling pathways. The abundance of mRNA and proteins contained in fully-grown oocytes from larger follicles (>6-8 mm) has a

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positive effect not only in cell maturation, but also on subsequent early embryonic development (Van Soom *et al.*, 2007). Therefore, the first designed strategy used to provide information on oocyte developmental capacity is the identification of a healthy gamete, because oocyte intrinsic viability is considered the most critical factor influencing *in vitro* maturation success.

The effect of the ovary donor and the follicle challenge

From studies conducted so far, oocytes destined to *in vitro* procedures are derived mostly from bitches at different stages of the estrous cycle (Otoi *et al.*, 2001; Hossein *et al.*, 2007) or different reproductive conditions (Rodrigues and Rodrigues, 2003). It has become clear that independent of the reproductive stage/status, within the ovaries retrieved from the suitable donor population, there is a restricted number of oocytes with maturational and developmental potential (Rodrigues *et al.*, 2007). Although *in vitro* meiosis of canine oocytes has been attributed by various authors to *in vivo* reproductive status of ovary donors (Yamada *et al.*, 1993; Luvoni *et al.*, 2001; Kim *et al.*, 2004), oocytes retrieved from ovaries of bitches at various reproductive phases of the estrous cycle and subjected to *in vitro* microenvironment can progress into meiosis (Songsasen and Wildt, 2005) and develop into early embryos up to and including the 8-cell stage (Rodrigues *et al.*, 2004).

The presence of larger (≥2 mm) follicles in ovaries of dogs during diestrous (Songsasen and Wildt, 2005) endorses our findings of good quality morphology embryos being produced at this phase of the estrous cycle (Rodrigues *et al.*, 2004, 2007). Nevertheless, more oocytes recovered from large (>2 mm) follicles have the capacity to mature *in vitro*, when development has been compared with that from oocytes derived from small antral (<1 mm) follicles (Songsasen and Wildt, 2005). Wesselowski (2008) showed that oocytes recovered from large follicles metabolize significantly more pyruvate, glutamine, and glucose (via glycolysis) than those from small ones. The same author demonstrated that oocytes collected from large follicles exhibit increased metabolic capabilities that may be responsible for oocyte higher developmental competence during culture.

Furthermore, the pre-ovulatory follicle is at high level of steroid secretion. Unfortunately, the *in vivo* pre-maturation preparative period, which is crucial for the acquisition of maturation, has been remarkably difficult to reproduce in *in vitro* studies with dog oocytes. Kim *et al.* (2005) revealed the improved capacity of *in vitro* matured oocytes from the follicular stage to progress to the metaphase II (MII) stage. It is worth noting that addition of estradiol-17β (E2) or progesterone (P4) dissociated significantly increased maturation of canine oocyte to MII and that a combination of the two hormones in medium further increased or decreased the oocyte developmental potential compared to E2 alone depending on P4 concentration.

As has been pointed out by others (Markides *et al.,* 1998), exogenous estrogens have antioxidant properties *in vitro* through their inhibition of 8-hydroxylation of DNA guanine bases. However, in an unnatural microenvironment even though exogenous steroid hormones into the culture medium act favoring cumulus oocyte complexes (COCs) metabolic reactions, a different profile of intracellular molecular cascades should be expected. Under such a static environmental condition, isotypes of the protein kinase (PKC) family, which are centrally involved in key developmental transitions, such as resumption of meiosis in the oocyte and regulation of spindle organization in meiosis I and II may be improperly activated or inhibited (Kalive *et al.*, 2010).

Serum progesterone concentration, which is high in estrous and in diestrous bitches, has been observed to mediate numerous physiological events on cumulus oocyte complexes during both *in vivo* and *in vitro* maturation (Kim *et al.*, 2005). However, in addition to the reported influence of *in vivo* progesterone (Willingham-Rocky *et al.*, 2003), other factors might be just as necessary for *in vitro* development of oocytes. During the luteal phase of reproductive cycle, the decrease of plasma progesterone concentrations is accompanied by a rise of prolactin (PRL). On a cellular level, PRL exerts mitogenic, morphogenic and secretory activities. This broad range of effects has led to the concept of a dual function of PRL, both as a circulating hormone and a cytokine (Ben-Jonathan *et al.*, 1996). In human *in vitro* fertilization, high concentrations of prolactin in follicular fluid are associated with maturation of the oocyte-cumulus complex, successful fertilization, and pregnancy (Laufer *et al.*, 1984). Yet there is no experimental data verifying the influence of PRL on *in vitro* ART in canines. However, in view of the aforementioned studies and comments, a comprehensive view of the PRL role in the *in vitro* maturation as well as development of dog oocytes could be advantageous in helping to explain the achievement of good quality embryos obtained by *in vitro* fertilization, in trials with canine oocytes derived from ovary donors at the luteal phase of both non-pregnant and pregnant cycles (Rodrigues, 2009, personal communication).

As documented by Kim *et al.* (2007), oocytes from follicular and luteal phases have higher glutathione (GSH) content when compared with those from the anestrous stage. GSH, an endogenous antioxidant, is the major non-protein sulphinyl compound in mammalian cells (Meister and Tate, 1976), and intracellular GSH content is linked to cytoplasmic maturation in the oocyte (Funahashi *et al*., 1994).

Similarities in the blood flow velocities and vascular impedance inside the ovaries of pregnant and

non-pregnant bitches by means of color-coded and pulsed Doppler ultrasonography were detected by Köster *et al.* (2001), who observed that ovulation and the early luteal phase were characterized by maximum ovarian blood perfusion. During luteinization of the follicle, granulosa cell activity plays an important role in the blood vessels recruitment and outgrowth from the thecal vascular plexus towards the inner compartments of the corpus luteum (Redmer and Reynolds, 1996). Follicles with the highest vascularity and blood flow velocities could contribute to the embryos with high developmental competence (Nargund, 2006) by regulating oxygen supply to the oocytes (Van Blerkom *et al.*, 1997). Thus, luteinized follicle vascularization, granulosa cell activity and possibly oocyte competence may be interrelated events (Picton *et al*., 1998).

Independent of the reproductive phase of the estrous cycle, follicle population in the ovary is heterogeneous with the topographical situation of the oocyte within the follicle being reported as linked to its potential to grow and to resume meiosis (Al-Mufti *et al.*, 1988).

A population of polyovular follicles is present in the ovaries of young, sexually mature bitches, at rates of 14% of the overall follicular population and have unknown viability (Telfer and Gosden, 1978; Wallner, 2007). It seems that polyovular follicles are formed in the same moment that primordial follicles emerge, when the future follicular cells surround more oocytes, enclosing them in the same follicle (Miclaus *et al.*, 2007). These follicles are comprised both by central and peripheric oocytes. Only the central oocytes are surrounded by a normally expanding cumulus; peripheral oocytes may resume meiosis, but the cumulus expansion is only partial and seems to depend on the topographical situation (Al-Mufti *et al.,* 1988). Despite the increase in both the overall ooplasmic mass and the granulosa cells, oocytes from polyovular follicles are smaller than those derived from uniovular counterparts (Telfer and Gosden, 1978). It has been suggested that the majority of the polyovulatory follicles are eliminated by atresia during the follicular growth cycle and the chance that one of them would reach ovulation is minimal (Miclaus *et al.*, 2007). Yet, the impact of the presence of canine polyovular follicles in IVM studies has not been assessed.

The effect of donor age on oocyte quality has also been investigated as a parameter in *in vitro* maturation studies. Over the past 10 years, canine ovaries have been obtained for *in vitro* procedures from shelters, private clinics and veterinary hospitals, preferably from young, healthy, and sexually mature bitches. However, due to the recognized benefits of spaying and neutering, a representative percentage of animals, included among which are strays and privately owned, has lately been sterilized at early ages. As a result, sexually immature bitches have been occasionally those providing the ovaries in IVM studies. It is important to stress that oocytes from pre-pubertal bitches do not mature well (Haenisch-Wohl *et al.,* 2003). These are oocytes characterized by accumulation of lipid yolk droplets in the ooplasm, high-energy metabolism, low protein synthesis, and high transcriptional activity in the cumulus cells (Haenisch-Woehl *et al.*, 2003). Although pre-pubertal bitches occasionally present tertiary follicles in the ovaries (Wallner, 2007), most oocytes obtained from these females are derived from small follicles, in which COCs express a deficiency of growth hormone receptors (Haenisch-Woehl *et al.*, 2003). Anguita *et al.* (2006) demonstrated that most oocytes with less than 110 µm diameter show DNA fragmentation before maturation. Continuing transcription, as observed in small bovine oocytes, indicates that oocyte growth is not complete (Fair *et al.*, 1995), and hence their complement of maternally derived mRNA necessary for early embryonic growth might also be incomplete (Cavilla *et al.*, 2008).

Accomplishing maturation: the "good" and the "bad" oocyte

Selection of oocytes for *in vitro* maturation is generally based on morphological criteria chosen for the purpose of ensuring oocyte viability. The appearance of the ooplasm and cumulus vestment is generally linked to the gamete maturation potential. Also, the ability to mature *in vitro* depends on the oocyte size, which varies in the bitch between 61.5 µm and 161.5 µm in diameter (Wallner, 2007). Canine tertiary follicles contain oocytes that average 96 µm (Wallner, 2007) and the early antral stage represents the condition where the oocyte is at maximal size (Songsasen *et al.*, 2009). High individual variations have been reported intra- and inter-female dogs with respect to size of oocytes comprised in the follicles (Theiss, 1997; Fujji *et al.*, 2000; Otoi *et al.*, 2000, 2001). As is common with other species, canine oocyte diameters > 100 µm are needed for *in vitro* meiosis resumption and completion of maturation (Hewitt and England, 1998; Srsen *et al.*, 1998; Otoi *et al.*, 2000; Songsasen *et al.*, 2005). In contrast, oocyte diameter size has been positively correlated with the incidence of apoptosis in COCs, and increasing levels of atresia in bovine COCs were reported to be accompanied by higher oocyte diameters (De Witt and Kruip, 2001).

Matters are further complicated by the fact that morphologically normal COCs are not necessarily those that will reach meiosis (Rodrigues and Rodrigues, 2006). These oocytes are probably bereft of molecular competence and incapable of successfully accomplishing embryo development. At the molecular level a reduced developmental competence of oocytes from pre-pubertal calves could be attributed to a deficient expression of glucose transporters and insufficient protein translation (Wrenzycki *et al.,* 2007).

According to Wrenzycki *et al.* (2005), the genes associated with developmental competence are known to be involved in the regulation of transcription and translation, post-translational modification of proteins, cell cycle regulation, folliculogenesis, oxidative stress defense, histone composition, gap junction signaling, prostaglandin synthesis, growth factor and cell signaling, extracellular matrix degrading components, metabolism, and transport systems.

Oocytes from grown follicles may not be fully sufficient for culture, because the follicle either might be undergoing atresia, its content might be inadequate by nature or composition (Murray *et al.*, 2008), or might be suffering from severe hypoxia. In humans, differences in oxygen content were registered between follicles of the same size from the same individual. Intrafollicular hypoxia may influence the normality of chromosomal organization and may inflict segregation disorders (anaphase lag, non-disjunction) in the oocyte (Van Blerkom *et al.*, 1997). Oocytes derived from such environments have their development compromised and may degenerate during culture. In general, degenerated or abnormally matured oocytes may be an expression of a defective *in vivo* environment that produces an oocyte with poor *in vitro* developmental capacity.

Thus, the diversity of substances in the follicular fluid (Kim *et al.*, 2006), the specific role of their presence such as the levels of the transforming growth factor-β superfamily (Wang and Sun, 2007), the role of signals arising from gonadotropin or steroidal actions within the follicle and the highly complex mechanisms controlled at the molecular level, have an effect on oocyte competence, its fertilization and further development. Following the observations of Murray *et al.* (2008) inappropriate exposure of the oocyte to steroids during follicle maturation may be detrimental to oocyte developmental competence and impact upon DNA methylation of the genome. Therefore, altered DNA methylation dynamics in the oocyte during its growth and maturation can negatively influence subsequent embryo development, though DNA methylation is a part of the mechanism involved in controlling normal expression patterns of imprinted genes.

In the near future, analysis of gene expression in granulosa and cumulus cells, as well as the oocyte, combined with physiology (functional genomics; Sirard *et al.*, 2007), is expected to provide clearer information about the acquisition of developmental competence of the canine gamete. In the meantime, different experiments have been conducted to identify and select high quality COCs by morphologic means. Besides the detection of glucose-6-phosphate dehydrogenase (G6PDH), there is as yet no reliable non-invasive method for oocyte selection (Van Soom *et al.*, 2007). G6PDH is an enzyme synthesized in growing oocytes. Whereas growing oocytes contain G6PDH, ones that have finished their growth show decreased G6PDH activity (Wu *et al.*, 2007). The activity in oocytes can be observed by using the brilliant cresyl blue (BCB) test, which is based on the capability of glucose-6-phosphate dehydrogenase (G6PDH) to convert the dye from blue to colorless. Thus, oocytes that have finished their growth will exhibit blue coloration (BCB+), whereas growing oocytes reduce the dye to a colorless solution (BCB-; Wu *et al.*, 2007). In species such as bovine (Pujol *et al*., 2000), swine (El Shourbagy *et al.*, 2006), and caprine (Rodríguez-González *et al.*, 2002), and more recently in murine (Wu *et al.*, 2007), the BCB test has been incorporated prior to culture for identifying fully-grown oocytes with the ability to mature to the MII stage. Also, we have used the BCB test to observe the level of blue color in grade 1 immature canine oocytes as an indirect quality and integrity indicator of nuclear chromatin configuration in COCs selected for *in vitro* maturation. Our findings showed that while few BCB+ stained oocytes (12%) were observed at the germinal vesicle breakdown (GVBD) stage, more oocytes were observed at the germinal vesicle (GV) stage, demonstrating that in dogs this is the most probable feature to be expected in grade 1 oocytes previously selected by visual morphological appearence (Rodrigues *et al.*, 2009a). In agreement with another study performed with mouse oocytes (Wu *et al*., 2007), we also observed that various grade 1 canine oocytes express an asynchrony in BCB impregnation between the cumulus cells and the ooplasm. The synchrony of BCB coloration between ooplasm and cumulus cells would suggest that the pentose phosphate pathway (PPP) metabolism of glucose is completely coupled between these cells (Wu *et al.*, 2007), and thus asynchrony in BCB coloration in COCs might suggest a metabolic uncoupling between the oocyte and its cumulus cells with impairment of GSH synthesis (de Matos *et al.*, 1997). The BCB absorbance asynchrony between cumulus cells and ooplasm might represent, as well, a physiological feature of canine COCs. The hypothesis can not be neglected, however this assertion remains to be investigated and proven.

Estimating oocyte viability by means of cumulus vestment

As morphologically indistinguishable grade 1 oocytes may differ in their capability to develop *in vitro*, it is very probable that oocyte competence is linked to both the qualitative (morphological integrity) and quantitative (maternal RNA stores, sufficient protein translation, etc.) traits. Parameters used as markers of oocyte maturation are nuclear morphology (Bolamba *et al.*, 2006; Santos *et al.*, 2006), redistribution of cortical granules (de Los Reyes *et al.*, 2007), cumulus cell expansion and embryonic development after *in vitro* fertilization (Rodrigues *et al.*, 2004, 2007). Usually, cumulus cells of dog oocytes matured *in vitro* expand to a medium degree. *In vitro* advanced cumulus expansion

in canine COCs is predominant in bitches with circulating progesterone concentrations greater than 2.5 ng/ml (Rodrigues *et al.*, 2009b). However, as previously reported (Rodrigues *et al*., 2007), degree of expansion in cumulus cells is still perceived as being an unreliable parameter in indicating canine oocyte developmental competence.

The occurrence and the recognition of signaling pathways in the COC compartments is a big issue, though the number of domains of interaction as well as the cooperation potential between the oocyte and its cumulus vestment may determine the destiny of the cell. Oocyte-specific genes have proved to be essential for normal oocyte, follicle, and embryo development. In a molecular biology study, Thélie *et al.*, (2007) have established that different genes may exert distinct roles during oocyte maturation and embryonic development. The expression profile of genes in COCs correlates to different outcomes. The abundance of gene transcripts in cumulus cells and the oocyte are implicated in the meiotic maturation stage *in vitro* (GVBD-MII). A high level of EP3 gene for example is found in grade 1 COCs, (for review see Wrenzycki *et al.* 2007). More recently it has been shown (Assou *et al*., 2008) that the up-regulation of Bcl-2-like protein 11 (BCL2L11) and phosphoenolpyruvate carboxykinase 1 (PCK1) genes in human cumulus cells results in successful pregnancy. These genes are respectively involved in apoptosis (programmed cell death) and regulation of gluconeogenesis.

Canine oocytes are particularly susceptible to degeneration, which begins after 24 h of *in vitro* culture (Saint Dizier *et al.*, 2001; Otoi *et al.*, 2007; Rodriguez *et al.*, 2008). Rates of degeneration of *in vitro* matured COCs reach values of 60-70% after 48 h of culture (Rodrigues and Rodrigues, 2003; Wesselowski, 2008). Larger intracellular spaces between the cumulus cells and the oocyte represent degenerative processes associated with atretic activation of the oocytes (Laurincik *et al.*, 1996). It is known that the cumulus cells and the oocyte are functionally and physically connected (Suzuki *et al*., 2000). Cumulus cells influence the developmental competence of oocytes (Bogliolo *et al.*, 2007) by facilitating transfer of nutrients to the internal compartments and protect the oocyte against oxidative stress-induced apoptosis through the enhancement of glutathione content in oocytes (Tatemoto *et al.*, 2000). Conversely, evidence exists of the role that oocytes play in preventing cumulus cell apoptosis by establishing a morphogenic gradient of oocyte-secreted factors. Hussein *et al.* (2005) used terminal deoxynucleotidyl transferasemediated dUTP nick end-labeling (TUNEL) assay together with quantitative confocal microscopy and showed that oocytes prevent apoptosis within cumulus cells by altering the ratio of Bcl-2 associated X Protein (Bax) to Apoptose Regulator Bcl-2 (Bcl 2) in favor of cell survival. In intact COCs, the authors showed that the incidence of apoptosis was lowest in the inner most layer of cumulus cells and increased with increasing distance from the oocyte.

From our observations by means Hoechst and propidium iodide staining, the rate of cell death is high both in pre-pubertal and adult bitches (100 vs. 87%) when viability of the cumulus cells is less than 50% (Rodriguez *et al.*, 2008). The degeneration of COC components, which are the somatic (cumulus cell) and the germ cell (oocyte), seems to follow a time amplifying dependent pattern (de Los Reyes *et al.*, 2005; Rodriguez *et al.*, 2008).

Reports in other species support the idea that degree of apoptosis in cumulus cells is negatively correlated with oocyte competence (Ikeda *et al.*, 2003; Yuan *et al.*, 2005). As observed recently in our laboratory, progression of nuclear maturation in dog oocytes is improved when more than 70% of cumulus cells in COCs are viable (Rodriguez *et al.*, 2008). Therefore, apoptosis influences the survival and/or developmental capacity of oocytes only after a defined threshold of cell death has occurred (Zeuner *et al.*, 2003).

Quantitative and qualitative features of apoptosis in the cumulus cells could be useful markers for predicting competence of COCs *in vitro*. Among the described features of apoptosis in cumulus cells of bovine oocytes (marginated chromatin, pyknotic appearance, multiple nuclear fragments, and apoptotic bodies; Yang and Rajamahendran, 2000), nuclear fragmentation was recently identified as a common sign of apoptosis in cumulus cells of canine COCs matured *in vitro* (Silva *et al.*, 2009b; Rodrigues *et al.*, 2009b). Fragmentation is reminiscent of cell division, and is a precisely timed event that takes place only in a mitotically active cell in response to altered cytoskeletal organization (Alikani *et al*., 2005). Many forms of cellular stress, included among which are environmental fluctuations during *in vitro* culture may trigger the process of fragmentation. The fragmented phenotype in cumulus mass is independent of degree of cumulus cell expansion and may be observed in COCs from bitches at various serum progesterone levels (Rodrigues *et al*., 2009b). Whereas numbers of apoptotic cells are predominant in cumulus cells from bitches with low (0-1 ng/ml) and high (>5 ng/ml) progesterone profiles, preovulatory to ovulatory circulating progesterone concentrations (2.6-5 ng/ml) seem to preserve the integrity of cells in cumulus mass in a more appropriate manner (Rodrigues *et al.*, 2009b). Therefore, threshold of apoptosis in cumulus cells may be listed among the parameters that could be used as a predictor of COC viability and possible developmental capacity.

A gene expression profile in human cumulus cells that correlated with embryo potential and successful pregnancy was identified by Assou *et al.* (2008). According to the authors (Assou *et al.*, 2008), the expression of the BCL2 family member BCL2L11,

PCK1 and Nuclear factor I B (NFIB) genes, which are linked to processes such as apoptosis, gluconeogenesis and embryogenesis, respectively, could be used as biomarkers for predicting pregnancy in embryo transfer programs.

Secondary factors influencing *in vitro* oocyte maturation

The culture medium

Hansen (2007) has outlined that many forms of cellular stress activate similar endpoints. The author (Hansen, 2007) also pointed out that factors that affect the quality of the oocyte, such as free radical formation, membrane destabilization, protein denaturation, DNA damage, and apoptosis, are known to influence embryo development and survival.

Oocytes removed from the follicle and exposed to an artificial environment (e.g., maturation medium) will experience changes associated with the action of compounds to which they are subjected. Moreover, spontaneous apoptosis occurs in COCs during suboptimal culture (Ikeda *et al.*, 2003; Esfandiari *et al.*, 2005; Yuan *et al.*, 2005). Aerobic metabolism is associated with the production of reactive oxygen species (ROS). ROS are formed as intermediary products of cellular metabolism; however *in vitro* environments usually increase the cell's production of ROS, which has been implicated as a main cause of cell damage. High ROS production or impaired antioxidant mechanisms results in oxidative stress with cellular DNA damage and apoptosis (Baka and Malamitsi-Puchner, 2006).

Specific commercial culture media can generate ROS depending on their composition, with media additives playing a role as ROS inducers (Agarwall *et al.*, 2006). Therefore, specific factors may interfere (positively or negatively) with the extent of either cell death or survival. A negative effect of bovine serum on the viability of canine COCs was observed in an experiment performed in our laboratory (Rodrigues *et al.* 2009b). High percentages of degenerated oocytes (73%) were observed in the serum-supplemented medium (10% (v/v) fetal calf serum) when compared to those cultured in defined high-glucose medium (11.0 mM glucose (56%)). Protein supplementation in the form of serum, which is commonly added to culture media, contains high levels of amine oxidase, favoring the increase in hydrogen peroxide (H_2O_2) production (Shannon, 1978), which in turn reduces the intracellular GSH level (Oyamada and Fukui, 2004). Also, serum has adverse effects on the structure of mitochondria by causing the accumulation of cytoplasmic lipids (Abe and Hoshi 2003).

Chemical challenge using glucose in the maturation medium, seems to enhance the rates of meiosis resumption (MR) and metaphase I (MI) stage of *in vitro* matured canine oocytes (31.4 and 24.3%, respectively; Silva *et al.*, 2009, personal communication). Glucose is the predominant energy substrate used by dog oocytes (Songsasen *et al.*, 2005; Wesselowsky, 2008). Over the course of oocyte maturation, a large proportion of total glucose is metabolized via the glycolytic pathway to provide substrates such as pyruvate for energy production (Sutton-McDowall *et al*., 2010). The capacity of the oocyte to utilize glucose is positively correlated with subsequent embryo developmental potential (Sutton *et al.*, 2003). However, glucose concentration in medium operates at an optimal level, whereby too much (>10 mM glucose) or too little (<2.3 mM glucose) produces negative effects during oocyte maturation (Thompson, 2006; Sutton-McDowall *et al*., 2010). An excessive concentration of glucose during *in vitro* maturation impaired the developmental competence of bovine (Hashimoto *et al.*, 2000) and hamster (Schini and Bavister, 1988) oocytes after *in vitro* fertilization. High glucose levels during IVM are associated with increased production of ROS, increased *O*-linked glycosylation via upregulation of the hexosamine biosynthesis pathway (HBP) and decreased concentrations of reduced GSH (Hashimoto *et al.*, 2000).

It was suggested by Reitzer *et al.* (1979) that the most important function of sugar for mammalian cell cultures may be to provide carbon for the pentose cycle metabolism. Glutamine utilization is a function of sugar metabolism pattern in medium. Glutamine oxidation provides ATP and the contribution to energy may vary according to the carbohydrate used as source of energy in medium (Reitzer *et al.*, 1979). According to Wongsrikeao *et al.* (2006), less oocytes complete nuclear maturation when fructose is the sole hexose source used during culture. Furthermore, the developmental competence of oocytes is improved by the presence of glucose and pyruvate combined (Downs and Hudson, 2000). Unfortunately, the optimal concentration of energy substrates that promote *in vitro* maturation is currently unknown. To date, only two studies were conducted specifically on dog oocyte metabolism (Songsasen *et al.*, 2005; Wesselowski, 2008). Furthermore, oocyte-mediated regulation of cumulus cell glycolysis has been viewed as a species-specific phenomenon (Sutton-McDowall *et al.*, 2010).

Cytoprotective mechanisms: oxygen tension

Oocytes are protected against oxidative stress by oxygen radical scavengers that are present in follicular fluid. Oviductal and uterine environments are characterized by an oxygen tension approximately one quarter to one third of atmospheric oxygen tensions (Esfandiari *et al.*, 2005). Reactive oxygen species (ROS) induce DNA damage and accelerate apoptosis. The extent of oxidative stress-induced damage depends on the amount, exposure duration, and type of ROS

involved (Combelles, 2009). Environmental factors, among which are oxygen tension and lack of protective antioxidant mechanisms present in follicular, oviductal and uterine environments (Agarwal and Allamaneni, 2006), act as variables influencing the generation of oxidative stress.

It seems likely that low oxygen tension mediates the redox state of mammalian oocytes and their activation potential (Iwamoto *et al.*, 2005). It was shown that excessive amounts of oxygen tension (hyperoxia) during the maturation period of the oocyte reduce the glutathione (GSH) effect of scavenging oxidative stress (Tatemoto *et al.*, 2000).

Songsasen *et al.* (2001) observed that achievement of nuclear oocyte maturation in the dog was not influenced by the oxygen concentration in medium. Nevertheless, we want to highlight that a low level of oxygen (O_2) tension (5%) may be necessary to maintain the viability of canine cumulus cells during IVM (Silva *et al.*, 2009b). Cumulus cells are able to synthesize high concentrations of GSH (Funahashi and Day, 1995), which is recognized as a participant in the process against oxidative damage (Kim *et al.*, 2004). Furthermore, these cells play an important role in enabling the matured oocyte to develop to the blastocyst stage (de Matos *et al*., 1997).

As reported by Silva *et al.* (2009b) canine oocytes cultured in high-glucose medium (11mM) resulted in less apoptosis in cumulus cells than those cultured in medium with fetal calf serum. These findings illustrate the way in which the effect of medium in conjunction with a low O_2 tension level positively influences the integrity of cumulus cells, its coupling with the oocyte and COC viability.

Cytoprotective mechanisms: antioxidants

Antioxidant enzymes can attenuate the effect of oxidative stress in different systems by scavenging ROS. Antioxidants counteract the fatal consequences of oxidative stress by enabling the redirection of metabolism flux from glycolysis to the pentose phosphate pathway. Several antioxidants, such as ascorbic acid (vitamin C), urate, isoflavones, taurine, hypotaurine (Alvarez and Storey, 1983), genistein, thiol compounds like cysteine and cysteamine (Pires, 2006; Hossein *et al.*, 2007; Cavalcante, 2009), α- tocopherol (vitamin E; Cavalcante, 2009), and β-mercaptoethanol (Songsasen *et al.*, 2002; Feugang *et al.*, 2004; Kim *et al.*, 2004) have been used as culture media supplements to reduce the risk of oxidative stress in dog oocytes. The amount of antioxidant in medium may contribute either to stimulatory or inhibitory effects in COCs.

Thiol compounds

In bovines, medium supplemented with cysteine has previously been shown to increase glutathione (GSH) synthesis in COCs (de Matos *et al.*, 1997). Nevertheless, in the literature, influence of cysteine in *in vitro* nuclear maturation of canine oocytes is presented with conflicting results, and reported either supporting nuclear maturation (Hossein *et al.*, 2007) or having no effects on meiosis (Pires, 2006; Cavalcante, 2009). The synergistic or antagonist actions of a compound in medium are dependent on a number of variables. For instance, the significance of a detrimental effect of cysteamine on IVM has been reported at certain dosages in medium (Guyader-Joly *et al.*, 1998). Since cysteine is rapidly metabolized in most cellular systems and readily oxidized to cystine in medium (Bannai, 1984), the availability of cysteine in medium is likely to influence the GSH concentration in COCs and, consequently in the rate of meiosis (Maedomari *et al.*, 2007).

Methods that increase cellular levels of GSH and therefore prevent oxidative stress include those based on administration of precursors of substrates for y-glutamylcysteine synthetase and GSH synthetase (Meister, 1994). Furthermore, *in vivo* matured canine oocytes were observed with higher concentrations of GSH when compared with their *in vitro* counterparts (Kim *et al*., 2007), where damage mainly affects the mitochondria responsible for transporting GSH from the cytosol (Meister, 1994).

Other cytoprotective compounds

A natural solution used for the preservation of gametes (Nunes, 1997; Cardoso *et al.*, 2006) and for culturing follicles (Martins *et al.,* 2005) *in vitro* is coconut (*Cocos nucifera*) water. Coconut water is rich in proteins, sugars, vitamins (ascorbic acid, folate, niacin, riboflavin), salt, neutral lipids (Marques, 1982), and aminoacids (glycine, cysteine, methionine, etc.). Also, coconut water is endowed with substances that can induce cellular division and electrolytes that promote the survival and viability of cryopreserved male and female gametes (Blume and Marques, 1994). Cytokines and zeatine ibozide (a substance that promotes growth in plants) have been isolated from coconut water. Also, the indole-3-acetic acid, which is an auxin (phytohormone), is present in coconut water. Auxin molecules play an essential role in the coordination of many growth and biological processes such as cell elongation and cell division in the plant life cycle. Indole-3-acetic acid was suggested to have binding properties to certain animal growth factors present in the ovarian tissue (Silva *et al.*, 2004).

A stable compound is powdered coconut water (ACP®; Salgueiro *et al.*, 2002). ACP® consists of a dehydrated form of coconut water in which the fruit is selected and its endospermic liquid is submitted to a heating treatment that alters its physical property into a thin and uniform powder. The powder is supplemented in medium used in *in vitro* systems designed for cell

culture. A study performed in our laboratory showed that high glucose (11mM) TCM 199 supplied with coconut powder (ACP-318; ACP Biotecnologia; Fortaleza, CE, Brazil) had little effect on nuclear maturation of dog oocytes in terms of developing to the MII stage. However, ACP medium with 5% powdered coconut water seems to exert a beneficial effect both on the maintenance of a typical chromatin configuration and on meiosis resumption in dog oocytes (Silva *et al.*, 2009a). It is thought that the efficiency of ACP-318 in medium is due to its antioxidant activity.

Other antioxidants tested in maturation medium for oocytes are vitamin C (ascorbic acid) and vitamin E (α-tocopherol). A beneficial role of vitamin C in protecting MII mouse oocyte spindle structure and chromosomal alignment against an oxidant (hydrogen peroxide)-induced damage was shown by Choi *et al.* (2007).

Results of Cavalcante (2009) showed that TCM 199 supplied with high levels of glucose (11 mM) and with 500 μM vitamin E contributed to the resumption of meiosis in dog oocytes at rates of 51.5 vs. 31.9% obtained in medium without vitamin E. Although the positive influence for meiosis resumption and a significantly higher percentage of metaphase MI-MII stages in oocytes cultured in medium supplemented with 500 μM α-tocopherol (22.9%; P = 0.014), in this study the concentration failed to enhance the extrusion of the first polar body (MII). This reinforces the complex biochemical pathway involved in the *in vitro* maturation of canine oocytes. One of the possible functions of α-tocopherol is preventing cumulus cell DNA from fragmentation (Tao *et al*., 2004) and therefore, maintaining the GSH synthesis in COCs.

Except for the study performed by Hossein *et al.* (2007), where addition of 0.5 mM cysteine and 100 microM cysteamine to the maturation medium improved IVM of canine oocytes, differences in the effects exerted by antioxidants in oocytes of dogs remain to be established.

As stated by Combelles (2009), the use and potential benefits of antioxidants during *in vitro* culture still remains a challenge. The author emphasized that the exact amounts of pro- and antioxidants that best support the somatic and germ cells of the COC still needs to be quantified. Similarly, further studies are needed to provide answers as to the usefulness and effectiveness of antioxidants for dog oocyte *in vitro* maturation.

Concluding remarks

Inadequacy of current systems for *in vitro* maturation of dog oocytes is due to specific requirements of the canine gamete to achieve full developmental competence. Many issues, among which are included those that can be used to elucidate intra and extracellular pathways controlling oocyte maturation in *in vitro* systems, need to be made clear. Major developments in the field of molecular biology will give valuable insights into different mechanisms underlying cell survival or death. We have come to understand the way to circumvent the dog oocyte's remarkable sensitivity to degeneration. Stimulating the appropriate *in vitro* metabolic pathways is probably among the more significant factors in predicting oocyte responsiveness, in preventing or fighting the damage imposed by the *in vitro* environment, and managing the situation as well. In conclusion, further studies deciphering the interaction/incorporation between COCs and *in vitro* environments will be informative for our understanding of the conundrum of the canine oocyte maturation and its developmental competence.

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