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THREE-DIMENSIONAL SCAFFOLDS FOR IN VITRO CULTURE OF FELINE OOCYTES

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INTRODUCTION

The assisted reproductive technologies (ARTs), oocyte in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro embryo culture (IVC), are aimed at the conservation of biologic and genetic biodiversity. The in vitro embryo production (IVEP) is crucial for threatened and endangered wild species, but its efficiency is still low because of the poor information on cross-species embryology (Pukazhenthi & Wildt, 2004; Pukazhenthi et al., 2006). Conversely, in the livestock animals, as ruminants, the IVEP gives good and repeatable results and is widely used for improving reproductive and genetic performances (Galli et al., 2014; Paramio & Izquierdo, 2014).

In the domestic carnivores, the embryo-related technologies are still at an experimental level because the efficiency and the clinical application are very limited. However, the availability of gonads from routinary surgery provides a source of gametes for experimental studies aimed at increasing the knowledge about reproductive biology and improving the ARTs in these small animals. Furthermore, domestic cats and dogs are optimal animal models for wild felids and canids.

The domestic cat (*Felis catus*) is also an optimal research model for studying human diseases, as anatomic, oncologic or genetic dysfunctions (e.g. Klinefelter's syndrome), and for improving fertility preservation procedures (Goodrowe et al.,

1988). The morphological and biological similarities between human and domestic cat reproductive features, as the ovarian tunica albuginea, the oocytes and the germinal vesicle (GV) characteristics, the timing of in vitro maturation (24 hours), and the reproductive conditions and environment have been well documented (Comizzoli et al., 2010).

Wildt and co-workers (2010) underlined other specific elements that attested the closest connection between these two species, as the male teratospermia or the female infertility syndromes, the asynchronous oocyte cytoplasmic and nuclear maturation, the ovarian hypersensitivity and the luteal dysfunction after gonadotropin therapy.

The increasing demand in the human and wildlife ARTs generates the growing interest in the reproductive biotechnologies of the domestic cat.

1.1. In vitro embryo production in the domestic cat

To ensure a successful IVEP, the availability of highly competent gametes is required.

Talking about the oocytes, the full competence is the inner ability to mature, be fertilized and develop to the late embryo stages. The cumulus-oocyte complex (COC), i.e. the oocyte surrounded by multiple layers of specialized cumulus cells

(CCs), is the functional unit for the in vitro biotechnologies. However, the recovered female gametes from isolated ovaries are a heterogeneous population made of competent and non-competent COCs derived from growing or atresic follicles. The selection of optimal COCs for IVEP is based on their morphological appearance that in the domestic cat and other carnivores (dog, leopard, tiger, and ferret) has been categorized in four distinct grades (Guraya, 1965; Wood & Wildt, 1997).

The grade I (=excellent) shows uniform, spherical and dark cytoplasm, due to the high intracellular lipid concentration, combined with a full complement of multiple and tightly compacted CCs layers (Fig. 1). The grade II (good) has the main characteristics of the grade I, but with fewer layers (2-4) of surrounding CCs. Conversely, the grade III (fair) shows less cytoplasmic uniformity visible as a diffused transparency, synonymous of large lipid droplets aggregation. These COCs show only a partial covering of proximal CCs "corona radiata" that lay adjacent to the zona pellucida (ZP). Finally, the grade IV (poor) presents elevated cytoplasm fragmentation and partial or total dissociation of corona radiata and CCs. It has been demonstrated that the excellent and good grades (I and II) COCs are characterized by high maturational and developmental rates, compared to the fair and the poor grades (III and IV) that show high levels of atresia (Wood & Wildt, 1997).

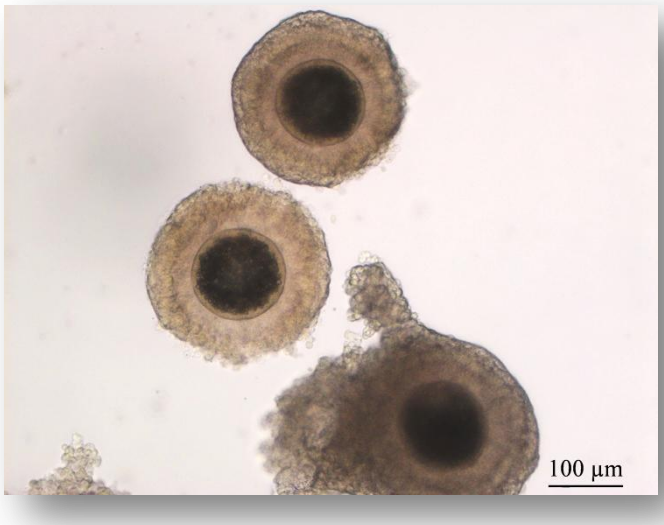

Figure 1. Domestic cat grade I cumulus-oocyte complexes (COCs).

Cumulus-oocyte complexes (COCs): the high competence ova

The presence of multiple layers of CCs surrounding the oocyte is an important and essential feature for an optimal IVEP. The intimate relationship between the germinal and the somatic compartments has been widely demonstrated. The oocyte and the surrounding CCs are closely associated during all stages of follicular development, primarily by numerous projections that cross the ZP and penetrate deeply into the ooplasma, very close to the oocyte nuclear envelope, as evidenced by electron microscopy in different mammalian species (Motta et al., 1994; Albertini et al., 2001). Different junctional complexes provide the structural anchorage for maintaining the compartments association and

ensuring the transfer of metabolites and messenger molecules, nutrients and hormones (Eppig, 1982; Buccione et al., 1990; Motta et al., 1994).

This strong and highly specialized interaction is of crucial importance during the growth, the maturation and the subsequent embryo development of female gametes (Eppig, 1982). It is well known that during the follicle development there is a dynamic alteration of the trans-zonal projections (TZPs), as their number increases and their form changes to coordinate the oocyte growth and the maturation events. Motta et al. (1994) showed that in the human preantral follicles the TZPs are numerous and adhesively connected to the oolemma, with deep invaginations reaching the oocyte nucleus. During the antral stage, the TZPs start to retract from the inner ooplasma, maintaining only fewer connections, since a complete retraction occurs during ovulation making the CCs totally uncoupled from the oocyte (Albertini et al., 2001).

The signaling pathway from oocytes and CCs also involves other specific junctional complexes, the gap junctions (GJs). They are intercellular membrane channels that allow the active transfer of inorganic ions, second messengers, and small metabolites (<1 kDa, Standring, 2009). A single GJ is composed by a channel of connexons, structurally constituted by a hexamer of connexins. The connexins are membrane proteins with different sizes and are produced by different specie-specific genes. They are composed by four membrane-spanning

domains, two extracellular and one cytoplasmic loops, and cytoplasmic –N and –C termini. The variable sequences of the cytoplasmic loop and of the –C terminal determine the specific biophysical and regulatory properties of the GJ in different tissue and organs (Bruzzone et al., 1996; Kidder & Mhawi, 2002).

The active participation of the GJs during the meiosis resumption of the fully grown mammalian oocytes has been documented by the observation of the dynamic changes in the connexin localization during IVM (Fagbohun & Downs, 1991).

For instance, in the bovine species, the connexin 43 (CX43) has been localized in the COCs by an immunohistochemical staining during 24 hours of IVM (Vozzi et al., 2001; Luciano et al., 2004). The CX43 fluorescent signals cross weakly the ZP at the beginning of IVM, and are strongly present in the CCs in large dots. After 3 h, the signals appear in the oocyte and CCs cytoplasm, and at the end of IVM, the signals increase in the inner oocyte cytoplasm with a contemporary decrease in the CCs directly in contact with the oocyte.

Meiotic balance: arrest and resumption

The oocytes are typically arrested at prophase I of the first meiotic division, until the surge of circulating gonadotropins induces the resumption of meiosis to complete the nuclear divisions and to achieve the metaphase II (MII) stage, prior to or soon after the ovulation (Dekel and Beers, 1980). The active coupling of the oocyte and the surrounding CCs is necessary for the acquisition of the full developmental competence (Luvoni et al., 2006). The prolongation of this coupling in the pre-ovulatory oocytes provides the transfer of some molecules in the ooplasma that maintain the block of the cell cycle, ensuring the completion of the cytoplasmic maturation, i.e. oocyte capacitation (Hyttel et al., 1997). In this process, an increase of the lipid storage, a reduction in the Golgi compartment, a redistribution of the ribosomes near the nucleus and the alignment of the cortical granules under the oolemma, are observed. In addition, the synthesis and the storage of different proteins and of specific maternal transcripts (mRNA) to provide the capability of supporting the monospermic fertilization, the pronuclear formation, and the early embryo development, occur (Tanghe et al., 2002; Diez et al., 2005).

In this scenario, the mediation of regulatory molecules via the oocyte-CCs GJs is essential (Fig. 2). Three major signals are involved in the meiotic arrest and resumption: calcium, cyclic adenosine 3',5'-monophosphate (cAMP), and

membrane potential. After the gonadotropin surge, the intracellular levels of calcium in the CCs increase and, due to the GJs coupling, this signal reaches the oocyte in few minutes, ensuring its activation. There are evidences that the absence of surrounding CCs or their active removal from the oocyte prevents the intra-oocyte calcium action, impairing the cytoplasmic maturation (Zuelke et al., 1991; Homa, 1995).

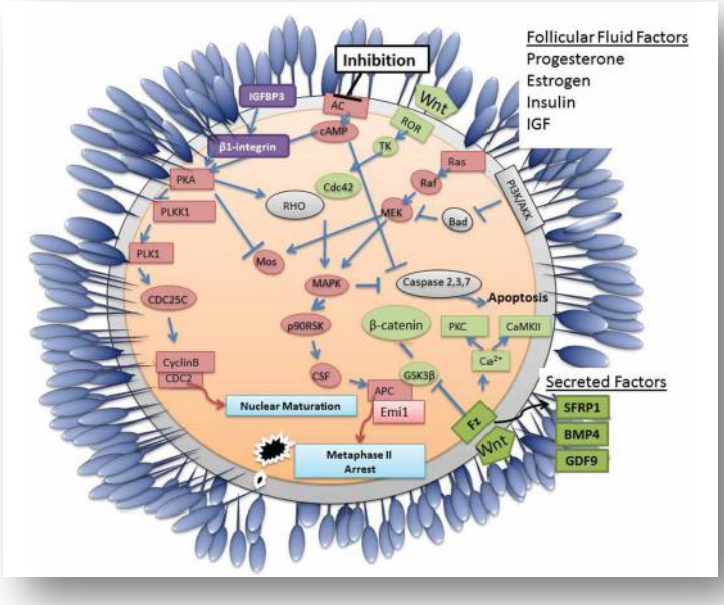

Figure 2. Molecular pattern for the achievement of full oocyte competence (O'Shea et al., 2012).

Another second messenger, the cAMP peaks in the CCs and in the oocyte at the same time. Before the gonadotropin stimulation, the oocyte meiotic arrest is ensured by the maintenance of a threshold level of this molecule modulated by its active synthesis by a specific protein complex (G-protein and adenylate

cyclase 2, ADK2), and by its degradation provided by the phosphodiesterase 3A (PDE3A). The cAMP is reported to be responsible for preventing the activation of the cyclin dependent kinase 1 (CDK1), the catalytic kinase subunit that together with the regulatory cyclin B1 subunit, forms the maturation promoting factor (MPF). The MPF is directly involved in the signal pathway leading to the breakdown of the nuclear envelope and to the occurrence of germinal vesicle break down (GVBD stage, Tsafriri et al., 1996; Vaccari et al., 2009; Adhikari & Liu, 2014). Due to the diffusion through the oocyte-CCs GJs of some specific regulatory molecules, as the cyclic guanosine monophosphate (cGMP) that modulates the PDE3A activity, the cAMP threshold level is ensured.

Finally, a membrane depolarization occurs in the somatic cells, particularly in the CCs, due to the action of specific enzymes. The CCs-GJs coupling extends the depolarization to the oocyte, influencing its meiotic resumption (Mattioli & Barboni, 2000).

After the gonadotropin induction, the oocyte meiosis resumption is actively coordinated by theca and granulosa cells (GCs) that, after a ligand-receptor link, are induced to secrete specific factors in the follicular fluid. The CCs also participate in the transfer of signals to the oocyte, as specific LH-receptors are present on their membrane (Peng et al., 1991; Mattioli, 1994; Mattioli et al., 1998).

After the gonadotropin surge some paracrine factors, known as the cumulus expansion-enabling factors (CEEFs, e.g. growth differentiation factor 9, GDF9 and bone morphogenetic factor 15, BMP15), are produced in the GCs and CCs in order to propagate the gonadotropin signal and to promote a specific process, so-called "cumulus expansion" (Dragovic et al., 2007; Nagyova, 2012; Nevoral et al., 2014). This event involves an extensive rearrangement of the CCs cytoskeleton and the active synthesis of proteoglycans and glycosaminoglycans, as the hyaluronic acid (HA), to transform the strictly packed cumulus complex in a mucified extracellular matrix (ECM) with a higher viscosity. During this phenomenon, the CCs lose their contact with each other, moving outward the oocyte along the hyaluronan polymeric backbone. The uncoupling of the GJs interrupts the communication between the somatic and the germinal compartments, enabling the oocyte to resume the meiosis. The diffusion of extracellular cGMP is stopped and the degradation of cAMP by the PDE3A within the oocyte increases, triggering a pathway of phosphorylations and dephosphorylations of MPF and mitogen-activated protein kinase (MAPK) to induce the GVBD and the achievement of the mature stage of MII (Aberdam et al., 1987; Nurse, 1990). When an abnormal uncoupling of the GJs between the somatic and the germinal compartments occurs, the achievement of the full maturational and developmental competence is negatively affected (carnivores, Luvoni et al., 2001; horses, Colleoni et al., 2004; bovine, Luciano et al., 2004).

Cumulus-denuded oocytes (CDOs): the poor competence ova

As previously mentioned, the oocytes with poor morphological characteristics (grade III and IV) are often associated with low rates of IVM and embryo development (Davachi et al., 2012; Auclair et al., 2013). Among these, oocytes without CCs are commonly referred to as cumulus-denuded oocytes (CDOs, Fig. 3). The absence of CCs and related secreted factors negatively affects the metabolism and the functionality of these oocytes, leading to an aberrant cytoplasmic maturation and an impaired cumulus-related lipid metabolism.

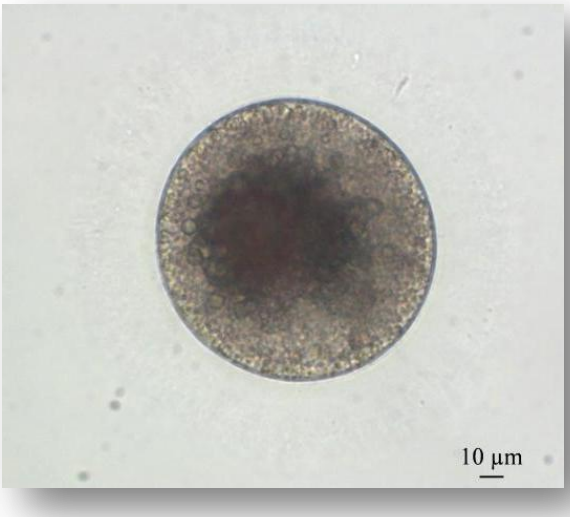

Figure 3. Domestic cat cumulus-denuded oocyte (CDO).

Improving the IVEP of low competence oocytes is an emerging goal and there are pragmatic reasons for exploring their in vitro developmental potential.

Gametes as the CDOs could represent an interesting option for widening the germinal pool in the case of high value individuals with very precious genetic material (Puzakenthi et al., 2006), and could be the only genetic source when the selected COCs (grade I) are cryopreserved.

The oocyte cryobanking is the main tool for long-term preservation of genetic biodiversity (Luvoni, 2006). However, the exposure to non-physiological conditions, as the sub-zero temperatures of liquid nitrogen, the presence of cryoprotectant agents, and the osmolality differences between the extra- and intracellular environments, could lead to severe collateral effects on the cell structures (Parks, 1997). The cold-induced effects to the surrounding CCs is due to the different size, structure and permeability of the somatic cells compared to the germinal cell, which make the CCs more susceptible to the cryoprotectant agents and to the cold temperatures (Luvoni, 2006; Songsasen & Comizzoli, 2009). As already mentioned, the presence of CCs is necessary for the proper oocyte maturation, but after thawing, the competence of the oocytes might decrease for the absence of CCs.

The in vitro culture of CDOs could also be necessary during the procedures of gamete reconstruction, as the GV transfer (GVT). The removal of surrounding CCs from the immature GV oocytes is essential for the micro-injection of the donor nucleus (karyoplast at GV stage) into the enucleated recipient (cytoplast) to obtain the artificial GVT oocyte. This innovative

technique offers the opportunity to investigate the role of maternal epigenetic modifications during the oocyte growth and the timing of the genomic imprinting. These studies might clarify the interspecific nuclear and cytoplasmic factors which regulate the oocyte meiotic progression (Franciosi et al., 2010).

Therefore, searching the optimal enriched culture condition for oocytes without the CCs support (CDOs) would greatly facilitate such biotechnological procedures.

COCs & CDOs in vitro embryo production

The history of domestic cat IVEP started in the 1977 with the first successful IVF of in vivo matured COCs with epididymal spermatozoa (Bowen, 1977). The better knowledge of the domestic cat endocrinology and physiology prompted the development of the in vitro reproductive biotechnologies. Induced superovulation, after gonadotropin treatments, ensured the chance to recover a population of in vivo matured COCs for IVF/IVC and embryo transfer (ET) into synchronized recipients (Goodrowe et al., 1988). In the '90s, some researchers focused their attention on the study of IVM protocols (Johnston et al., 1989 - 1993; Goodrowe et al., 1991) and the first full in vitro procedure of IVEP was documented in the 1997 by Pope and co-workers. In

this work, IVM of immature COCs followed by IVF with ejaculated spermatozoa, IVC of presumptive zygotes, and subsequent ET (at morulae and blastocyst stages) were successfully performed. The same scientific team performed the first intra-cytoplasmic sperm injection (ICSI) in the domestic cat with in vivo (Pope et al., 1998) and in vitro matured COCs (Gómez et al., 2000), reporting that this technique could be successfully applied in this species.

The cryopreservation of the domestic cat gametes and embryos was the main challenge of the third millennium. In the 2000, Luvoni and Pellizzari reported the successful embryo development after the IVF of cryopreserved in vitro matured cat oocytes and in the 2012 the important goals of live birth kittens from vitrified in vitro matured COCs were obtained (Pope et al., 2012; Tharasanit et al., 2012).

Domestic cat COCs have also been used to produce cloned embryos of domestic and non-domestic felids by innovative techniques as the somatic cell nuclear transfer (SCNT). Some reports demonstrated that the cat COCs can reprogram the nuclei of several endangered felid cells, as confirmed by the in vitro development until the blastocyst stage and/or the production of live offspring (Gómez et al., 2006, 2009).

Regarding the IVEP with CDOs, the studies were mainly focused on other mammalian species, as cattle and mice, than the domestic cat. In vitro culture of bovine and murine CDOs with autologous or heterologous isolated GCs or CCs, as monolayer – suspensions - clumps or conditioned medium, has been performed (Hashimoto et al., 1998; Ikeda et al., 2000; Tanghe et al., 2003; Ge et al., 2008; Zhao et al., 2014). The presence of companion cells promoted the maturation and the meiotic progression of the murine CDOs by the improvement of different quality parameters (spindle assembly, mitochondrial congregation, glutathione intracellular levels and dynamics of MPF), normally impaired when CDOs were cultured separately. In the bovine species, the CDOs embryo development until blastocyst stage was also enhanced by the presence of companion cells, although at lower frequencies than that of the competent COCs.

The COCs themselves have been used as companion cells for the CDOs in vitro culture. A beneficial effect on the restoration of full developmental capability of low competence oocytes, although at lower rates compared to the COCs control, was observed (Luciano et al., 2005). The oocyte maturation rate, the glutathione level, the embryo development capacity, the blastocyst quality and the genetic expression of some transcripts were also found significantly improved than those of the CDOs cultured separately (pig, Lin et al., 2016).

In the domestic cat, Chigioni and co-workers (2005) found that the co-culture of CDOs with unattached CCs clumps or with competent COCs during IVM enhanced the meiosis resumption of the low competence gametes to a similar level of that of COCs control. Another effort to improve the cat CDOs developmental capability was later investigated by Godard and co-workers (2009). The authors found that the presence of COCs in the same culture condition with CDOs during both IVM and IVF improved the maturation rates and the embryonic developmental stages compared to that of the CDOs cultured separately.

The mammalian oocytes are known to produce a broad range of secreted factors, referred to as oocyte-secreted factors (OSFs) which exert their action on both oocyte and CCs. Many authors report that the OSFs released in the culture media are promoting molecules for the oocyte overall competence (Table I; Gilchrist et al., 2004; Gilchrist et al., 2006; Gilchrist et al., 2008). Some of those secreted factors, as GDF9 and BMP15, are known to regulate the differentiation, activities and genetic expression of the CCs in different species (Hussein et al., 2006; Gilchrist et al., 2008; Yeo et al., 2008; De Los Reyes et al., 2013).

In particular, the GDF9 plays an important role in the CCs expansion and in the over-expression of some cumulus related genes (cumulus matrix genes) as the hyaluronic acid synthase 2 (HAS2) and the cyclooxygenase 2 (COX2)

throughout the "small mother against decapentaplegic" (SMAD) pathway. Furthermore, it is involved in the steroidogenesis process, promoting the progesterone (P4) synthesis and blocking the expression of LH membrane receptors on cumulus cells (Gilchrist et al., 2008).

Table I. Effect of the oocyte-secreted factors (OSFs) on mural granulosa cells (MGCs), cumulus cells (CCs) or oocyte functions (modified from Gilchrist et al., 2008).

Signaling cascades	Activation of GCs/CCs SMAD signaling Activation of MAPK signaling
Oocyte growth	Stimulation or suppression of KitL
CCs/MGCs proliferation	Stimulation of Ccnd2 Stimulation of GCs/CCs DNA synthesis, cell number or follicle growth Interaction with IGF-I Stimulation of CCs Ar
CCs apoptosis	Prevention of CCs apoptosis
CCs/MGCs luteinization	Regulation of MGCs/CCs progesterone or estradiol production Suppression of FSH-induced Lhcgr Regulation of MGCs inhibin – follistatin - activin production Stimulation of CCs Amh Suppression of CCs Cd44
CCs metabolism	Stimulation of CCs glycolysis Stimulation of CCs aminoacid transport
CCs expansion	Enabling FSH/EGF-induction of expansion genes and secretion of hyaluronic acid Regulation of plasminogen activator
Oocyte quality	IVM additive increasing blastocyst development and fetal survival

SMAD, small mother against decapentaplegic; MAPK, mitogen-activated protein kinase; KitL, protein-tyrosine kinase; Ccnd2, cyclin D2; IGF-I, insulin growth factor-I; Ar, androgen receptor; FSH, follicle stimulation hormone; Lhcgr, luteinizing hormone/choriogonadotropin receptor; Amh, anti-müllerian hormone, Cd44, CD44 molecule (indian blood group); EGF, epidermal growth factor.

1.2. In vitro culture systems: 2D vs 3D

Traditionally, the in vitro culture of mammalian follicles and oocytes is performed in two-dimensional systems (2D, or non-spherical), as microdrops in petri dishes, multiwell plates, and membranes coated with ECM proteins. Although these systems provide a good environment for the cell culture, their flat surfaces cause the adhesion of the surrounding cells of follicles and oocytes to the substrata, causing the perturbation of spatial arrangements (Fig. 4). The disruption of the communication network between the somatic and the germinal compartments and the high risk of phenotypic changes of the CCs can also occur (Desai et al., 2010). Therefore, these conditions could lead to a distorted cell-cell orientation and to an abnormal diffusion of the paracrine signals away from the target cell-surface receptors (Cukierman et al, 2002; Vanhoutte, 2009). It has been reported that the 2D systems can also influence the physiological polarity of the cells, modifying the GCs secretion of different factors, such as P4 and 17β estradiol (Kreeger et al., 2006). In the human reproductive biotechnologies, these conditions could not be adequate for the culture of frozen/thawed ovarian cortex follicles. These biological structures require longer time to achieve the full development in vitro and the traditional 2D systems could lead to aberrant conformations and functionalities of the cultured follicles (Abir et al., 2006).

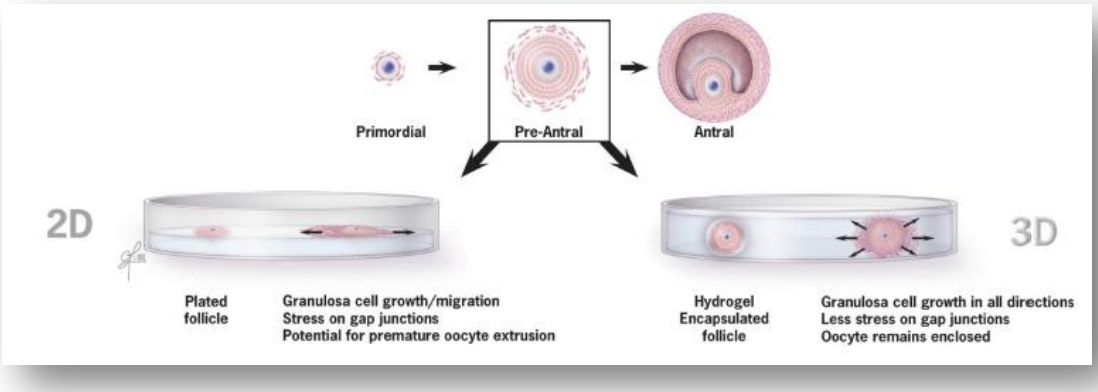

Figure 4. Comparison of in vitro culture in 2D or in 3D systems (modified from Desai et al., 2010).

In the past years, many studies were focused on the development of innovative three-dimensional (3D, or spherical) culture systems: the scaffolds. They mimic the biological shape and structure of the tissue ECM in which the cells are physiologically immersed. Their action is to drive the cell spatial organization and to stimulate their adhesion, growth, proliferation and production of secreted factors, essential for tissue repairing (Desai et al., 2010). In these particular 3D conditions, the cells can maintain their physiological architecture and can recreate their specific microenvironment as in vivo (Altman et al., 2003). It has been documented that the cell behavior, growth, response to stimuli, signaling and gene expression profiles in the 3D scaffolds, compared to the traditional 2D systems, are most resembling those observed in living cells (Cukierman et al., 2002; Kreeger et al., 2006). Furthermore, a different pattern of

specific receptors for cell adhesion to the artificial ECM is activated in the 3D scaffolds than in the 2D substrata (Cukierman et al., 2001).

To guarantee the physiological cell architecture, the scaffolds should be composed by biomaterials, defined as "*any substance (other than a drug) or combination of substances synthetic or natural in origin, which can be used any time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ or function of the body*" (Von Recum & LaBerge, 1995).

The main characteristics of the biomaterials are listed below (Lloyd, 2002; Dischер et al., 2005; Desai et al., 2010).

- Biocompatibility: integration in the organism without evoking a sustained inflammatory or toxic response during in vivo implantation, ensuring cell viability for short and long culture period;
- acceptable shelf life;
- biodegradability: degradation time linear with healing or regeneration processes;
- non cytotoxicity: the degradation products should be non-toxic, for easy metabolism and clearing from the organism;
- permeability: to allow adequate gas exchange, diffusion of nutrients and removal of cellular waste;
- elasticity: to allow the spatial growth and expansion of the cells;

- mechanical properties, as viscosity, modeling and rigidity: specific for indicated application and compatible with healing or regeneration processes.

A wide range of biomaterials with different physical and chemical characteristics were tested for specific applications (Williams, 2009). For tissue regeneration, synthetic or natural polymers were widely used.

Synthetic polymers are characterized by the absence of cellular component, viability, and self-repair due to their inorganic properties (Zhong et al., 2010). These polymers show poor integration with the biological environment and a high production of toxic molecules during the in situ degradation, with a decreased of in vivo biocompatibility. Conversely, the natural polymers composed by the biological molecules of the ECM are characterized by heterogeneity, viability, low organic toxicity and self-repair (Fan et al., 2015). For instance, different component of natural ECM, as collagen, HA and silk fibroin have been successfully applied in tissue regeneration as skin grafts (Altman et al., 2003; Dieckmann et al., 2010). Among these natural polymers, alginate is an interesting biomaterial for biotechnological applications.

Alginate is an anionic polymer produced by the alginic acid of the cellular wall of brown algae (*Phaeophyceae*), particularly of the genus *Laminaria*. It is the main component of the algae skeleton and it confers resistance and flexibility.

Alginic acid is composed by two types of uronic acids, β-$_D$-mannuronic acid (M) and α–$_L$-guluronic acid (G), that are converted to mannuronate and guluronate salts to forming alginate after a neutralization process (Brito et al., 2014). The alginate skeleton contains blocks of purely G, purely M and alternating G and M monomers, that in the presence of divalent or multivalent cations (Ca_{2+} or Ba_{2+}) interact ionically with G blocks between two different chains forming a 3D network (Fig. 5).

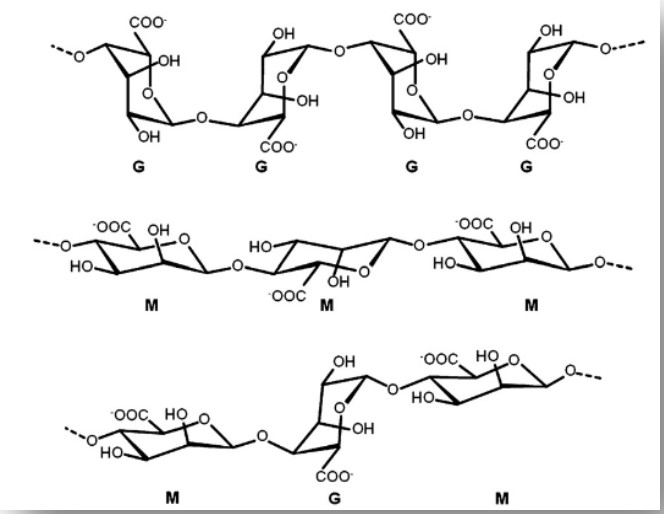

Figure 5. Chemical structures of G-block, M-block and alternating blocks (3D network) of alginate (Lee & Mooney, 2012).

The features of the alginate 3D network are reported to be strictly dependent on the alginate molecular weight, the M/G ratio and the G-block length (Vigo et al., 2004; Lee & Mooney, 2012; Brito et al., 2014) The interesting properties of alginate, as high biocompatibility – biodegradability – no specifically interaction

with mammalian cells – easy handling, make this polymer a valid biomaterial for cell encapsulation in the regenerative medicine and in the tissue culture techniques (Gombotz & Wee, 1998; Huang et al., 2012; Lee & Mooney, 2012).

Follicles, oocytes & embryos in a new dimension: the 3D system

Other than in the regenerative medicine, the 3D engineering was successfully applied in the reproductive biotechnologies and the efficacy of the 3D systems for the IVC of mammalian follicles and oocytes has been confirmed (Ghidoni et al., 2008).

The 3D technologies prompted the modern concept of microencapsulation. The entrapment of follicles and oocytes in microstructures (microcapsules) that provide an adequate microenvironment to maintain differentiated functions is the main challenge in the contemporary ARTs.

In case of restoring the fertility potential of young cancer patients, this micro-technology could ensure the in vitro growth of immature primordial follicles until primary or secondary stages and the subsequent achievement of oocyte full development for IVF (Xu et al., 2009). In the domestic animals, the IVC of canine and feline follicles in different 3D scaffolds has been also evaluated. The encapsulation in gel or in microcapsules maintained the structural

integrity of the follicles that augmented in size and secreted hormones, as in the in vivo conditions (Songsasen et al., 2011; Fujihara et al., 2012).

The alginate as biocompatible 3D scaffold is considered suitable for mammalian follicles (West et al., 2007). An encouraging follicle development in pure or modified alginate scaffolds were obtained in different species (mouse, Kreeger et al., 2006; human, Amorim et al., 2009; domestic cat, Fujihara et al., 2012; macaque, Xu et al., 2013) and the proliferation and differentiation of theca and GCs, the steroid secretions and the specific markers expression were observed (Xu et al., 2006; Shikanov et al., 2011). The suitability of the 3D alginate scaffolds has been recently validated in the mouse model by Parrish and co-workers (2011). The authors found that almost 60% of the gene pattern expression of follicles, as well as some oocyte-specific genes (Figlα, Jag1, Mater, Nobox, and Vasa), were similar between the in vitro and the in vivo conditions, although some differences in the endocrine-related and growth-related genes (BMP15, Tgfβ) were found.

Different biomaterials (collagen, agarose, and alginate) have also been used for the 3D IVC of mammalian oocytes in different species (dog, Otoi et al., 2006; pig, Munari et al., 2007; human, Combelles et al., 2005). All natural polymers promoted the oocytes maturation until the MI/MII stage, ensuring the achievement of the full competence for further embryo development. In order

to enhance the meiosis resumption of entrapped immature oocytes, the co-culture with autologous CCs or GCs was also successfully performed (mouse, Pangas et al., 2003; human, Torre et al., 2006).

The embryo culture in 3D scaffolds has been poorly investigated.

In the swine species, the efficiency of the 3D alginate systems in sustaining the preimplantation embryo elongation, an important step for a successful ET, was evaluated. The expression gene profiles of some steroidogenic transcripts (STAR, CYPY11A1 and CYP19A1) and immune response transcripts (IL1B) showed that the entrapped embryos underwent morphological changes similar to the in vivo counterparts (Sargus-Patino et al., 2014). In the bovine species, the embryos cultured in alginate capsules showed similar developmental rates compared to the control group cultured in microdrops (Yaniz et al., 2002). In another study, the capability of post-hatching bovine embryos to undergo morphologic changes when cultured in 3D alginate capsules was found similar to the in vivo embryos (Zhao et al., 2015).

Since few information are available on the IVEP from domestic cat CDOs, the application of innovative culture systems, as the alginate scaffolds, could be of high interest to evaluate the performances of low competence oocytes in the 3D space and to improve the traditional in vitro techniques in this species.

OBJECTIVES

The domestic cat is an excellent animal model for wild felids reproductive biotechnologies, but in this species only few studies were focused on the improvement of the in vitro efficiency of CDOs and on the use of 3D systems for oocyte culture.

This thesis was aimed at improving the in vitro performances of the domestic cat low competence CDOs. For this purpose, an enriched culture system represented by 3D microcapsules of barium alginate (BA) in association with fresh cumulus-oocytes complexes (COCs), was developed.

The specific aims were to investigate:

(a) the suitability of a 3D system (barium alginate microcapsules) for domestic cat oocytes in vitro culture **(Paper I)**;

(b) the efficiency of the 3D system in improving the in vitro maturation of CDOs co-cultured with COCs or cultured separately **(Paper I)**;

c) the efficiency of the association of the 3D system and the co-culture with competent COCs in a commercial medium during in vitro maturation on the domestic cat CDOs in vitro embryo development **(Paper II)**.

MATERIALS & METHODS

These studies were approved by the Ethical Committee of the Università degli Studi di Milano (December 9th, 2014), and all animals were enrolled following written consent by the owner.

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

3.1 Animals and experimental designs

Cumulus-oocyte complexes were collected from 123 healthy queens (*Felis catus*) at random stages of the estrous cycle during routine ovariectomy at the veterinary clinics of the Department of Health, Animal Science and Food Safety of this University. After surgery, ovaries were immediately placed in a phosphate buffered saline (PBS) with a mixture of antibiotics (AB) and antimycotics (100 IU/ml of penicillin G sodium, 0.1 mg/ml of streptomycin sulfate, and 0.25 μg/ml of amphotericin B), and transported to the laboratory at room temperature (RT) where they were processed. Cat COCs were obtained by mincing of the ovaries in PBS and AB with 0.1% (w/v) polyvinyl alcohol (PVA) and only grade I COCs were selected for the experiments.

The CDOs were obtained by mechanical deprivation, with a small bore pipette, of COCs' cumulus cells.

In **Paper I**, the samples were collected from 65 queens.

In Experiment I, to investigate the suitability of a 3D system for feline oocytes, barium alginate (BA) microcapsules were prepared with different working conditions and a scoring method was applied to evaluate the following physical properties of the obtained microcapsules:

- dimensions (mm): length, width;
- shape: R (round), E (elongated);
- consistency: E (excellent), G (good), L (low).

Ninety one fresh COCs were in vitro matured in the 3D system or in the traditional microdrops of medium (2D system) for 24 h in a controlled atmosphere. At the end of the in vitro maturation, viability and maturation rates of feline COCs were evaluated.

In Experiment II, 216 fresh feline CDOs were co-cultured with COCs [CDOs(+)] or cultured separately [CDOs(-)] in 3D (Fig. 6) or 2D system to verify whether the BA microcapsules would improve the in vitro maturation of CDOs. After 24 h, the viability and maturation rates of CDOs(+), CDO(-) and COCs co-cultured with CDOs [COCs(+)] were evaluated.

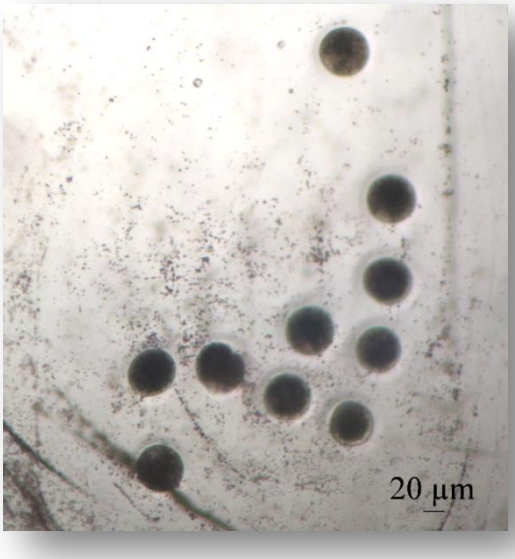

Figure 6. Domestic cat cumulus-denuded oocytes (CDOs) entrapped in barium alginate microcapsules (3D system, objective 10X).

In **Paper II**, the samples were collected from 58 queens.

In Experiment I, 144 fresh feline CDOs were co-cultured with COCs [CDOs(+)] or cultured separately [CDOs(-)] in a commercial medium in 3D BA microcapsules or in 2D microdrops. A control group of COCs [COCs(-)] was in vitro matured in 3D (Fig. 7) or 2D conditions. After 24 h, the viability and maturation rates of CDOs(+), CDO(-), COCs co-cultured with CDOs [COCs(+)] and COCs control [COCs(-)] were compared.

In Experiment II, 115 fresh feline CDOs were in vitro matured with COCs [CDOs(+)] in a commercial medium in 3D or in 2D system, as in experiment I. After in vitro fertilization with chilled epididymal feline spermatozoa, presumptive zygotes were in vitro cultured separately in 3D or in 2D system,

according to the IVM conditions. A control group of COCs [COCs(-)] was in vitro matured, fertilized and cultured in 3D or in 2D system. Embryonic development was recorded during 7 days of in vitro culture.

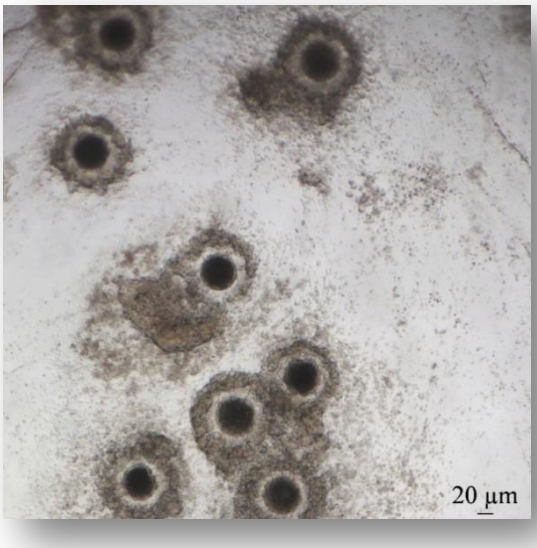

Figure 7. Domestic cat cumulus-oocyte complexes (COCs) entrapped in barium alginate microcapsules (3D system, objective 10X).

3.2 In vitro maturation in 3D and 2D systems (Paper I & Paper II)

The feline oocytes were in vitro matured for 24 h at 38.5°C and 5% CO_2 in air in modified Kreb's Ringer bicarbonate buffered salt solution with AB (b-mKRB) supplemented with 3 mg/mL of bovine serum albumin (BSA), 0.5 IU/mL of equine chorionic gonadotropin (eCG), 1 IU/mL of human chorionic gonadotropin (hCG), 10 ng/mL of epidermal growth factor (EGF), 0.6 mM cysteine (complete maturation medium, c-mKRB) **(Paper I)**, and in Quinn's

Advantage Protein Plus Blastocyst (SBP, SAGE® In Vitro Fertilization, Trumbull, Connecticut, USA) medium (b-SBP) supplemented with 75 IU FSH + 75 IU LH (Menogon®, Ferring Pharmaceuticals, Switzerland), 10 ng/ml of EGF, AB and 0.6 mM cysteine (complete maturation medium, c-SBP) **(Paper II)**.

3D system

For the 3D system, a two-steps encapsulation technique in BA was developed, as a modification of the protocol previously described for living-cell (Conte et al. 1999; Vigo et al. 2004). The Na-alginate powder (0.5%) was dissolved into the different solutions reported below, to obtain the melting solution (MS) at medium viscosity (3.500 cP, centipose). A saturated solution of $BaCl_2$ was then added to an aliquot of a different medium (see below) to obtain the dropping solution (DS) of $BaCl_2$ (40 mM) that was dropped at RT with a 25G needle into the MS maintained stirred for 30-40 minutes. The microcapsules were then collected, washed twice in PBS and suspended in the c-mKRB or c-SBP for immediate use, or maintained at 4°C in a petri dish with PBS until use.

To obtain the BA microcapsules, the following working conditions were tested **(Paper I; Exp. I)**: 1: MS with b-mKRB and DS with b-mKRB. 2: MS with c-mKRB and DS with c-mKRB. 3: MS with sterile water and DS with b-mKRB. 4. MS with sterile water and DS with c-mKRB. Based on the results of

the Exp. I, in **Exp. II** and in **Paper II**, MS with sterile water and DS with b-SBP was used.

The feline oocytes [CDOs(+); CDOs(-); COCs(+); COCs(-)] were injected into the inner core of the microcapsule by a small bore pipette (Fig. 8A) and subsequently immersed in the c-mKRB **(Paper I)** or in the c-SBP **(Paper II)** in a multiwell dish.

2D system

For the 2D culture system, traditional microdrops (50-100µl) of c-mKRB **(Paper I)** or c-SBP **(Paper II)** were placed in a petri dish and covered by mineral oil (Fig. 8B).

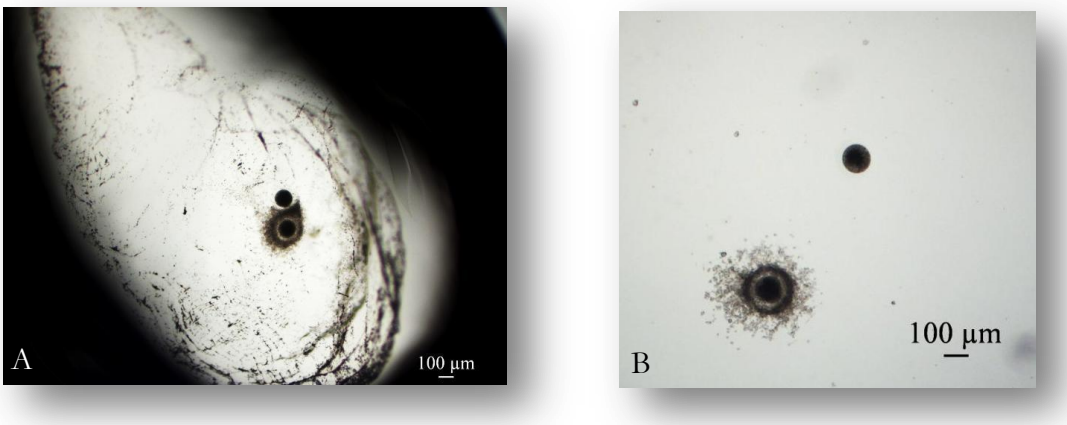

Figure 8. Domestic cat cumulus-denuded oocyte (CDO) in co-culture with cumulus-oocyte complex (COC) in barium alginate microcapsule (A) and in microdrop of medium (B) (2.5X objective).

3.3 Epididymal sperm recovery, in vitro fertilization and embryo culture (Paper II; Exp. II)

In vitro fertilization was performed with chilled feline epididymal spermatozoa obtained from isolated testicles after orchiectomy at veterinary clinics. The epididymides were isolated from the testicles and placed in a 35 mm Petri dish containing 1 ml of Ham's F-10 medium supplemented with 2 mM L-glutamine, 5% (v/v) fetal calf serum (FCS) and AB. Epididymal spermatozoa were released by mincing with a scalpel blade. Tissue debris were removed by forceps, and the remaining sperm suspension was centrifuged (300 g, 5 min) and diluted 1:2 with Tris egg yolk buffer for chilling at 4°C degree for 24 h. Before use, the spermatozoa were washed by centrifugation and a swim-up treatment was performed by gently layering 50 µl of b-SBP on the sperm pellet. After 30-45 min at 38.5°C, the supernatant was collected and concentration and motility were determined in the sperm suspension.

After 24 h of maturation, the oocytes were washed twice and transferred into 90 µl drops of fresh b-SBP supplemented with 5% of FCS and AB (c-SBP2, National Institutes of Health, Bethesda, MD, USA). Immediately prior to insemination, the sperm suspension was diluted in c-SBP2 to a final concentration of 0.75-1 x 10^6 motile spermatozoa/ml, and 10 µl were added to each fertilization drop containing the oocytes.

At 18-24 h post insemination, cumulus cells of COCs and unbound spermatozoa were removed from the oocytes using a small bore glass pipette. After washing, the presumptive zygotes were in vitro cultured for 7 days in c-SBP2, in 3D or 2D system according to the IVM conditions. Fresh culture medium (c-SBP2) was added every two days and the embryonic development was recorded.

3.4 Assessment of viability, maturation rates and embryonic development (Paper I & Paper II)

After 24 h of IVM, CDOs and COCs were evaluated for overall viability and nuclear maturation rates **(Paper I & Paper II: Exp. I)**. Sequential stainings with fluorescein diacetate/propidium iodide (FDA/PI) for viability and bis-benzimide (Hoechst 33342) for chromatin configuration were performed.

For the viability, the oocytes were maintained at dark in 50 µl of the staining solution (PI: 10 mg/ml; FDA: 5 mg/ml) for 5 min and then evaluated under a fluorescent microscope (Axiovert 100, Zeiss, Arese, Italy). This differential staining allowed the evaluation of viable (bright green fluorescence) or dead cells (red fluorescence).

After washing, CDOs and COCs (deprived of cumulus cells by mechanical displacement with a small bore glass pipette) were placed on a slide with a

minimum amount of medium and then covered by 10 µl of Hoechst solution. After 5 min of incubation in the dark, the Hoechst solution was removed and the oocytes were covered with an anti-fade reagent (Fluoromount™ Acqueous Mounting Medium). The fixed oocytes were then observed under a fluorescent microscope (Axiovert) at 400x magnification for nuclear evaluation.

The chromatin configurations were classified as follows (Bolamba et al. 1998; Hewitt and England 1999):

- germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- germinal vesicle break down-Anaphase I (GVBD–AI): identification of different patterns of chromatin condensation (GVBD) or identification of bivalents (AI);
- telophase I-metaphase II (TI–MII): identification of two groups of chromosomes moving to opposite ends of meiotic spindle (TI) or two sets of chromosomes clearly visible (MII);
- degenerated: collapsed nucleus or irregular nuclear conformation.

For the assessment of embryo development **(Paper II: Exp. II)**, cleaved embryos, 8-16 cells, morulae and blastocysts stages were recorded along 7 days of culture

3.5 Statistical analysis

Data for the microcapsules physical properties were reported as mean value and standard deviation (SD); viability, maturation and embryonic development rates of CDOs(+), CDOs(-), COCs(+) and COCs(-) were analyzed by Chi-square test and the level of significance was set at $p<0.05$.

RESULTS

4.1. Paper I

4.1.1. Experiment I

To obtain the 3D BA microcapsules for the encapsulation of feline oocytes, different working conditions were tested. The working condition 3 showed the best physical properties (dimensions, shape and consistency, Table 2). The dissolution of Na-alginate powder in sterile water and the dropping of $BaCl_2$ in basic medium (b-mKRB) was the proper combination (Fig. 9).

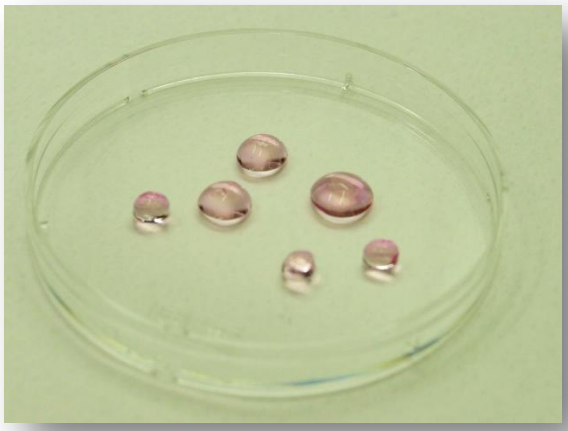

Figure 9. Three-dimensional barium alginate microcapsules in Petri dish.

Although the dropping solution made with complete medium (c-mKRB, working condition 4) allowed the formation of BA microcapsules, their shape and consistency were not as good as in the working condition 3. On the other hand, the dissolution of Na-alginate powder in b- or c-mKRB (working conditions 1 and 2) did not allow the formation of any microcapsule.

Table 2. Physical properties of the barium alginate (BA) microcapsules in different working conditions.

Working conditions	Width, mm (mean±SD)	Lenght, mm (mean±SD)	Shape (R,E)	Consistency (E,G,L)
1 MS with b-mKRB DS with b-mKRB	0	0	-	-
2 MS with c-mKRB DS with c-mKRB	0	0	-	-
3 MS with sterile water DS with b-mKRB	15.8 ± 1.81	24.12 ± 4.12	R	E
4 MS with sterile water DS with c-mKRB	15.03 ± 2.47	31.52 ± 3.98	R and E	G and L

MS, melting solution of Na-alginate (0.5%); DS, dropping solution of $BaCl_2$ (40 mM); b-mKRB, modified Kreb's Ringer bicarbonate buffered salt solution with antibiotics; c-mKRB, b-mKRB supplemented with 3 mg/mL of bovine serum albumin (BSA), 0.5 IU/mL of equine chorionic gonadotropin (eCG), 1 IU/mL of human chorionic gonadotropin (hCG), 10 ng/mL of epidermal growth factor (EGF) and 0.6 mM cysteine ; shape:, R (round), E (elongated); consistency: E (excellent), G (good), L (low).

The results of COCs IVM in 3D and 2D systems (Table 3) showed that a similar high viability ($p>0.05$) was maintained and no differences were found in the meiotic resumption. Full maturation (TI-MII, Fig. 10) rates were also similar in the two systems (3D: 7/47=14.9% vs 2D: 7/44=15.9%; $p>0.05$).

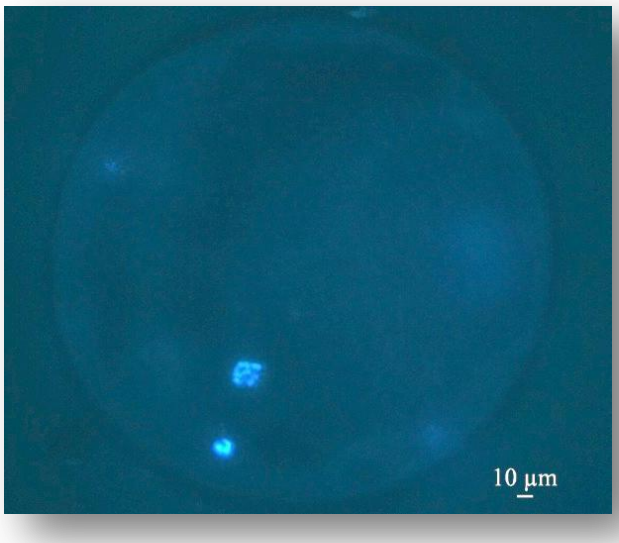

Figure 10. Domestic cat cumulus-oocyte complex (COC) at metaphase II, after in vitro maturation in 2D system (Hoechst 33342, 40X objective).

Table 3. Viability and nuclear status of feline cumulus-oocyte complexes (COCs) after in vitro maturation in 3D or 2D system.

System	Viability No. of oocytes (%)	Immature (GV) No. of oocytes (%)	Meiotic resumption (GVBD-MII) No. of oocytes (%)	Degenerated No. of oocytes (%)
3D	41/47 (87.2)	8/47 (17)	32/47 (68.1)	7/47 (14.9)
2D	37/44 (84)	9/44 (20.5)	26/44 (59.1)	9/44 (20.5)

No statistical differences.

3D, barium alginate microcapsules; 2D, microdrops of maturation medium; GV, germinal vesicle; GVBD, germinal vesicle break down; MII, metaphase II.

4.1.2. Experiment II

The results presented in Table 4 showed that the overall viability was similar in 3D and 2D systems ($p>0.05$), but in the 3D BA microcapsules the presence of COCs resulted in a higher viability of CDOs(+), than that obtained without COCs [CDOs(-)] or in 2D microdrops ($p=0.007$ and $p=0.002$, respectively; Fig. 11).

Table 4. Viability of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) cultured in 3D or 2D system.

Groups	Viability in 3D system No. of oocytes (%)	Viability in 2D system No. of oocytes (%)
CDOs(+)	51/56 (91.1) ^{a,x}	35/52 (67.3) ^{b,x}
COCs(+)	45/47 (95.7) ^{x}	44/48 (91.7) ^{y}
CDOs(-)	37/52 (71.2) ^{y}	46/56 (82.1) ^{x,y}
Overall Viability	133/155 (85.8)	125/156 (80.1)

^{a,b} Different superscripts indicate significant differences within rows ($p<0.05$)
^{x,y} Different superscripts indicate significant differences within columns ($p<0.05$)
CDOs(+), CDOs co-cultured with COCs; COCs(+), COCs co-cultured with CDOs; CDOs(-), CDOs cultured separately; 3D, barium alginate microcapsules; 2D, microdrops of maturation medium.

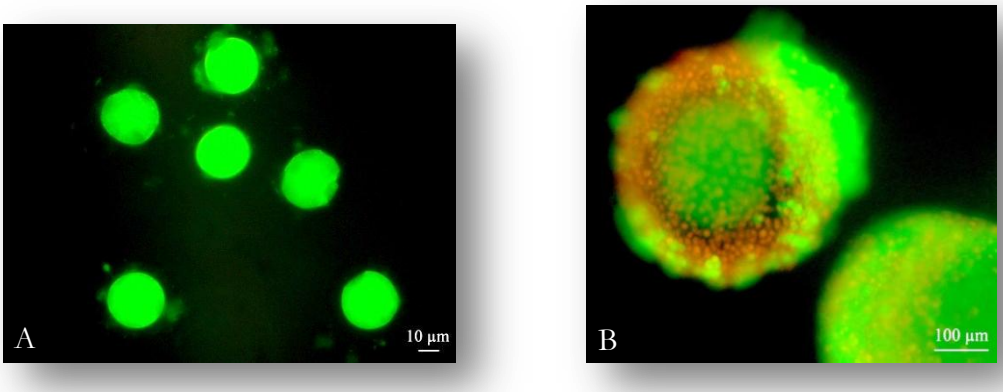

Figure 11. Viability of domestic cat cumulus-denuded oocytes (CDOs, A) after in vitro maturation in 3D system with cumulus-oocyte complexes (COCs, B; FDA-PI staining, 20X objective).

The 3D BA microcapsules were able to support the meiotic resumption of COCs and CDOs, as well as the 2D microdrops (Table 5).

The group of CDOs(+) did not benefit from the co-culture in 3D microcapsules, as the percentages of meiotic resumption were similar of those of CDO(-).The highest rates of meiosis resumption were reached by COCs(+) in both 3D and 2D systems ($p<0.05$). These gametes achieved better results of full maturational (TI-MII) stages than the CDOs(+) (3D: 19/47=40.4% vs 4/56=7.1%; $p=0.005$. 2D: 25/48=52.1% vs. 4/52=7.7%; $p=0.001$) and the CDOs(-) (3D: 3/52=5.8%; $p=0.003$. 2D: 2/56=7.1%; $p=0.001$).

Table 5. Nuclear status of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) cultured in 3D or 2D system.

Groups	Immature (GV) No. of oocytes (%)		Meiotic resumption (GVBD-MII) No. of oocytes (%)		Degenerated No. of oocytes (%)	
	3D	2D	3D	2D	3D	2D
CDOs(+)	18/56 (32.1)[x]	18/52 (42.9)[x]	31/56 (55.4)[x]	27/52 (51.9)[x]	7/56 (12.5)	7/52 (13.5)
COCs(+)	3/47 (6.4)[y]	5/48 (10.4)[y]	39/47 (83)[y]	40/48 (83.3)[y]	5/47 (10.6)	3/48 (6.3)
CDOs(-)	21/52 (40.4)[x]	20/56 (35.7)[x]	21/52 (40.4)[x]	23/56 (41.1)[x]	10/52 (19.2)	13/56 (23.2)

No differences within rows.

[x,y] Different superscripts indicate significant differences within columns ($p<0.05$).

CDOs(+), CDOs co-cultured with COCs; COCs(+), COCs co-cultured with CDOs; CDOs(-), CDOs cultured separately; 3D, barium alginate microcapsules; 2D, microdrops of maturation medium; GV, germinal vesicle; GVBD, germinal vesicle break down; MII, metaphase II.

4.2. Paper II

4.2.1. Experiment I

In all the treatment groups, the 3D system of BA microcapsules were able to sustain the viability (range: 87.9% - 100%) and the meiosis resumption of domestic cat CDOs and COCs in the commercial Quinn's Advantage Protein Plus Blastocyst (SBP) medium, as the 2D microdrops ($p>0.05$).

The resumption of meiosis (Table 6) of COCs co-cultured (+) or cultured separately (-) was significantly higher ($p<0.001$) in 3D and 2D system than that of CDOs(+) and CDOs(-). The rate of GVBD-MII stage of CDOs(+) did not differ from that of CDOs(-). No differences in degeneration rates were observed.

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Table 6. Meiotic progression of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) after in vitro maturation in 3D or 2D system.

Groups	No. of oocytes		Meiotic resumption (GVBD-MII) No. of oocytes (%)		*GVBD-AI* *No. of oocytes (%)*		*TI-MII* *No. of oocytes (%)*		Degenerate No. of oocytes (%)	
	3D	2D	3D	2D	*3D*	*2D*	*3D*	*2D*	3D	2D
CDOs(+)	39	33	13 (33.3)[a]	16 (48.5)[a]	*8 (20.5)[a]*	*12 (36.4)[a]*	*5 (12.8)[a]*	*4 (12.1)[a]*	4 (10.3)	2 (6.1)
COCs(+)	39	33	34 (87.2)[b]	28 (84.8)[b]	*20 (51.3)[b]*	*15 (45.5)[a]*	*14 (35.9)[b]*	*13 (39.4)[b]*	2 (5.1)	2 (6.1)
CDOs(-)	36	36	16 (44.4)[a]	13 (36.1)[a]	*3 (8.3)[a]*	*7 (19.4)[a,b]*	*13 (36.1)[b]*	*6 (16.7)[a]*	6 (16.7)	1 (2.8)
COCs(-)	35	35	30 (85.7)[b]	32 (91.4)[b]	*8 (22.9)[a,x]*	*2 (5.7)[b,y]*	*22 (62.9)[c,x]*	*30 (85.7)[c,y]*	0 (0)	1 (2.9)

[x,y] Different superscripts indicate significant differences within rows ($p<0.05$).

[a,b,c] Different superscripts indicate significant differences within columns ($p<0.05$).

CDOs(+), CDOs co-cultured with COCs; COCs(+), COCs co-cultured with CDOs; CDOs(-), CDOs cultured separately; COCs(-), COCs cultured separately (control group); 3D, barium alginate (BA) microcapsules; 2D, microdrops of maturation medium; GV, germinal vesicle; GVBD, germinal vesicle break down; MII, metaphase II.

4.1.1. Experiment II

The embryo development in terms of cleavage, 8-16 cells and morulae and blastocysts rates was similar in the oocytes matured and cultured in 3D BA microcapsules and in the 2D microdrops ($p>0.05$, Table 7). The CDO(+) matured in the co-culture with COCs developed at lower rates than the associated COCs ($p<0.05$) in both systems. However, the CDOs(+) achieved the 8-16 cells and the late embryo stages (morulae and blastocysts) at similar rates of COCs(-). The proportions of the morulae and blastocysts (Fig. 12) on the total number of cleaved embryos showed that CDOs(+) embryonic development was similar to that of associated COCs(+) and COCs control.

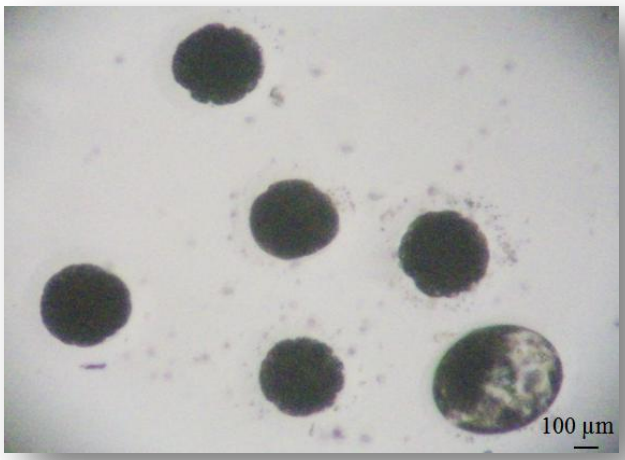

Figure 12. Domestic cat morulae and blastocysts at day 7 of in vitro culture in microdrops of medium (2D system, objective 5X

Table 7. Embryonic developmental rates of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) matured and cultured in 3D or 2D system.

Groups	No. of oocytes		Cleavage No. (%)		8-16 cells No. (%)		Morulae + blastocysts No. (%)		*Morulae + blastocysts/cleaved* *No. (%)*	
	3D	2D	3D	2D	3D	2D	3D	2D	3D	2D
CDOs(+)	56	59	7 (12.5)[a]	10 (16.9)[a]	4 (7.1)[a]	8 (13.6)[a]	4 (7.1)[a]	6 (10.2)[a]	4 (57.1)[a,b]	6 (60)
COCs(+)	57	57	30 (52.6)[b]	28 (49.1)[b]	25 (43.9)[b]	22 (38.6)[b]	23 (40.4)[b]	22 (38.6)[b]	23 (76.7)[b]	22 (78.6)
COCs(-)	45	57	14 (31.1)[c]	12 (21.1)[a]	6 (13.3)[a]	12 (21.1)[a]	3 (6.7)[a]	11 (19.3)[a]	3 (21.4)[a,x]	11 (91.7)[y]

[a,b,c] Different superscripts indicate significant differences within columns ($p<0.05$)

[x,y] Different superscripts indicate significant differences within rows ($p<0.05$)

CDOs(+), CDOs co-cultured with COCs during IVM; COCs(+), COCs co-cultured with CDOs during IVM; COCs(-), COCs cultured separately (control group); 3D, barium alginate (BA) microcapsules during IVM and IVC; 2D, microdrops of culture medium during IVM and IVC.

GENERAL DISCUSSION

This thesis was aimed at developing an enriched culture system to improve the in vitro performances of low competence domestic cat CDOs. At very low rates these gametes reach the full developmental competence because the functional and metabolic support of their somatic cells is missed (Tanghe et al. 2002; Luciano et al. 2005).

For this purpose the enriched culture system, consisted in the 3D microcapsules of barium alginate (BA) in association with fresh cumulus-oocytes complexes (COCs), was tested.

In **Paper I**, the dissolution of Na-alginate powder in sterile water and the subsequent dropping of $BaCl_2$ dissolved in the basic maturation medium (b-mKRB), was the best protocol to obtain round microcapsules with a solid inner core useful for feline oocytes encapsulation. The working conditions that involved media with hormones and growth factors supplementation (c-mKRB), as melting or dropping solution, seemed to inhibit the complete dissolution of Na-alginate powder and the ionic interactions with $BaCl_2$, compromising the effective creation and use of the microcapsules. However, the metabolic effect of nutrients, growth factors and hormones on the encapsulated oocytes was ensured by the immersion of the BA microcapsules in complete media. The effective exchange of different molecules through these systems has been documented (Vigo et al. 2004).

A similar viability and maturation rate of feline COCs cultured in these proper BA microcapsules compared to those cultured in 2D microdrops, proved that the 3D system was a suitable culture condition for feline oocytes (**Exp. I**).

The CDOs co-cultured with COCs showed a higher viability in 3D than in 2D system, but no beneficial effects of this association was observed in meiosis resumption and full maturation (TI-MII stages) rates. Conversely, the associated COCs had the highest viability and maturation rates in both systems (**Exp. II**). Present data differed from previous studies in the domestic cat and in other species (Luciano et al. 2005; Ge et al. 2008; Godard et al. 2009) in which the presence of COCs during in vitro maturation and in vitro fertilization seemed to promote the achievement of MII stage and of the subsequent embryo development of CDOs. In these experiments, fresh domestic cat COCs were mechanically deprived of the surrounding CCs to obtain the CDOs. This method, that differs from those of the aforementioned studies in which vortex or incubation with hyaluronidase were used, could have influenced the results. It remains to investigate how the oocytes without CCs, and not denudated ad hoc, behave in the same culture conditions.

It is notable that the presence of CDOs seemed to enhance the meiotic competence of the associated COCs. In bovine and murine model, the positive effect of denuded oocytes in the same culture condition as companion cells of

COCs has been already reported (Hussein et al. 2006; Gilchrist et al. 2008). It is well known that the oocytes produce some specific paracrine factors, known as the oocyte-secreted factors (OSFs), which act specifically on surrounding CCs, regulating their differentiation, functional activity and gene expression. These factors could presumably have provided some beneficial support to ameliorate the maturation rates of COCs in both 3D and 2D culture conditions.

In **Paper II**, the domestic cat CDOs achieved the full maturational competence and developed until morula and blastocyst stages at the same proportions of the competent COCs. However, the enriched conditions represented by 3D barium alginate microcapsules during maturation and culture did not improve the results, as similar rates of CDOs viability, meiotic progression and embryonic development were obtained in 3D and 2D systems. Differently from other studies in which bovine embryos in vitro-derived from competent oocytes developed at higher rates in 3D system compared to traditional 2D microdrops (Zhao et al., 2015), in this study the embryo development of feline COCs did not differ in the two systems. In addition, the enrichment of the in vitro maturation with competent COCs did not exert a beneficial effect for the CDOs performances. No differences were found in the maturation (**Exp. I**) and in the subsequent embryonic development (**Exp. II**) when the CDOs were in vitro matured with or without the associated COCs, in

both 3D and 2D systems. The results of IVM confirm what has been found in the **Paper I**, but differ from other studies, which demonstrated a positive effect of the co-culture with COCs on bovine and domestic cat CDOs (Luciano et al., 2005; Godard et al., 2009).

The presence of own CCs is crucial for the oocyte quality. The CCs are involved in many cellular processes, as the metabolism of different substrates (i.e. glucose, fatty acids, carbohydrates, and amino acids), and provide the specialized microenvironment for cytoplasmic and nuclear oocyte maturation and development (Sutton-McDowall et al., 2010). The deprivation of CCs has a strong and negative impact on the developmental competence of the oocyte in different species (Auclair et al., 2013). For instance, the absence of CCs and related specific secreted factors, lead to aberrant cytoplasmic maturation, including an impaired cumulus-related lipid metabolism compared to that of the competent COCs (Auclair et al., 2013). In the domestic cat, no information were available regarding the lipid metabolism of CDOs. A more detailed investigation of the different metabolism in high and low competence oocytes should be of high interest to design a proper enriched culture condition for this species.

The Quinn's Advantage Protein Plus Blastocyst (SBP) medium, specifically designed for the IVC of human embryos, was used in both experiments (**Exp. I, II**). This commercial medium is easy to handle, pathogen free, high reproducible and routinely used for assisted reproductive techniques. Present data suggest that

the SBP could support the full maturation and development of COCs and CDOs.

The COCs benefit from the association with CDOs, as their embryo development rates were higher than that of COCs cultured separately. The positive effect of the association presumably depends on the paracrine effects the OSFs. The presence of CDOs in the same culture with COCs during maturation has been shown not only to improve their achievement of full maturational stages, as previously mentioned, but also to promote their embryo development in different species (Hussein et al., 2006).

CONCLUSIONS

The 3D barium alginate microcapsules are suitable systems for the in vitro culture of domestic cat oocytes, as viability, maturation and embryo development rates were similar to that obtained in the traditional 2D microdrops (**Paper I & Paper II**).

The enriched culture system based on the association with competent COCs did not improve the in vitro meiosis resumption of the domestic cat CDOs, as their full maturational (TI-MII) rates were similar to that of CDOs cultured separately. The domestic cat low competence oocytes need more specific and designed ad hoc in vitro conditions, as well as the formulation of specific maturation media **(Paper I)**.

However, the domestic cat CDOs could fully mature and develop until blastocyst stage at similar proportion to the associated COCs and COCs control. The commercial Quinn's Advantage Protein Plus Blastocyst (SBP) medium specifically designed for human embryos, resulted adequate for the domestic cat in vitro culture, although it did not enhance the CDOs full competence **(Paper II)**.

The presence of CDOs in the co-culture had beneficial effects on the in vitro performances of competent COCs, as their viability, maturation and

embryonic development rates were higher than those obtained when cultured separately as control group (**Paper I & Paper II**).

The more physiological microenvironment, as the oocyte architecture maintained by the 3D culture, represents an enriched condition that deserves further investigations. A better knowledge of the expression profiles of potential oocyte quality markers, as the OSFs, and how they could differ from COCs and CDOs in the 3D and 2D systems could help the design of the optimal enriched culture conditions for the domestic cat low competence oocytes.

REFERENCES

- Aberdam E, Hanski E, Dekel N.
  Maintenance of meiotic arrest in isolated rat oocytes by the invasive adenylate cyclase of Bordetella pertussis.
  Biology of Reproduction 1987; 36: 530–5.

- Abir R, Nitke S, Ben-Haroush A, Fisch B.
  In vitro maturation of human primordial ovarian follicles: Clinical significance, progress in mammals, and methods for growth evaluation.
  Histology and Histopathology 2006; 21: 887-98.

- Adhikari D, Liu K.
  The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes.
  Molecular and Cellular Endocrinology 2014; 382: 480-7.

- Albertini DF, Combelles CMH, Benecchi E, Carabatsos MJ.
  Cellular basis for paracrine regulation of ovarian follicle development.
  Reproduction 2001; 121: 647-53.

- Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen J, Lu H, Richmond J, Kaplan DL.
  Silk-based biomaterials.
  Biomaterials 2003; 24: 401-16.

- Amorim CA, Langendonckt AV, David A, DolmansM, Donnez J.
  Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix.
  Human Reproduction 2009; 24: 92–9.

- Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R, Uzbekova S.
  Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. American Journal of Physiology.
  Endocrinology and Metabolism 2013, 67: 599–613.

- Bolamba D, Borden-Russ KD, Durrant BS.
  In vitro maturation of domestic dog oocytes cultured in advanced preantral and early antral follicles.
  Theriogenology 1998; 49: 933-42.

- Bowen RA.
  Fertilization in vitro of feline ova by spermatozoa from the ductus deferens.
  Biology of Reproduction 1977; 17: 144–7.

- Brito IR, Lima IMT, Xu M, Shea LD, Woodruff TK, Figueiredo JR.
  Three-dimensional systems for in vitro follicular culture: overview of alginate-based matrices.
  Reproduction, Fertility and Development 2014; 26: 915–30.

- Bruzzone R, White TW, Paul DL.
  Connections with connexins: the molecular basis of direct intercellular signaling.
  European Journal of Biochemistry 1996; 238: 1–27.

- Buccione R, Schroeder AC, Eppig JJ.
  Interactions between somatic cells and germ cells throughout mammalian oogenesis.
  Biology of Reproduction 1990; 43: 543–7.

- Chigioni S, Perego L, Luvoni GC.
  Companion cumulus-oocyte complexes for in vitro culture of denuded oocytes in the cat.
  In: Farstad W, Arus J (ed.), Proc 4th Ann Congr EVSSAR. Amsterdam (The Netherlands), 14 April 2005, pp. 24-25.

- Colleoni S, Luciano AM, Gandolfi F.
  Cumulus–oocyte communications in the horse: role of the breeding season and of the maturation medium.
  Reproduction in Domestic Animals 2004; 39, 70–5.

- Combelles CMH, Fissore RA, Albertini DF, Racowsky C.
  In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system.
  Human Reproduction 2005; 20: 1349-58.

- Comizzoli P, Songsasen N, Wildt DE.
  Protecting and extending fertility for females of wild and endangered mammals.
  Cancer Treatment and Research 2010; 156: 87–100.

- Conte U, Torre ML, Maggi L, Giunchedi P, Vigo D, Maffeo G, Russo V. Microcapsules containing seminal material for artificial insemination in pigs. *European Patent Application EP0922451 (16/6/1999) CAS 131, 49474v.*

- Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science 2001; 23; 294: 1708-12.*

- Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. *Current Opinion in Cell Biology 2002; 14: 633-9.*

- Davachi ND, Kohram H, Zainoaldini S. Cumulus cell layers as a critical factor in meiotic competence and cumulus expansion of ovine oocytes. *Small Ruminant Research 2012; 102: 37-42.*

- De Los Reyes M, Rojas C, Parraguez VH, Palomino J. Expression of growth differentiation factor 9 (GDF-9) during in vitro maturation in canine oocytes. *Theriogenology 2013; 80: 587-96.*

- Dekel N, Beers WH. Development of the rat oocyte in vitro: inhibition and induction of maturation in the presence or absence of the cumulus oophorus. *Developmental Biology 1980; 75: 247–54.*

- Desai N, Alex A, AbdelHafez F, Calabro A, Goldfarb J, Fleischman J, Falcone T. Three-dimensional in vitro follicle growth: overview of culture models, biomaterials, design parameters and future directions. *Reproduction, Biology, and Endocrinology 2010; 8: 119-31.*

- Dieckmann C, Renner R, Milkova L and Simon JC. Regenerative medicine in dermatology: biomaterials, tissue engineering, stem cells, gene transfer and beyond. *Experimental Dermatology 2010; 19: 697–706.*

- Diez C, Duque P, Gomez E, Hidalgo CO, Tamargo C, Rodrıguez A, Fernandez L, De La Varga S, Fernandez A, Faca N, Carbajo M.
  Bovine oocyte vitrification before or after meiotic arrest: effects on ultrastructure and developmental ability.
  Theriogenology 2005; 64: 317–33.

- Discher DE, Janmey P, Wang YL.
  Tissue cells feel and respond to the stiffness of their substrate.
  Science 2005; 18; 310: 1139-43.

- Dragovic RA, Ritter LJ, Schulz SJ, Amato F, Thompson GJ, Armstrong DT, Gilchrist RB.
  Oocyte-secreted factor activation of SMAD 2/3 signaling enables initiation of mouse cumulus cell expansion.
  Biology of Reproduction 2007; 76, 848–57.

- Eppig JJ.
  The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion.
  Developmental Biology 1982; 89: 268–72.

- Fagbohun CF, Downs SM.
  Metabolic coupling and ligand-stimulated meiotic maturation in the mouse cumulus–oocyte complex.
  Biology of Reproduction 1991; 45: 851-59.

- Fan Q, Ma J, Xu Q, Zhang J, Simion D, Carmen G, Guo C.
  Animal-derived natural products review: focus on novel modifications and applications.
  Colloids and Surfaces. B, Biointerfaces 2015; 128: 181–90.

- Franciosi F, Perazzoli F, Lodde V, Modina SC, Luciano AM.
  Developmental competence of gametes reconstructed by germinal vesicle transplantation from fresh and cryopreserved bovine oocytes.
  Fertility and Sterility 2010; 93: 229-38.

- Fujihara M, Comizzoli P, Wildt DE, Songsasen N.
  Cat and dog primordial follicles enclosed in ovarian cortex sustain viability after in vitro culture on agarose gel in a protein-free medium.
  Reproduction in Domestic Animals 2012; 47: 102-8.

- Galli C, Duchi R, Colleoni S, Lagutina I, Lazzari G.
  Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice.
  Theriogenology 2014; 81: 138-51.

- Ge L, Sui HS, Lan GC, Liu N, Wang JZ, Tan JH.
  Coculture with cumulus cells improves maturation of mouse oocytes denuded of the cumulus oophorus: observations of nuclear and cytoplasmic events.
  Fertility and Sterility 2008; 90: 2376–88.

- Ghidoni I, Chlapanidas T, Bucco M, Crovato F, Marazzi M, Vigo D, Torre ML, Faustini M.
  Alginate cell encapsulation: new advances in reproduction and cartilage regenerative medicine.
  Cytotechnology 2008; 58: 49–56.

- Gilchrist RB, Lane M, Thompson JG.
  Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality.
  Human Reproduction Update 2008; 14: 159–77.

- Gilchrist RB, Ritter LJ, Armstrong DT.
  Oocyte–somatic cell interactions during follicle development in mammals.
  Animal Reproduction Science 2004; 82–83: 431–46.

- Gilchrist RB, Ritter LJ, Myllymaa S, Kaivo-Oja N, Dragovic RA, Hickey TE, Ritvos O, Mottershead DG.
  Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation.
  Journal of Cell Science 2006; 119: 3811-21.

- Godard N, Pukazhenthi BS, Wildt DE, Comizzoli P.
  Paracrine factors from cumulus-enclosed oocytes ensure the successful maturation and fertilization in vitro of denuded oocytes in the cat model.
  Fertility and Sterility 2009; 91: 2051-60.

- Gombotz WR, Wee SF.
  Protein release from alginate matrices.
  Advanced Drug Delivery Reviews 1998; 31: 267–85.

- Gómez MC, Pope CE, Harris R, Davis A, Mikota S, Dresser BL.
  Births of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured in vitro.
  Reproduction, Fertility, and Development 2000; 12: 423-33.

- Gómez MC, Pope CE, Ricks DM, Lyons J, Dumas C, Dresser BL.
  Cloning endangered felids using heterospecific donor oocytes and interspecies embryo transfer.
  Reproduction, Fertility, and Development 2009; 21: 76-82.

- Gómez MC, Pope CE, Dresser BL.
  Nuclear transfer in cats and its application.
  Theriogenology 2006; 66: 72–81.

- Goodrowe KL, Hay M, King WA.
  Nuclear maturation of domestic cat ovarian oocytes in vitro.
  Biology of Reproduction 1991; 45: 466-70.

- Goodrowe KL, Wall RJ, O'Brien SJ, Schmidt PM, Wildt DE.
  Developmental competence of domestic cat follicular oocytes after fertilization in vitro.
  Biology of Reproduction 1988; 39: 355-72.

- Guraya SS.
  A histochemical analysis of lipid yolk deposition in the oocytes of cat and dog.
  The Journal of Experimental Zoology 1965; 160: 123-36.

- Hashimoto S, Saeki K, Nagao Y, Minami N, Yamada M, Utsumi K.
  Effects of cumulus cell density during in vitro maturation of the developmental competence of bovine oocytes.
  Theriogenology 1998; 49: 1451–63

- Hewitt DA, England GCW.
  Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation in vitro.
  Animal Reproduction Science 1999; 55: 63-75.

- Homa ST.
  Calcium and meiotic maturation of the mammalian oocyte.
  Molecular, Reproduction and Development 1995; 40: 122–34.

- Huang X, Zhang X, Wang X, Wang C, Tang B.
  Microenvironment of alginate-based microcapsules for cell culture and tissue engineering.
  Journal of Bioscience and Bioengineering 2012; 114: 1-8.

- Hussein TS, Thompson JG, Gilchrist RB.
  Oocyte-secreted factors enhance oocyte developmental competence.
  Developmental Biology 2006; 296: 514–21.

- Hyttel P, Fair T, Callesen H, Greve T.
  Oocyte growth capacitation and final maturation in cattle.
  Theriogenology 1997; 47: 23–32.

- Ikeda S, Ichihara-Tanaka K, Azuma T, Muramatsu T, Yamada M.
  Effects of midkine during in vitro maturation of bovine oocytes on subsequent developmental competence.
  Biology of Reproduction 2000; 63: 1067–74.

- Johnston LA, Donoghue AM, O'brien SJ, Wildt DE.
  Influence of culture medium and protein supplementation on in vitro oocyte maturation and fertilization in the domestic cat.
  Theriogenology 1993; 40: 829-39.

- Johnston LA, O'Brien SJ, Wildt DE.
  In vitro maturation and fertilization of domestic cat follicular oocytes.
  Gamete Research 1989; 24: 343-56.

- Kidder GM, Mhawi AA.
  Gap junctions and ovarian folliculogenesis.
  Reproduction 2002; 123: 613-20.

- Kreeger PK, Deck JW, Woodruff TK, Shea LD.
  The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels.
  Biomaterials 2006; 27: 714–23.

- Lee KY, Mooney DJ.
  Alginate: properties and biomedical applications.
  Progress in Polymer Science 2012; 37: 106–12.

- Lin T, Oqani RK, Lee JE, Shin HY, Jin DI.
  Coculture with good-quality COCs enhances the maturation and development rates of poor-quality COCs.
  Theriogenology 2016; 85: 396-407.

- Lloyd AW.
  Interfacial bioengineering to enhance surface biocompatibility.
  Medical Device Technology 2002: 13; 18-21.

- Luciano AM, Lodde V, Beretta MS, Colleoni S, Lauria A, Modina S.
  Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione.
  Molecular, Reproduction and Development 2005; 71: 389–97.

- Luciano AM, Modina S, Vassena R, Milanesi E, Lauria A, Gandolfi F.
  Role of intracellular cyclic adenosine 3',5'-monophosphate concentration and oocyte-cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte.
  Biology of Reproduction 2004; 70: 465–72.

- Luvoni GC.
  Gamete cryopreservation in the domestic cat.
  Theriogenology 2006; 66: 101–11.

- Luvoni GC, Chigioni S, Perego L, Lodde V, Modina S, Luciano AM.
  Effect of gonadotropins during in vitro maturation of feline oocytes on oocyte–cumulus cells functional coupling and intracellular concentration of glutathione.
  Animal Reproduction Science 2006; 96: 66–78.

- Luvoni GC, Luciano AM, Modina S, Gandolfi F.
  Influence of different stages of the oestrous cycle on cumulus–oocyte communications in canine oocytes: effects on the efficiency of in vitro maturation.
  Journal of Reproduction and Fertility 2001; 57: 141–6.

- Luvoni GC, Pellizzari P.
  Embryo development in vitro of cat oocytes cryopreserved at different maturation stages.
  Theriogenology 2000; 53: 1529-40.

- Mattioli M, Barboni B.
  Signal transduction mechanism for LH in the cumulus–oocyte complex.
  Molecular and Cellular Endocrinology 2000; 161:19–23.

- Mattioli M, Gioia L, Barboni B.
  Calcium elevation in sheep cumulus- oocyte complexes after luteinising hormone stimulation.
  Molecular, Reproduction and Development 1998; 50: 361–9.

- Mattioli M.
  Transduction mechanisms for gonadotrophin-induced oocyte maturation in mammals.
  Zygote 1994; 2: 347–49.

- Motta PM, Makabe S, Naguro T, Correr S.
  Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy.
  Archives of Histology and Cytology 1994; 57: 369-94.

- Munari E, Asti A, Colombani C, Benzoni E, Faustini M, Torre ML, Riccardi A, Villani S, Sesso L, Conte U, Vigo D.
  Competence of swine oocytes matured by three-dimensional gonadotropin free co-culture.
  Veterinary Research Communications 2007; 31: 181-4.

- Nagyova E.
  Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation.
  Endocrine Regulations 2012; 46: 225–35.

- Nevoral J, Orsák M, Klein P, Petr J, Dvořáková M, Weingartová I, Vyskočilová A, Zámostná K, Krejčová T, Jílek F.
  Cumulus cell expansion, its role in oocyte biology and perspectives of measurement: a review.
  Scientia Agriculturae Bohemica 2014; 45: 212–25.

- Nurse P.
  Universal control mechanism regulating onset of M-phase.
  Nature 1990; 344: 503–8.

- O'Shea LC, Mehta J, Lonergan P, Hensey C, Fair T.
  Developmental competence in oocytes and cumulus cells: candidate genes and networks.
  Systems Biology in Reproductive Medicine 2012; 58: 88-101.

- Otoi T, Shimizu R, Naoi H, Wongsrikeao P, Agung B, Taniguchi M.
  Meiotic competence of canine oocytes embedded in collagen gel.
  Reproduction in Domestic Animals 2006; 41: 17–21.

- Pangas SA, Saudye H, Shea LD, Woodruff TK.
  Novel approach for the three-dimensional culture of granulosa cell–oocyte complexes.
  Tissue Engineering 2003; 9: 1013-21.

- Paramio MT, Izquierdo D.
  Current status of in vitro embryo production in sheep and goats.
  Reproduction in Domestic Animals 2014; 49: 37-48.

- Parks JE.
  Hypothermia and mammalian gametes.
  In: Karow AM, Critser JK, editors. Reproductive tissue banking. Scientific principles. San Diego, CA: Academic Press; 1997. p. 229–61

- Parrish EM, Siletz A, Xu M, Woodruff TK, Shea LD.
  Gene expression in mouse ovarian follicle development in vivo versus an ex vivo alginate culture system.
  Reproduction 2011; 142: 309-18.

- Peng XR, Hsueh AJ, LaPolt PS, Bjersing L, Ny T.
  Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation.
  Endocrinology 1991; 129: 3200-7.

- Pope CE, Gómez MC, Kagawa N, Kuwayama M, Leibo SP, Dresser BL.
  In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer.
  Theriogenology 2012; 77: 531-38.

- Pope CE, Johnson CA, McRae MA, Keller GL, Dresser BL.
  Development of embryos produced by intracytoplasmic sperm injection of cat oocytes.
  Animal Reproduction Science 1998; 53: 221-36.

- Pope CE, McRae MA, Plair BL, Keller GL, Dresser BL.
  In vitro and in vivo development of embryos produced by in vitro maturation and in vitro fertilization of cat oocytes.
  Journal of Reproduction and Fertility 1997; 51: 69-82.

- Pukazhenthi BS, Comizzoli P, Travis AJ, Wildt DE.
  Applications of emerging technologies to the study and conservation of threatened and endangered species.
  Reproduction, Fertility, and Development 2006; 18: 77–90.

- Pukazhenthi BS, Wildt DE.
  Which reproductive technologies are most relevant to studying, managing and conserving wildlife?
  Reproduction, Fertility, and Development 2004; 16: 33–46.

- Sargus-Patino CN, Wright EC, Plautz SA, Miles JR, Vallet JL, Pannier AK.
  In vitro development of preimplantation porcine embryos using alginate hydrogels as a three-dimensional extracellular matrix.
  Reproduction, Fertility and Development 2014; 26: 943–53.

- Shikanov A, Xu M, Woodruff TK, Shea LD.
  A method for ovarian follicle encapsulation and culture in a proteolytically degradable 3 dimensional system.
  Journal of Visualized Experiments 2011; 49: 1-9.

- Songsasen N, Comizzoli P.
  A historic overview of embryos and oocyte preservation in the world of mammalian in vitro fertilization and biotechnology.
  In: Borini A, Coticchio G, editors. Preservation of human oocytes. London: Informa Healthcare; 2009, p. 1-11.

- Songsasen N, Woodruff TK, Wildt DE.
  In vitro growth and steroidogenesis of dog follicles as influenced by the physical and hormonal microenvironment.
  Reproduction 2011; 142: 113-22.

- Standring S.
  Struttura e funzioni fondamentali delle cellule.
  In: Anatomia del Gray. Le basi anatomiche per la pratica clinica. Ed. Elsevier/Masson Milano, 2009; 40: 7-8.

- Sutton-McDowall ML, Gilchrist RB, Thompson JG.
  The pivotal role of glucose metabolism in determining oocyte developmental competence.
  Reproduction 2010; 139: 685-95.

- Tanghe S, Van Soom A, Mehrzad J, Maes D, Duchateau L, de Kruif A.
  Cumulus contributions during bovine fertilization in vitro.
  Theriogenology 2003; 60: 135–49.

- Tanghe S, Van Soom A, Nauwynck H, Coryn M, De Kruif A.
  Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization.
  Molecular Reproduction and Development 2002; 61: 414-24.

- Tharasanit T, Buarpung S, Manee-In S, Thongkittidilok C, Tiptanavattana N, Comizzoli P, Techakumphu M.
  Birth of kittens after the transfer of frozen-thawed embryos produced by intracytoplasmic sperm injection with spermatozoa collected from cryopreserved testicular tissue.
  Reproduction in Domestic Animals 2012; 47: 305-8.

- Torre ML, Munari E, Albani E, Levi-Setti PE, Villani S, Faustini M, Conte U, Vigo D.
  In vitro maturation of human oocytes in a follicle-mimicking three-dimensional coculture.
  Fertility and Sterility 2006, 86: 572-576.

- Tsafriri A, Chun SY, Zhang R, Hsueh AJ, Conti M.
  Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors.
  Developmental Biology 1996; 178: 393–402.

- Vaccari S, Weeks II JL, Hsieh M, Menniti, FS, Conti M.
  Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes.
  Biology of Reproduction 2009; 81: 595-604.

- Vanhoutte L, Nogueira D, Dumortier F, De Sutter P.
  Assessment of a new in vitro maturation system for mouse and human cumulus-enclosed oocytes: three-dimensional prematuration culture in the presence of a phosphodiesterase 3-inhibitor.
  Human Reproduction 2009; 24: 1946-59.

- Vigo D, Torre ML, Faustini M, Munari E, Russo V, Norberti R, Villani S, Asti A, Bini PP, Conte U.
  Barium alginate capsules for 3D immobilisation of living cells: morphology, membrane properties and permeability.
  Journal of Drug Delivery Science and Technology 2004: 14; 167-72.

- Von Recum AF, LaBerge M.
  Educational goals for biomaterials science and engineering: prospective views.
  Journal of Applied Biomaterials 1995; 6: 137-44.

- Vozzi C, Formenton A, Chanson A, Senn A, Sahli R, Shaw P, Nicod P,. Germond M, Haefliger JA.
  Involvement of connexin 43 in meiotic maturation of bovine oocytes.
  Reproduction 2001: 122; 619–28.

- West ER, Shea LD, Woodruff TK.
  Engineering the Follicle Microenvironment.
  Seminars in Reproductive Medicine 2007; 25: 287–99.

- Wildt DE, Comizzoli P, Pukazhenthi B, Songsasen N.
  Lessons from biodiversity-the value of nontraditional species to advance reproductive science, conservation, and human health.
  Molecular Reproduction and Development 2010; 77: 397-409.

- Williams DF.
  On the nature of biomaterials.
  Biomaterials 2009; 30: 5897–909.

- Wood TC, Wildt DE.
  Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro.
  Journal of Reproduction and Fertility 1997; 110: 355-60.

- Xu J, Lawson MS, Yeoman RR, Molskness TA, Ting AY, Stouffer RL, Zelinski MB.
  Fibrin promotes development and function of macaque primary follicles during encapsulated three-dimensional culture.
  Human Reproduction 2013; 28: 2187–2200.

- Xu M, Barret S, West-Farrel E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK.
  In vitro grown human ovarian follicles from cancer patients support oocyte growth.
  Human Reproduction 2009; 24: 2531–40.

- Xu M, West E, Shea LD, Woodruff TK.
  Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development.
  Biology of Reproduction 2006; 75: 916-23.

- Yaniz JL, Santolaria P, Lopez-Gatius F.
  In vitro development of bovine embryos encapsulated in sodium alginate.
  Journal of Veterinary Medicine 2002; 49: 393–5.

- Yeo CX, Gilchrist RB, Thompson JG, Lane M.
  Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice.
  Human Reproduction 2008; 23: 67–73.

- Zhao S, Liu ZX, Gao H, Wu Y, Fang Y, Wu SS, Li MJ, Bai JH, Liu Y, Evans A, Zeng SM.
  A three-dimensional culture system using alginate hydrogel prolongs hatched cattle embryo development in vitro.
  Theriogenology 2015; 84: 184–92.

- Zhao XM, Ren JJ, Du WH, Hao HS, Liu Y, Qin T, Wang D, Zhu HB.
  Effect of mouse cumulus cells on the in vitro maturation and developmental potential of bovine denuded germinal vesicle oocytes.
  Zygote 2014; 22: 348-55.

- Zhong SP, Zhang YZ and Lim CT.
  Tissue scaffolds for skin wound healing and dermal reconstruction.
  Nanomedicine and Nanobiotechnology 2010; 2: 510–25.

- Zuelke KA, Keith CH, Brackett BG.
  LH induction of calcium oscillations in immature bovine oocytes.
  Biology of Reproduction 1991; 44: 141.

SUMMARY

The cumulus-denuded oocytes (CDOs) are not commonly involved in the assisted reproductive techniques (ARTs), as the absence of their surrounding cumulus cells negatively affects their maturational and developmental competence in vitro.

In some cases these gametes could represent an interesting option for widening the germinal pool of high value individuals with very precious genetic material and could be the only genetic source when the selected cumulus-oocyte complexes (COCs, grade I) are cryopreserved.

Therefore, enriched culture conditions to improve the CDOs in vitro full competence should be adopted.

The innovative three-dimensional (3D) scaffolds, derived from the bioengineering and nanotechnology research, ensure the optimal culture conditions to maintain the cells physiological conformation and behavior as in the in vivo environment.

In this thesis, the association of 3D barium alginate microcapsules with competent COCs was used to improve the in vitro performances of domestic cat CDOs.

The results showed that the 3D BA microcapsules are suitable systems for the in vitro culture of feline oocytes, as their viability and in vitro maturation

rates and embryonic development were similar to those obtain in the traditional 2D system.

The enriched co-culture condition did not improve the in vitro competence of CDOs, as their full maturational (TI-MII) rates and late embryo stages (morulae and blastocysts) were similar to those of the CDOs cultured separately or to those of the COCs control.

However, the presence of CDOs in the co-culture with COCs improved significantly the in vitro developmental rates of the high competence oocytes, presumably for the paracrine action of some specific oocyte-secreted factors (OSFs).

A better knowledge of the expression profiles of potential oocyte quality markers, as the OSFs, and how they could differ from COCs and CDOs in the 3D and 2D systems could help the design of the optimal enriched culture conditions for the domestic cat low competence oocytes.

PAPER I

A THREE-DIMENSIONAL ALGINATE SYSTEM FOR IN VITRO CULTURE OF CUMULUS-DENUDED FELINE OOCYTES

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A THREE-DIMENSIONAL ALGINATE SYSTEM FOR IN VITRO CULTURE OF CUMULUS-DENUDED FELINE OOCYTES.

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Abstract

In the case of high valuable individuals with very precious genetic material, widening the genetic pool including gametes with poor morphological characteristics, as cumulus-denuded oocytes (CDOs), could be an option.

To improve the in vitro culture of low competence feline CDOs, an enriched three-dimensional (3D) system of barium alginate microcapsules in association with competent cumulus-oocyte complexes (COCs) was developed. The overall viability and the meiotic progression of feline CDOs co-cultured with COCs or cultured separately in 3D or in 2D (traditional microdrops) system were compared. The 3D system was able to support viability and meiotic resumption of the feline oocytes, as well as the 2D microdrops. In 3D microcapsules the presence of COCs resulted in a higher viability of CDOs, than that obtained without COCs or in 2D microdrops, but the percentages of meiotic resumption were similar of those of CDOs cultured separately. It is notable that the presence of CDOs seemed to enhance the meiotic progression of the associated COCs.

In conclusion, the 3D barium alginate microcapsules are a suitable system for feline oocytes in vitro culture, but more specific enriched conditions should be developed to improve the CDOs full competence in vitro.

Keywords

Domestic cat; denuded oocytes; in vitro maturation; 3D system.

Abridged title: 3D in vitro culture of feline oocytes.

Introduction

Cumulus-denuded oocytes (CDOs) are generally not included in the in vitro procedures due to their poor nuclear and cytoplasmic competence caused by the lack of surrounding cumulus oophorus cells. These closely associated cumulus cells (CCs) form an intimate network with the oocyte, thus the somatic-germinal two-way transfer of different small molecules is ensured through the highly specialized projection through the zona pellucida, i.e. gap junctions (Eppig 1982). The structural integrity of CCs and the functional coupling between the two compartments is of crucial importance for the successful subsequent embryo development (Fagbohun and Downs 1991; Tanghe et al. 2002; Luciano et al. 2004).

Several attempts have been made to improve the in vitro performances of oocytes with poor developmental potential, as well as oocytes deprived of CCs. The co-culture with companion cumulus-oocyte complexes (COCs) seemed to have beneficial effects on the CDOs in vitro outcomes. In the bovine species, the presence of intact COCs during both in vitro maturation and fertilization promoted the restoration of CDOs competence, although the blastocyst rates

remained low (Luciano et al. 2005). Co-culture of feline CDOs with cumulus cells clumps enhanced the resumption of meiosis, although the frequency of complete nuclear maturation was lower than that of competent COCs (Chigioni et al. 2005).

In the case of high valuable individuals with very precious genetic material, widening the genetic pool including gametes with poor morphological characteristics, as CDOs, could be an option. Therefore enriched conditions for the culture of these low competence oocytes should be further developed.

The traditional culture systems for follicles and oocytes are based on microdrops of medium, but this condition seemed to lead to a non-physiological cells conformation and biological activity. To mimic more faithfully the in vivo follicular architecture and cellular spatial arrangement, bioengineering and nanotechnology researches have been focused on developing different in vitro conditions. With the support of natural or synthetic polymers, three-dimensional (3D) innovative culture systems were developed to enhance the adhesion, the proliferation and the release of secreted factors by cultured cells (Desai et al. 2010; Antoni et al. 2015). The 3D environment also resulted in cell behavior, signaling and gene expression profiles most resemble those observed in living cells (Cukierman et al. 2002). It has been demonstrated that the encapsulation of follicles and oocytes in biocompatible three-dimensional systems allows the

maintenance of their physiological structure and functional integrity in different species (mouse, Pangas et al. 2003; human, Combelles et al. 2005, pig, Munari et al. 2007).

The domestic cat is an excellent animal model for wild felids reproductive biotechnologies, but in this species only few studies were focused on the improvement of the in vitro performances of low competence oocytes and on the use of 3D systems for oocyte culture (Godard et al. 2009; Fujihara et al. 2012).

Thus, the present study was performed to investigate: (a) the suitability of a 3D system (barium alginate microcapsules) for feline oocytes in vitro culture, and (b) whether 3D system would improve in vitro maturation of CDOs co-cultured with COCs or cultured separately.

Material and methods

The study was approved by the Ethical Committee of the Università degli Studi di Milano (December 9th, 2014), and all animals were enrolled following written consent by the owner.

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Experimental design

Experiment I

To investigate the suitability of a 3D system, barium alginate (BA) microcapsules were prepared with different working conditions and a scoring method was applied to evaluate the physical properties of the obtained microcapsules.

Fresh feline COCs were in vitro cultured in the 3D system or in traditional microdrops of medium (2D system) for 24 h. At the end of the in vitro maturation, viability and maturation rates of feline COCs were evaluated.

Experiment II

To verify whether the 3D system would improve the in vitro maturation of cumulus-denuded oocytes, fresh feline CDOs were co-cultured with COCs [CDOs(+)] or cultured separately [CDOs(-)] in 3D or 2D system. After 24 h, the viability and maturation rates of CDOs(+), CDO(-) , and COCs co-cultured with CDOs [COCs(+)] were evaluated.

Animals and collection of feline oocytes

Ovaries from domestic cats were harvested at random stages of the estrous cycle during routine ovariectomy at veterinary clinics. After surgery, ovaries were immediately placed in a phosphate buffered saline (PBS) with a mixture of antibiotics (AB) and antimycotics (100 IU/ml of penicillin G sodium, 0.1 mg/ml

of streptomycin sulfate, and 0.25 μg/ml of amphotericin B), and transported to the laboratory at room temperature (RT) where they were processed.

Feline COCs were obtained by mincing of the ovaries in PBS and AB with 0.1% (w/v) polyvinyl alcohol (PVA) and only grade I COCs were selected for the experiments.

The CDOs were obtained by mechanical deprivation, with a small bore pipette, of COCs' cumulus cells.

In vitro maturation in 3D and 2D systems

The feline oocytes were matured in vitro for 24 h in a controlled atmosphere (38.5°C and 5% CO_2 in air) in modified Kreb's Ringer bicarbonate buffered salt solution with AB (b-mKRB) supplemented with 3 mg/mL of bovine serum albumin (BSA), 0.5 IU/mL of equine chorionic gonadotropin (eCG), 1 IU/mL of human chorionic gonadotropin (hCG), 10 ng/mL of epidermal growth factor (EGF) and 0.6 mM cysteine (complete maturation medium, c-mKRB).

For the 3D system, a two-steps encapsulation technique in BA was developed, as a modification of the protocol previously described for living-cell (Conte et al. 1999; Vigo et al. 2004). The Na-alginate powder (0.5%) was dissolved into the different solutions reported below, to obtain the melting solution (MS) at medium viscosity (3.500 cP, centipose). A saturated solution of $BaCl_2$ was then added to an aliquot of a different medium (see below) to obtain the dropping

solution (DS) of $BaCl_2$ (40 mM) that was dropped at RT with a 25G needle into the MS maintained stirred for 30-40 minutes. The microcapsules were then collected, washed twice in PBS and suspended in the c-mKRB for immediate use, or maintained at 4°C in a petri dish with PBS until use.

To obtain the BA microcapsules, the following working conditions were tested:

1: MS with b-mKRB and DS with b-mKRB. 2: MS with c-mKRB and DS with c-mKRB. 3: MS with sterile water and DS with b-mKRB. 4. MS with sterile water and DS with c-mKRB.

The feline COCs, the CDOs(+), the CDOs(-) and the COCs(+) were injected into the inner core of the microcapsule by a small bore pipette and subsequently immersed in the c-mKRB in a multiwell dish.

For the 2D culture system, traditional microdrops of c-mKRB (50-100µl) were placed in a petri dish and covered by mineral oil.

Assessment of viability and maturation rates

After 24 h of in vitro maturation, COCs and CDOs were evaluated for overall viability and nuclear maturation rates. Sequential stainings with fluorescein diacetate/propidium iodide (FDA/PI) for viability and bis-benzimide (Hoechst 33342) for chromatin configuration were performed.

For the viability, the oocytes were maintained at dark in 50 µl of the staining solution (PI: 10 mg/ml; FDA: 5 mg/ml) for 5 min and then evaluated under a

fluorescent microscope (Axiovert 100, Zeiss, Arese, Italy). This differential staining allowed the evaluation of viable (bright green fluorescence) or dead cells (red fluorescence).

After washing, CDOs and COCs (deprived of cumulus cells by mechanical displacement with a small bore glass pipette) were placed on a slide with a minimum amount of c-mKRB, and then covered by 10 µl of Hoechst solution. After 5 min of incubation in the dark, the Hoechst solution was removed and the oocytes were covered with an anti-fade reagent (Fluoromount™ Acqueous Mounting Medium). The fixed oocytes were then observed under a fluorescent microscope at 400x magnification for nuclear evaluation.

The chromatin configurations were classified as follows (Bolamba et al. 1998; Hewitt and England 1999):

- germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- germinal vesicle break down-Anaphase I (GVBD–AI): identification of different patterns of chromatin condensation (GVBD) or identification of bivalents (AI);
- telophase I-Metaphase II (TI–MII): identification of two groups of chromosomes moving to opposite ends of meiotic spindle (TI) or two sets of chromosomes clearly visible (MII);
- degenerated: collapsed nucleus or irregular nuclear conformation.

Statistical analysis

Data for physical properties of the microcapsules were reported as mean value and standard deviation (SD), the viability and maturation rates of COCs, CDOs(+), CDOs(-) and COCs(+) were analyzed by Chi-square test and the level of significance was set at $p<0.05$.

Results

A total of 131 feline ovaries were processed for this experiment and 402 COCs of grade I were assigned to the treatments.

Experiment I

For the physical evaluation of the microcapsules, the following properties were recorded:

- dimensions (mm): length, width;
- shape: R (round), E (elongated);
- consistency: E (excellent), G (good), L (low).

The working condition 3 showed the best physical properties of BA microcapsules for feline oocytes encapsulation (Table 1).

Table 1. Physical properties of the barium alginate (BA) microcapsules in different working conditions.

Working conditions	Width, mm (mean±SD)	Lenght, mm (mean±SD)	Shape (R,E)	Consistency (E,G,L)
1 MS with b-mKRB DS with b-mKRB	0	0	-	-
2 MS with c-mKRB DS with c-mKRB	0	0	-	-
3 MS with sterile water DS with b-mKRB	15.8 ± 1.81	24.12 ± 4.12	R	E
4 MS with sterile water DS with c-mKRB	15.03 ± 2.47	31.52 ± 3.98	R and E	G and L

MS: Melting solution of Na-alginate (0.5%)
DS: Dropping solution of $BaCl_2$ (40 mM)
b-mKRB: modified Kreb's Ringer bicarbonate buffered salt solution with antibiotics;
c-mKRB: b-mKRB supplemented with 3 mg/mL of bovine serum albumin (BSA), 0.5 IU/mL of equine chorionic gonadotropin (eCG), 1 IU/mL of human chorionic gonadotropin (hCG), 10 ng/mL of epidermal growth factor (EGF) and 0.6 mM cysteine.
Shape: R (round), E (elongated).
Consistency: E (excellent), G (good), L (low)

The dissolution of Na-alginate powder in sterile water and the dropping of $BaCl_2$ in b-mKRB was the proper combination. Although the dropping solution made with c-mKRB (working condition 4) allowed the formation of BA microcapsules, their shape and consistency were not as good as in working condition 3. On the other hand, the dissolution of Na-alginate powder in b- or

c-mKRB (working conditions 1 and 2) did not allow the formation of any microcapsule.

The results of in vitro maturation of the COCs cultured in both 3D and 2D systems, showed that they maintained a similar high viability ($p>0.05$), and no differences were found in their meiotic resumption (Table 2), nor full maturation (TI-MII stages) rates (3D: 7/47=14.9% vs 2D: 7/44=15.9%; $p>0.05$).

Table 2. Viability and nuclear status of feline cumulus-oocyte complexes (COCs) after in vitro maturation in 3D or 2D system.

System	Viability No. of oocytes (%)	Immature (GV) No. of oocytes (%)	Meiotic resumption (GVBD-MII) No. of oocytes (%)	Degenerated No. of oocytes (%)
3D	41/47 (87.2)	8/47 (17)	32/47 (68.1)	7/47 (14.9)
2D	37/44 (84)	9/44 (20.5)	26/44 (59.1)	9/44 (20.5)

No statistical differences.

3D: barium alginate microcapsules

2D: microdrops of maturation medium

GV, germinal vesicle; GVBD, germinal vesicle break down; MII, metaphase II

Experiment II

The results presented in Table 3, showed that the overall viability was similar in 3D and 2D systems ($p>0.05$). In 3D microcapsules the presence of COCs

resulted in a higher viability of CDOs(+), than that obtained without COCs [CDOs(-)] or in 2D microdrops (p=0.007 and p=0.002, respectively).

Table 3. Viability of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) cultured in 3D or 2D system.

Groups	Viability in 3D system No. of oocytes (%)	Viability in 2D system No. of oocytes (%)
CDOs(+) COCs(+)	51/56 (91.1) [a,x] 45/47 (95.7) [x]	35/52 (67.3) [b,x] 44/48 (91.7) [y]
CDOs(-)	37/52 (71.2) [y]	46/56 (82.1) [x,y]
Overall Viability	133/155 (85.8)	125/156 (80.1)

[a,b] Different superscripts indicate significant differences within rows (p<0.05)
[x,y] Different superscripts indicate significant differences within columns (p<0.05)
CDOs(+): CDOs co-cultured with COCs
COCs(+): COCs co-cultured with CDOs
CDOs(-): CDOs cultured separately
3D: barium alginate microcapsules
2D: microdrops of maturation medium

The 3D system was able to support the meiotic resumption of the feline oocytes, as well as the 2D microdrops (Table 4). The group of CDO(+) did not benefit from the co-culture in 3D microcapsules, as the percentages of meiotic resumption were similar of those of CDO(-). The highest values were reached by COCs(+) in both 3D and 2D system (p<0.05). This group achieved better results of full maturation (TI-MII stages) than the CDOs(+) (3D: 19/47=40.4% vs 4/56=7.1%; p=0.005. 2D: 25/48=52.1% vs. 4/52=7.7%; p=0.001) and the CDOs(-) (3D: 3/52=5.8%; p=0.003. 2D: 2/56=7.1%; p=0.001).

Table 4. Nuclear status of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) cultured in 3D or 2D system.

Groups	Immature (GV) No. of oocytes (%)		Meiotic resumption (GVBD-MII) No. of oocytes (%)		Degenerated No. of oocytes (%)	
	3D	2D	3D	2D	3D	2D
CDOs(+)	18/56 (32.1)[x]	18/52 (42.9)[x]	31/56 (55.4)[x]	27/52 (51.9)[x]	7/56 (12.5)	7/52 (13.5)
COCs(+)	3/47 (6.4)[y]	5/48 (10.4)[y]	39/47 (83)[y]	40/48 (83.3)[y]	5/47 (10.6)	3/48 (6.3)
CDOs(-)	21/52 (40.4)[x]	20/56 (35.7)[x]	21/52 (40.4)[x]	23/56 (41.1)[x]	10/52 (19.2)	13/56 (23.2)

No differences within rows.
[x,y] Different superscripts indicate significant differences within columns ($p<0.05$).
CDOs(+): CDOs co-cultured with COCs
COCs(+): COCs co-cultured with CDOs
CDOs(-): CDOs cultured separately
3D: barium alginate microcapsules
2D: microdrops of maturation medium
GV, germinal vesicle; GVBD, germinal vesicle break down; MII, metaphase II

Discussion

The present study was aimed at developing an enriched culture system to improve the in vitro performances of low competence feline oocytes that lost their surrounding cumulus cells (cumulus-denuded oocytes, CDOs). At very low rates, these gametes reach the full cytoplasmic and nuclear competence because the functional and metabolic support of their somatic cells is missed. The subsequent in vitro fertilization and embryo development is also highly compromised (Tanghe et al. 2002; Luciano et al. 2005).

The enriched culture system used for CDOs in this work consisted in a three-dimensional microcapsules of barium alginate (BA) in association with fresh feline cumulus-oocytes complexes (COCs).

To obtain the 3D BA microcapsules different working conditions were tested. The results showed that the dissolution of Na-alginate powder in sterile water and the subsequent dropping of $BaCl_2$ dissolved in the basic maturation medium (b-mKRB), was the best protocol to obtain round microcapsules with a solid inner core useful for feline oocytes encapsulation. The working conditions that involved media with hormones and growth factors supplementation (c-mKRB), as melting or dropping solution, seemed to inhibit the complete dissolution of Na-alginate powder and the ionic interactions with $BaCl_2$, compromising the effective creation and use of the microcapsules. However, the metabolic effect of nutrients, growth factors and hormones on the encapsulated oocytes was

ensured by the immersion of the BA microcapsules in c-mKRB. The effective exchange of different molecules through these systems has been documented (Vigo et al. 2004).

A similar viability and maturation rate of feline COCs cultured in these proper BA microcapsules compared to those cultured in 2D microdrops, proved that the 3D system was a suitable culture condition for feline oocytes.

Many authors reported the efficiency of 3D systems for mammalian follicles and oocytes in vitro culture. In vitro growth and development (i.e. the proliferation and the differentiation of theca and granulosa cells, the steroid secretion and markers expression) of murine and human follicles were enhanced after in vitro culture in 3D alginate microcapsules, and the achievement of the full competence of the inner oocytes was also obtained (Xu et al. 2006; Xu et al. 2009; Shikanov et al. 2011). In addition, the encapsulation of human or swine oocytes in a collagen gel or in a BA capsules helped the in vitro meiosis progression until MII stage (Combelles et al. 2005; Munari et al. 2007).

In the present study, the CDOs co-cultured with COCs showed a higher viability in 3D than in 2D system, but no beneficial effects of this association was observed in meiosis resumption and full maturation (TI-MII stages) rates. Instead, the associated COCs had the highest viability and maturation rates in both systems.

Present data differed from previous studies in the domestic cat and in other species (Luciano et al. 2005; Ge et al. 2008; Godard et al. 2009) in which the presence of COCs during in vitro maturation and in vitro fertilization seemed to promote the achievement of MII stage and of the subsequent embryo development of CDOs.

In this study, fresh feline COCs were mechanically deprived of the surrounding cumulus cells to obtain the CDOs. This method, that differs from those of the aforementioned studies in which vortex or incubation with hyaluronidase were used, could have influenced the results. It remains to investigate how the oocytes without cumulus cells, and not denudated *ad hoc,* behave in the same culture conditions.

It could also be hypothesized that the feline CDOs need different conditions to improve their performances in vitro, as the formulation of more specific maturation media.

It is notable that the presence of CDOs seemed to enhance the meiotic competence of the associated COCs. In bovine and murine model, this positive effect of denuded oocytes in the same culture condition as companion cells of COCs has been already reported (Hussein et al. 2006; Gilchrist et al. 2008). It is well known that the oocytes themselves produce some specific paracrine factors, known as the oocyte-secreted factors (OSFs), which act specifically on surrounding cumulus cells, regulating their differentiation, functional activity and

gene expression. In the present study, these factors could have provided some beneficial support to ameliorate the maturation rates of COCs in both 3D and 2D culture conditions.

In conclusion, the 3D barium alginate microcapsules could support the in vitro culture of the feline oocytes, as well as the traditional 2D system. Since the in vitro maturation rates of CDOs remain low, more specific and designing ad hoc in vitro conditions for these low competence oocytes should be adopted.

The more physiological microenvironment, i.e. the maintenance of oocytes architecture ensured by the 3D culture, represents an enriched condition that might also modulate the molecular expression of some oocyte quality markers, as the OSFs. The genetic expression of these factors in oocytes cultured in 3D or 2D system should be investigated for improving the feline oocytes in vitro performances.

Author contributions

MGM, VG and GCL contributed to design the study, analyse the data and draft the paper. MGM and SC performed the experiments. All authors have approved the final version.

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

Antoni D, Burckel H, Josset E, Noel G, 2015: Three-dimensional cell culture: a breakthrough in vivo. Int J Mol Sci Suppl **16**, 5517-5527.

Bolamba D, Borden-Russ KD, Durrant BS, 1998: In vitro maturation of domestic dog oocytes cultured in advanced preantral and early antral follicles. Theriogenology Suppl **49**, 933-942.

Chigioni S, Perego L, Luvoni GC, 2005: Companion cumulus-oocyte complexes for in vitro culture of denuded oocytes in the cat. In: Farstad W, Arus J (ed.), Proc 4th Ann Congr EVSSAR. Amsterdam (The Netherlands), 14 April 2005, pp. 24-25.

Combelles C, Fissore RA, Albertini DF, Racowsky C, 2005: In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system. Hum Reprod Suppl **20,** 1349-1358.

Conte U, Torre ML, Maggi L, Giunchedi P, Vigo D, Maffeo G, Russo V, 1999: Microcapsules containing seminal material for artificial insemination in pigs. European Patent Application EP0922451 (16/6/1999) CAS 131, 49474v.

Cukierman E, Pankov R, Yamada KM, 2002: Cell interactions with three-dimensional matrices. Curr Opin Cell Biol Suppl **14**, 633-639.

Desai N, Alex A, AbdelHafez F, Calabro A, Goldfarb J, Fleischman J, Falcone T, 2010: Three-dimensional in vitro follicle growth: overview of culture models, biomaterials, design parameters and future directions. Reprod Biol Endocrinol **8**, 119-131.

Eppig JJ, 1982: The relationship between cumulus cell–oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol Suppl **89,** 268–272.

Fagbohun CF, Downs SM, 1991: Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte–cumulus cell complex. Biol Reprod Suppl **45**, 851–859.

Fujihara M, Comizzoli P, Wildt DE, Songsasen N, 2012: Cat and dog primordial follicles enclosed in ovarian cortex sustain viability after in vitro culture on agarose gel in a protein-free medium. Reprod Domest Anim Suppl **47**, 102–108.

Ge L, Sui HS, Lan GC, Liu N, Wang JZ, Tan JH, 2008: Coculture with cumulus cells improves maturation of mouse oocytes denuded of the cumulus oophorus: observations of nuclear and cytoplasmic events. Fertil Steril Suppl **90**, 2376-2388.

Gilchrist RB, Lane M, Thompson JG, 2008: Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update Suppl **14**, 159–177.

Godard N, Pukazhenthi BS, Wildt DE, Comizzoli P, 2009: Paracrine factors from cumulus-enclosed oocytes ensure the successful maturation and fertilization in vitro of denuded oocytes in the cat model. Fertil Steril Suppl **91**, 2051-2060.

Hewitt DA, England GCW, 1999: Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation in vitro. Anim Reprod Sci Suppl **55**, 63-75.

Hussein TS, Thompson JG, Gilchrist RB, 2006: Oocyte-secreted factors enhance oocyte developmental competence. Dev Biol Suppl **296**, 514–521.

Luciano AM, Lodde V, Beretta MS, Colleoni S, Lauria A, Modina S, 2005: Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. Mol Reprod Dev Suppl **71**, 389–397.

Luciano AM, Modina S, Vassena R, Milanesi E, Lauria A, Gandolfi F, 2004: Role of intracellular cyclic adenosine 3',5'-monophosphate concentration and oocyte–cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte. Biol Reprod Suppl **70**, 465–4672.

Munari E, Asti A, Colombani C, Benzoni E, Faustini M, Torre ML, Riccardi A, Villani S, Sesso L, Conte U, Vigo D, 2007: Competence of swine oocytes matured by three-dimensional gonadotropin free co-culture. Vet Res Commun Suppl **31**, 181-184.

Pangas SA, Saudye H, Shea LD, Woodruff TK, 2003: Novel approach for the three-dimensional culture of granulosa cell–oocyte complexes. Tissue Eng Suppl **9**, 1013-1021.

Shikanov A, Xu M, Woodruff TK, Shea LD, 2011: A method for ovarian follicle encapsulation and culture in a proteolytically degradable 3 dimensional system. J Vis Exp **49**, 1-9.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, De Kruif A, 2002: Mini review: functions of the cumulus oophorus during oocyte maturation, ovulation and fertilization. Mol Reprod Dev Suppl **61**, 414-424.

Vigo D, Torre ML, Faustini M, Munari E, Russo V, Norberti R, Villani S, Asti A, Bini PP, Conte U, 2004: Barium alginate capsules for 3D immobilisation of living cells: morphology, membrane properties and permeability. J Drug Deliv Sci Technol Suppl **14**, 167-172.

Xu M, Barret S, West-Farrel E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK, 2009: In vitro grown human ovarian follicles from cancer patients support oocyte growth. Hum Reprod Suppl **24**, 2531–2540.

Xu M, West E, Shea LD, Woodruff TK, 2006: Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development. Biol Reprod Suppl **75**, 916-923.

PAPER II

EMBRYO DEVELOPMENT OF CUMULUS-DENUDED FELINE OOCYTES IN VITRO MATURED IN A THREE-DIMENSIONAL ALGINATE SYSTEM

EMBRYO DEVELOPMENT OF CUMULUS-DENUDED FELINE OOCYTES IN VITRO MATURED IN A THREE-DIMENSIONAL ALGINATE SYSTEM.

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Abstract

To improve the in vitro full competence of oocytes with poor morphological characteristics, different culture conditions were evaluated. Domestic cat cumulus-denuded oocytes (CDOs) were in vitro matured in an enriched system of three-dimensional (3D) barium alginate microcapsules in association with cumulus-oocyte complexes (COCs) in a commercial medium. After in vitro fertilization with chilled feline spermatozoa, the subsequent embryonic development in 3D system was evaluated. The results were compared with those obtained after in vitro maturation and culture in a traditional culture system (two-dimensional, 2D, microdrops). The feline CDOs achieved the in vitro full maturation (TI-MII stages) and developed until late embryo stages (morulae and blastocysts) at the same proportions of the high competence COCs. The enriched conditions during maturation and culture represented by 3D barium alginate microcapsules did not improve the results, as similar rates of CDOs viability, meiotic progression and embryonic development were obtained in 3D and 2D systems.

In conclusion, although in this study the in vitro embryo production from feline CDOs was obtained, the optimal culture conditions for these low competence oocytes deserve further investigations.

Key words: Domestic cat; denuded oocytes; embryo development; 3D system.

Introduction

The poor maturational and developmental competence of oocytes that lost their surrounding cumulus cells (CCs) is ascertained. The lack of CCs negatively affects the metabolism and the functionality of the oocytes because the bi-directional communication between germinal and somatic compartments, through the exchange of different molecules, nutrients and secreted factors, is crucial for the regulation of oocyte meiotic arrest and meiosis resumption (Eppig, 1982; Fagbohun & Downs, 1991; Tanghe et al., 2002). Only cumulus-oocyte complexes (COCs) surrounded by multiple layers of CCs are selected for in vitro embryo production, but in some cases the cumulus-denuded oocytes (CDOs) represent the only available genetic source.

For instance, cryopreserved COCs generally lose their CCs due to the intrinsic cold-damages of freezing or vitrification procedures. The different size, structure and permeability of these somatic cells compared to the germinal cell, are responsible of their high susceptibility to the cryoprotectant agents and to the cold temperature that are detrimental for their survival. This affects the maturational and developmental full competence of thawed oocytes in vitro (Songsasen & Comizzoli, 2009).

Widening the germinal pool with oocytes with poor morphological characteristics could also be of high interest for increasing the chance of fertility

preservation in case of limited numbers of female gametes, as in wild felids (Puzakenthi et al., 2006).

Some attempts of enriched cultures aimed at improving the maturation rates and the subsequent embryo development of low competence oocytes have been reported. The in vitro association of CDOs with competent COCs enhanced the achievement of the metaphase II (MII) stage and the embryo development until blastocyst stage in different species including cats (Ikeda et al., 2000; Luciano et al., 2005; Godard et al., 2009). The domestic cat is an optimal animal model for reproductive biotechnologies of wild felids and for comparative studies on human fertility preservation medicine (Comizzoli et al., 2010)

Recently, the co-culture of cat CDOs with competent COCs in an innovative culture system of 3D barium alginate microcapsules was described for the first time (Morselli et al., 2015). The results showed that the 3D system was able to support the viability and the meiosis resumption of feline oocytes, but their in vitro embryo development was not investigated. The 3D in vitro culture system mimics more faithfully the conformation of biological extra-cellular matrix, and its efficiency in maintaining the viability and promoting the growth of follicles and oocytes, other than supporting the embryo development, has been demonstrated in different mammalian species (Kreeger et al., 2006; Torre et al., 2006; Munari et al., 2007; Zhao et al., 2015).

This study was developed to investigate whether the association of a 3D system and a co-culture with competent COCs in a commercial medium during in vitro maturation could sustain the feline CDOs in vitro embryo development.

Material and methods

The study was approved by the Ethical Committee of the University of Milan (December 9th, 2014), and all animals were enrolled following written consent by the owner.

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Experimental design

Experiment I

Fresh feline CDOs were co-cultured with COCs [CDOs(+)] or cultured separately [CDOs(-)] in a commercial medium in 3D (barium alginate microcapsules) or 2D (microdrops) system. A control group of COCs [COCs(-)] was in vitro matured in 3D or in 2D conditions. After 24 h, the viability and

maturation rates of CDOs(+), CDO(-) , COCs co-cultured with CDOs [COCs(+)] and COCs control [COCs(-)] were compared.

Experiment II

Fresh feline CDOs were in vitro matured with COCs [CDOs(+)] in a commercial medium in 3D or 2D system, as in experiment I. After in vitro fertilization with chilled epididymal feline spermatozoa, presumptive zygotes were in vitro cultured separately in 3D or 2D system, according to the IVM conditions. A control group of COCs [COCs(-)] was in vitro matured, fertilized and cultured in 3D or 2D system. Embryonic development was recorded during 7 days of in vitro culture.

Animals and collection of feline oocytes

Ovaries from domestic cats were harvested at random stages of the estrous cycle during routine ovariectomy at veterinary clinics. After surgery, ovaries were immediately placed in a phosphate buffered saline (PBS) with a mixture of antibiotics (AB) and antimycotics (100 IU/ml of penicillin G sodium, 0.1 mg/ml of streptomycin sulfate, and 0.25 μg/ml of amphotericin B), and transported to the laboratory at room temperature (RT) where they were processed.

Feline COCs were obtained by mincing of the ovaries in PBS and AB with 0.1% (w/v) polyvinyl alcohol (PVA) and only grade I COCs were selected for the experiments.

The CDOs were obtained by mechanical deprivation, with a small bore pipette, of COCs' cumulus cells.

In vitro maturation and in vitro culture in 3D and 2D systems

The feline oocytes were in vitro matured for 24 h in a controlled atmosphere (38.5°C and 5% CO_2 in air) in Quinn's Advantage Protein Plus Blastocyst (SBP, SAGE® In Vitro Fertilization, Trumbull, Connecticut, USA) medium (b-SBP) supplemented with 75 IU FSH + 75 IU LH (Menogon®, Ferring Pharmaceuticals, Switzerland), 10 ng/ml of epidermal growth factor (EGF), AB and 0.6 mM cysteine (c-SBP).

After fertilization (see below), the presumptive zygotes were in vitro cultured for 7 days in a controlled atmosphere (38.5°C and 5% CO2 in air) in b-SBP supplemented with 5% of fetal calf serum (FCS) and AB (c-SBP2, National Institutes of Health, Bethesda, MD, USA).

For the 3D system, a two-steps encapsulation technique in barium alginate (BA) was performed as previously described (Morselli et al., 2015; Vigo et al., 2004; Conte et al., 1999). Briefly, the sodium alginate powder (0.5%) was dissolved into sterile water to obtain the melting solution at medium viscosity (3.500 cP, centipose). A saturated solution of $BaCl_2$ was then added to an aliquot of b-SBP medium to obtain the dropping solution of $BaCl_2$ (40 mM) that was dropped at RT with a 25G needle into the melting solution maintained stirred for 30-40

minutes. The microcapsules were then collected, washed twice in PBS, and suspended in the b-SBP medium for immediate use, or maintained at 4°C in a Petri dish with PBS until use.

The feline CDOs(+), the CDOs(-), the COCs(+) and the COCs of the control group [COCs(-)] were injected into the inner core of the microcapsule by a small bore pipette and subsequently immersed in the culture medium (c-SBP) in a multiwell dish.

For the 2D culture system, traditional microdrops of c-SBP (50-100 μl) were placed in a Petri dish and covered by mineral oil.

Epididymal sperm recovery and in vitro fertilization

In vitro fertilization was performed with chilled feline epididymal spermatozoa obtained from isolated testicles after orchiectomy at veterinary clinics. The epididymides were isolated from the testicles and placed in a 35 mm Petri dish containing 1 ml of Ham's F-10 medium supplemented with 2 mM L-glutamine, 5% (v/v) FCS and AB. Epididymal spermatozoa were released by mincing with a scalpel blade. Tissue debris were removed by forceps, and the remaining sperm suspension was centrifuged (300 g, 5 min) and diluted 1:2 with Tris egg yolk buffer for chilling at 4°C degree for 24 h. Before use, the spermatozoa were washed by centrifugation and a swim-up treatment was performed by gently layering 50 μl of b-SBP on the sperm pellet. After 30-45 min at 38.5°C, the

supernatant was collected and concentration and motility were determined in the sperm suspension.

After 24 h of maturation, the oocytes were washed twice in c-SBP2 and transferred into 90 μl drops of fresh c-SBP2. Immediately prior to insemination, the sperm suspension was diluted in c-SBP2 to a final concentration of 0.75-1 x 10^6 motile spermatozoa/ml, and 10 μl were added to each fertilization drop containing the oocytes.

At 18-24 h post insemination, cumulus cells of COCs and unbound spermatozoa were removed from the oocytes using a small bore glass pipette. After washing, the presumptive zygotes were in vitro cultured for 7 days in c-SBP2, in 3D or 2D system according to the IVM conditions. Fresh culture medium (c-SBP2) was added every two days and the embryonic development were recorded.

Assessment of viability, maturation rates and embryonic development

Sequential stainings with fluorescein diacetate/propidium iodide (FDA/PI) for viability assessment and with bis-benzimide (Hoechst 33342) for the evaluation of chromatin configuration were performed in Experiment I.

Briefly, the oocytes were maintained at dark in 50 μl of the staining solution (PI: 10 mg/ml; FDA: 5 mg/ml) for 5 min and then evaluated under a fluorescent microscope (Axiovert 100, Zeiss, Arese, Italy). This differential staining allowed the evaluation of viable (bright green fluorescence) or dead cells (red

fluorescence). After washing, CDOs, COCs (after removal of cumulus cells) were placed on a slide with a minimum amount of medium, and then covered by 10 µl of Hoechst solution. After 5 min of incubation in the dark, the Hoechst solution was removed and the oocytes were covered with an anti-fade reagent (Fluoromount ™Acqueous Mounting Medium). The fixed oocytes were then observed under a fluorescent microscope at 400x magnification for the evaluation of nuclear stages.

The chromatin configurations of the oocytes were classified as follows (Bolamba et al., 1998; Hewitt and England, 1999):

- germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- germinal vesicle break down-Anaphase I (GVBD–AI): identification of different patterns of chromatin condensation (GVBD) or identification of bivalents (AI);
- telophase I-Metaphase II (TI–MII): identification of two groups of chromosomes moving to opposite ends of meiotic spindle (TI) or two sets of chromosomes clearly visible (MII);
- - degenerated: collapsed nucleus or irregular nuclear conformation.

In Experiment II for the assessment of embryo development, cleaved embryos, 8-16 cells, morulae and blastocysts stages were recorded along 7 days of culture.

Statistical analysis

Data for viability, maturation and embryonic development rates of CDOs(+), CDOs(-), COCs(+) and COCs control [COCs(-)] were analyzed by Chi-square test and the level of significance was set at $p<0.05$.

Results

A total of 115 feline ovaries were processed for this study and 617 grade I COCs were assigned to the treatments.

Experiment I

In all the treatment groups, the BA microcapsules of 3D system were able to sustain the viability (range: 87.9% - 100%,) and the meiosis resumption of feline oocytes, as the 2D microdrops ($p>0.05$).

The resumption of meiosis (Table 1) of COCs co-cultured (+) or cultured separately (-) was significantly higher ($p<0.001$) in 3D and 2D system than that of CDO(+) and CDO(-). The rate of GVBD-MII stage of CDOs(+) did not differ from that of CDO(-). No differences in degeneration rates were observed.

Experiment II

The results reported in the Table 2 showed that the embryo development, in terms of cleavage, 8-16 cell and morula and blastocyst rates, was similar in oocytes matured and cultured in 3D BA microcapsules and in the 2D microdrops ($p>0.05$). The CDO(+) matured in the co-culture with COCs developed at lower rates than the associated COCs ($p<0.05$) in both systems. However, the CDOs(+) achieved the 8-16 cells and the late embryo stages (morulae and blastocysts) at similar proportions of COCs(-). The proportions of morulae and blastocysts on the total number of cleaved embryos, showed that CDOs(+) embryonic development was similar to that of associated COCs(+) and COCs(-).

Table 1. Meiotic progression of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) after in vitro maturation in 3D or 2D system.

Groups	No. of oocytes		Meiotic resumption (GVBD-MII) No. of oocytes (%)		*GVBD-AI* *No. of oocytes (%)*		*TI-MII* *No. of oocytes (%)*		Degenerate No. of oocytes (%)	
	3D	2D	3D	2D	*3D*	*2D*	*3D*	*2D*	3D	2D
CDOs(+)	39	33	13 (33.3)[a]	16 (48.5)[a]	*8 (20.5)[a]*	*12 (36.4)[a]*	*5 (12.8)[a]*	*4 (12.1)[a]*	4 (10.3)	2 (6.1)
COCs(+)	39	33	34 (87.2)[b]	28 (84.8)[b]	*20 (51.3)[b]*	*15 (45.5)[a]*	*14 (35.9)[b]*	*13 (39.4)[b]*	2 (5.1)	2 (6.1)
CDOs(-)	36	36	16 (44.4)[a]	13 (36.1)[a]	*3 (8.3)[a]*	*7 (19.4)[a,b]*	*13 (36.1)[b]*	*6 (16.7)[a]*	6 (16.7)	1 (2.8)
COCs(-)	35	35	30 (85.7)[b]	32 (91.4)[b]	*8 (22.9)[a,x]*	*2 (5.7)[b,y]*	*22 (62.9)[c,x]*	*30 (85.7)[c,y]*	0 (0)	1 (2.9)

[x,y] Different superscripts indicate significant differences within rows ($p<0.05$).

[a,b,c] Different superscripts indicate significant differences within columns ($p<0.05$).

CDOs(+), CDOs co-cultured with COCs; COCs(+), COCs co-cultured with CDOs; CDOs(-), CDOs cultured separately; COCs(-), COCs cultured separately (control group); 3D, barium alginate (BA) microcapsules; 2D, microdrops of maturation medium; GV, germinal vesicle; GVBD, germinal vesicle break down; MII, metaphase II.

Table 2. Embryonic developmental rates of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) matured and cultured in 3D or 2D system.

Groups	No. of oocytes		Cleavage No. (%)		8-16 cells No. (%)		Morulae + blastocysts No. (%)		*Morulae + blastocysts/cleaved No. (%)*	
	3D	2D	3D	2D	3D	2D	3D	2D	3D	2D
CDOs(+)	56	59	7 (12.5)[a]	10 (16.9)[a]	4 (7.1)[a]	8 (13.6)[a]	4 (7.1)[a]	6 (10.2)[a]	4 (57.1)[a,b]	6 (60)
COCs(+)	57	57	30 (52.6)[b]	28 (49.1)[b]	25 (43.9)[b]	22 (38.6)[b]	23 (40.4)[b]	22 (38.6)[b]	23 (76.7)[b]	22 (78.6)
COCs(-)	45	57	14 (31.1)[c]	12 (21.1)[a]	6 (13.3)[a]	12 (21.1)[a]	3 (6.7)[a]	11 (19.3)[a]	3 (21.4)[a,x]	11 (91.7)[y]

[a,b,c] Different superscripts indicate significant differences within columns ($p<0.05$)

[x,y] Different superscripts indicate significant differences within rows ($p<0.05$)

CDOs(+), CDOs co-cultured with COCs during IVM; COCs(+), COCs co-cultured with CDOs during IVM; COCs(-), COCs cultured separately (control group); 3D, barium alginate (BA) microcapsules during IVM and IVC; 2D, microdrops of culture medium during IVM and IVC.

Discussion

The achievement of in vitro-derived embryos from low competence oocytes, as those without cumulus cells, represents a current challenge for improving the chance of fertility preservation.

In the present study, the feline cumulus-denuded oocytes achieved the full maturational competence and developed until morula and blastocyst stages at the same proportions than the competent COCs.

However, the enriched conditions represented by 3D barium alginate microcapsules during maturation and culture did not improve the results. Similar rates of CDOs viability, meiotic progression and embryonic development were obtained in 3D and 2D systems. Differently from other studies in which bovine embryos in vitro-derived from competent oocytes developed at higher rates in 3D system compared to traditional 2D microdrops (Zhao et al., 2015), in this study the embryo development of feline COCs did not differ in the two systems.

In addition, the enrichment of the in vitro maturation with competent COCs did not exert a beneficial effect for the CDOs performances. No differences were found in the maturation and in the subsequent embryonic development when the CDOs were in vitro matured with or without the associated COCs, in both 3D and 2D systems. These results of maturation confirm what has been found in a previous work (Morselli et al., 2015), but differ from other studies, which

demonstrated a positive effect of the co-culture with COCs on bovine and domestic cat CDOs (Luciano et al., 2005; Godard et al., 2009).

The presence of own cumulus cells is crucial for the oocyte quality. The CCs are involved in many cellular processes, as the metabolism of different substrates (i.e. glucose, fatty acids, carbohydrates, and amino acids), and provide the specialized microenvironment for cytoplasmic and nuclear oocyte maturation and development (Sutton et al., 2010). The deprivation of CCs has a strong and negative impact on the developmental competence of the oocyte in different species (Auclair et al., 2013). For instance, the absence of CCs and related specific secreted factors, lead to aberrant cytoplasmic maturation, including an impaired cumulus-related lipid metabolism compared to that of the competent COCs (Auclair et al., 2013). In the domestic cat, no information were available regarding the lipid metabolism of CDOs. A more detailed investigation of the different metabolism in high and low competence oocytes should be of high interest to design a proper enriched culture condition for this species.

In the present study, the Quinn's Advantage Protein Plus Blastocyst (SBP) medium, specifically designed for the in vitro culture of human embryos, was used in both experiments. This commercial medium is easy to handle, pathogen free, high reproducible and routinely used for assisted reproductive techniques. Present data suggest that the SBP could support the full maturation and development of COCs and CDOs.

As already observed in the previous work (Morselli et al., 2015), the COCs benefit from the association with CDOs, as their embryo development rates were higher than that of COCs cultured separately. The positive effect of the association presumably depends on the paracrine effects of some specific growth factors, known as the oocyte-secreted factors (OSFs). These diffusible molecules are produced by the oocytes themselves and up-regulates the expression of some CCs-related genes, regulating their functions. The presence of CDOs in the same culture with COCs during maturation has been shown to improve their achievement of full maturational stages and to promote their embryo development in different species (Hussein et al., 2006).

In conclusion, the feline CDOs could fully mature and develop until blastocyst stage, but the enriched culture conditions (3D BA microcapsules and association with competent COCs) during in vitro maturation did not improve the results. The potential benefit of the maintenance of the feline oocyte architecture in the 3D culture deserves further investigations. A better knowledge of specific expression profiles and how they could differ from COCs and CDOs in the 3D and 2D systems could help the design of the optimal enriched culture conditions for the low competence oocytes.

Author contributions

MGM, GCL and PC contributed to design the study, analyse the data and draft the paper. MGM performed the experiments. All authors have approved the final version.

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R, Uzbekova S. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. Am J Physiol Endocrinol Metab 2013, 67: 599–613.

Bolamba D, Borden-Russ KD, Durrant BS. In vitro maturation of domestic dog oocytes cultured in advanced preantral and early antral follicles. Theriogenology 1998; 49 (5): 933-942.

Comizzoli P, Songsasen N, Wildt DE. Protecting and extending fertility for females of wild and endangered mammals. Cancer Treat Res 2010; 156: 87-100.

Conte U, Torre ML, Maggi L, Giunchedi P, Vigo D, Maffeo G, Russo V. Microcapsules containing seminal material for artificial insemination in pigs. European Patent Application EP0922451 (16/6/1999) CAS 131, 49474v.

Eppig JJ. The relationship between cumulus cell–oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol 1982; 89 (1): 268–272.

Fagbohun CF, Downs SM. Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte–cumulus cell complex. Biol Reprod 1991; 45 (6): 851–859.

Godard N, Pukazhenthi BS, Wildt DE, Comizzoli P. Paracrine factors from cumulus-enclosed oocytes ensure the successful maturation and fertilization in vitro of denuded oocytes in the cat model. Fertil Steril 2009; 91 (5): 2051-2060.

Hewitt DA, England GCW. Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation in vitro. Anim Reprod Sci 1999; 55 (1): 63-75.

Hussein TS, Thompson JG, Gilchrist RB. Oocyte-secreted factors enhance oocyte developmental competence. Dev. Biol. 2006; 296: 514-521.

Ikeda S, Ichihara-Tanaka K, Azuma T, Muramatsu T, Yamada M. Effects of midkine during in vitro maturation of bovine oocytes on subsequent developmental competence. Biol Reprod 2002; 63: 1067-1074.

Kreeger PK, Deck JW, Woodruff TK, Shea LD. The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels. Biomaterials 2006; 27 (5): 714–723.

Luciano AM, Lodde V, Beretta MS, Colleoni S, Lauria A, Modina S. Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. Mol Reprod Dev 2005: 71 (3): 389–397.

Morselli MG, Canziani S, Vigo D, Luvoni GC. A three-dimensional alginate system for in vitro culture of cumulus-denuded feline oocytes. Reprod Fert Develop 2015: submitted.

Munari E, Asti A, Colombani C, Benzoni E, Faustini M, Torre ML, Riccardi A, Villani S, Sesso L, Conte U, Vigo D. Competence of swine oocytes matured by three-dimensional gonadotropin free co-culture. Vet Res Commun 2007; 31 (1): 181-184.

Pukazhenthi B, Comizzoli P, Travis AJ, Wildt DE. Applications of emerging technologies to the study and conservation of threatened and endangered species. Reprod Fertil Dev. 2006; 18(1-2): 77-90

Songsasen N, Comizzoli P. A historic overview of embryos and oocyte preservation in the world of mammalian in vitro fertilization and biotechnology. In: Borini A, Coticchio G, editors. Preservation of human oocytes. London: Informa Healthcare; 2009, p. 1-11.

Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction 2010; 139: 685-695.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, De Kruif A. Mini review: Functions of the cumulus oophorus during oocyte maturation, ovulation and fertilization. Mol Reprod Dev 2002; 61 (3): 414-24.

Torre ML, Munari E, Albani E, Levi-Setti PE, Villani S, Faustini M, Conte U, Vigo D. In vitro maturation of human oocytes in a follicle-mimicking three-dimensional coculture. Fertility and Sterility 2006, 86 (3): 572-576.

Vigo D, Torre ML, Faustini M, Munari E, Russo V, Norberti R, Villani S, Asti A, Bini PP, Conte U. Barium alginate capsules for 3D immobilisation of living cells: morphology, membrane properties and permeability. J Drug Deliv Sci Technol 2004: 14 (3); 167-172.

Zhao S, Liu ZX, Gao H, Wu Y, Fang Y, Wu SS, Li MJ, Bai JH, Liu Y, Evans A, Zeng SM. A three-dimensional culture system using alginate hydrogel prolongs hatched cattle embryo development in vitro. Theriogenology 2015, 84: 184–192.

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APPENDIX

CO-AUTHORSHIP

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Sperm ubiquitination in epididymal feline semen

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ABSTRACT

Ubiquitin is a 8.5-kDa peptide that tags other proteins for proteasomal degradation. It has been proposed that ubiquitination might be responsible for the elimination of defective spermatozoa during transit through the epididymis in humans and cattle, but its exact biological function in seminal plasma has not yet been clarified. In the domestic cat (*Felis catus*), the percentage of immature, unviable, and abnormal spermatozoa decreases during the epididymal transit, indicating the existence of a mechanism that removes defective spermatozoa. Magnetic cell separation techniques, based on the use of magnetic beads coated with anti-ubiquitin antibodies, may allow the selective capture of ubiquitinated spermatozoa from semen, thus contributing to the identification of a potential correlation between semen quality and ubiquitination process. Moreover, the selective identification of all the ubiquitinated proteins in different epididymal regions could give a better understanding of the ubiquitin role in feline sperm maturation. The aims of this study were as follows: (1) to verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads and identify the morphological and acrosomal differences between whole sample and unbound gametes, (2) to characterize all the ubiquitinated proteins in spermatozoa retrieved in the three epididymal regions by a proteomic approach. The data indicated the presence of ubiquitinated proteins in cat epididymal semen. However, a correlation between abnormal and ubiquitinated spermatozoa has not been found, and ubiquitin cannot be considered as a biomarker of quality of epididymal feline spermatozoa. To the author's knowledge, this is the first identification of all the ubiquitinated proteins of cat spermatozoa collected from different epididymal regions. The proteomic pattern allows a further characterization of cat epididymal semen and represents a contribute to a better understanding of the ubiquitin role in feline sperm maturation.

1. Introduction

Ubiquitin is a 8.5-kDa peptide that tags other proteins for proteasomal degradation, and it is also involved in the regulation of protein function. This protein is a normal component of human blood, ovarian follicular fluid, and seminal plasma [1], and its role in the elimination of defective spermatozoa during transit through the epididymis has been described in humans and cattle [2,3].

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The hypothesis of epididymal removal by ubiquitination of defective spermatozoa is based on the fact that proportions of ubiquitinated bovine spermatozoa decrease along the transit from the head to the corpus epididymis and that cultured epididymal epithelial cells are capable of spermiophagy [4].

The results demonstrated that the increase of sperm ubiquitin is inversely associated with spermatic concentration, motility, and normal morphology, indicating that ubiquitination could be a biomarker of poor semen quality [3]. Conversely, some authors [5] found a positive correlation between sperm ubiquitin and good semen parameters, suggesting a different role for sperm ubiquitination.

Therefore, the role played by ubiquitination of spermatozoa proteins and their function during the transit through the epididymis is still an open question, even in species whose semen properties have been extensively studied, such as human and bovine.

Regarding the domestic cat (*Felis catus*), only in one study ubiquitin staining of both normal and altered epididymal spermatozoa has been described [6].

It has been ascertained that in the cat, the percentage of immature, unviable, and abnormal spermatozoa decreases during epididymal transit, indicating the existence of a mechanism that removes defective spermatozoa [7,8]. Thus, the epididymis that is responsible for sperm development and maturation might also act as a quality control organ to prevent misshapen, genetically abnormal, or infertile spermatozoa from entering the ejaculate.

Magnetic cell separation techniques, based on the use of magnetic beads coated with anti-ubiquitin antibodies, may allow the selective capture of ubiquitinated spermatozoa from semen, thus contributing to the identification of a potential correlation between semen quality, spermatozoa maturation, and ubiquitination process. This would contribute to an understanding whether ubiquitin could be considered as a biomarker of quality of epididymal feline semen.

The selective identification of all the ubiquitinated proteins in spermatozoa of different epididymal regions could give a better understanding of the ubiquitin role in feline sperm maturation [9–12].

The evaluation of ubiquitination pattern of seminal proteins has been applied to humans [13,14], and today, with the advances of proteomic techniques, thousands of proteins have been described as being part of normal human semen [15]. The challenges for analyzing protein ubiquitination are largely due to low stoichiometry of ubiquitinated species in cells [16]. To overcome these limitations, it is essential to enrich for ubiquitinated proteins before mass spectrometry (MS) analysis. In the present study, this strategy, combined to a shot-gun proteomic approach, has been applied for the first time to semen of domestic cat treated with magnetic ubiquitin beads before MS/MS analysis to identify all the ubiquitinated proteins in spermatozoa of different epididymal tracts.

Therefore, the aims of the present study were as follows: (1) to verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads and identify the structural (morphological and acrosomal) differences between whole sample and unbound gametes, (2) to extensively characterize all the ubiquitinated proteins in spermatozoa retrieved in the three epididymal regions by a proteomic approach.

2. Materials and methods

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2.1. Animals and experimental design

Twenty-two healthy and pubertal cats presented to the Department for routine orchiectomy were included in this study.

In experiment 1, semen samples were obtained from 10 pairs of isolated testes by squeezing cauda epididymis and vasa deferentia in a warm (37 °C) PBS solution. Ubiquitination of the epididymal spermatozoa was evaluated by Western blot analysis as described in detail in the below section.

To verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads, the sample was divided into two aliquots: the first was processed with magnetic ubiquitin beads, and the second aliquot was not treated and used as control. Sperm parameters (concentration, motility, morphology, and acrosomal integrity) were evaluated in whole sample (control) and in the sample treated with beads (unbound spermatozoa, see Section 2.3).

In experiment 2, 12 pairs of epididymides were processed. The epididymis was dissected from each testis and pampiniform plexus using a scalpel blade. The small vessels were removed with scissors to reduce blood contamination. Each pair of epididymides was macroscopically divided into three anatomical portions, caput, corpus, and cauda, according to the previous studies [17,18]. Each pair of caput, corpus, and cauda was placed in a Petri dish containing 2 mL of PBS and minced with a scalpel blade. After 30 minutes of incubation at 37 °C, 1 mL of the suspension was collected from each dish. Each sample was processed with magnetic ubiquitin beads, and proteomic analysis was assessed in spermatozoa bound to the beads to extensively characterize all the ubiquitinated proteins in the three regions of the epididymis.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two-dimensional electrophoresis, and Western blot analysis

Whole semen samples were suspended in 0.5 M Tris–HCl, pH 6.8, 10% glycerol, and 10% sodium dodecyl sulfate (SDS). The protein content was determined by Bradford method [19] and separated on an homemade 11% polyacrylamide gel according to Laemmli [20]. Two-dimensional (2D) electrophoresis was carried out as described in Tedeschi et al. [21]. For the first dimension, proteins were applied to rehydrated Immobilized pH Gradient strips (70 mm, 3–10 Non Linear) (Amersham Biosciences, Cologno Monzese, Italy). Isoelectric focusing was performed at 15 °C as follows: 600 V for 10 minutes, 900 V for 15 minutes, 1500 V for 15 minutes, 2500 V for 15 minutes, and 3500 V for 5 hours and 15 minutes. Before

the second dimension, each strip was rinsed with buffer (6 M urea in 0.375 M Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, bromophenol blue). The second dimension was performed on homemade 12% SDS minigels (8.5 × 6 × 0.15 cm) at 20 mA/gel.

Proteins in the gel (either 1D or 2D) were stained with ammonium silver stain or transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Bedford, MA, USA) by electrophoretic elution at 180 mA for 1 hour. The transfer buffer was 10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid and 10% methanol. Membranes were then probed for immunoreactivity as follows: for ubiquitin detection, the membrane was blocked with 5% dry milk in Tris-buffered saline (TBS), washed five times with TBS-T 0.25%, incubated in rabbit anti-ubiquitin polyclonal antibody (AbCam, Cambridge, UK) to 1:1000 in 2% dry milk in TBS-T 0.25%. The membrane was washed five times with TBS-T 0.25%, incubated in anti-IgG rabbit HRP conjugated (Calbiochem, Darmstadt, Germany) 1:1000 in 2% dry milk in TBS-T 0.25%.

2.3. Sperm separation with magnetic ubiquitin beads

Magnetic ubiquitin beads (Li Starfish S.r.l., Cernusco S/N, Milan, Italy) suspended in 10 mM phosphate (pH 7.5) with 0.02% sodium azide were added to semen aliquots (80 μL beads/mL of semen) in a 1.5-mL tube. The tube was gently mixed for 20 minutes to allow contact between the magnetic beads and the targeted spermatozoa before placing it in a magnetic field for 10 minutes. The separation procedure is a negative depletion in which the magnetic beads attach to the targeted surface marker and are collected against the wall of the tube by application of an external laboratory magnet. The separated sample was decanted and collected while the tube was still in the magnetic field, whereas the ubiquitinated spermatozoa bound to the beads remained attached to the wall of the tube as long as the magnet was in place. Thus, two fractions were obtained: spermatozoa bound to the beads and spermatozoa unbound in the tube.

2.4. Spermatozoa evaluation

As previously mentioned, sperm parameters were evaluated in whole sample (control) and in the sample treated with beads (unbound spermatozoa). Sperm concentration was determined with a Bürker chamber. Motility was subjectively assessed under a light microscope (×40) with a heated stage at 38 °C. Spermatozoa were considered to be motile only if they exhibited progressive motility of a score of at least 3 or 4 on a scale of 0 to 4 (0, absent; 1, weak or sluggish; 2, definite; 3, good; and 4, vigorous) [22].

The morphology of spermatozoa was assessed after staining of the smear with Bengal Rose and Victoria Blue B. A total of 100 spermatozoa were evaluated under light microscope with oil immersion objective at ×100 magnification. Normal spermatozoa and site of defects in abnormal spermatozoa (head, neck/midpiece, and tail) were recorded [23]. The acrosome status was evaluated by staining the spermatozoa with peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the procedure described for stallion spermatozoa [24]. Staining solution was prepared with 90 μL of FITC–PNA (40 μg/mL in PBS) added with 10 μL of PI (340 μM in PBS).

An amount of 10 μL of sperm suspension was smeared on a microscope slide and fixed in 96% ethanol for 30 seconds. The slide was dried in dark, and then a droplet of 20 μL of FITC–PNA/PI was added to the slide. The slide was incubated in a moist chamber at 4 °C, and after 30 minutes it was rinsed with 4 °C distilled water and air dried at 4 °C in dark overnight. At least 100 spermatozoa were evaluated under fluorescent microscope (Axiovert 100, Zeiss, Oberkochen, Germany). The intact acrosome was stained green, whereas the head of the sperm was stained red.

The observed fluorescence images of ethanol-permeabilized spermatozoa, stained with FITC–PNA/PI, were classified into three patterns—(1) intact acrosome: spermatozoa displaying intensively bright fluorescence of the acrosomal cap; (2) abnormal acrosome: spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap or swollen acrosomal cap; and (3) absent acrosome: spermatozoa displaying a fluorescent band at the equatorial segment or displaying no fluorescence.

Mean ± SD of sperm characteristics were analyzed by Student's t-test ($P < 0.05$).

2.5. Mass spectrometry analysis

To increase the homogeneity of the sample and overcome the inter- and intrasample variability, epididymal spermatozoa of different cats collected from caput (A), corpus (B), or cauda (C) and bound to the magnetic beads were pooled.

The ubiquitin-conjugate proteins were analyzed by liquid chromatography-tandem mass spectrometry to determine the identities and the ubiquitination sites after tryptic digestion, and missed tryptic cleavage at the modified site. In particular, the spermatozoa bound to the magnetic beads were collected and lysed in a buffer containing 7 M urea, 2 M thiourea, 50 mM ammonium bicarbonate and the complete protease inhibitor cocktail (Roche, Basel, Switzerland).

On sonication, the lysates were clarified by centrifugation at 15,000 rpm for 15 minutes, and the total protein concentration was determined using the Bradford method [19]. Protein samples were reduced with 45 mM dithiothreitol, alkylated with 100 mM iodoacetamide, and subsequently digested with sequencing grade modified trypsin overnight at 37 °C. Digestion was stopped by adding 1 μL of 98% formic acid. The proteolytic digests were desalted on a $ZipTipC_{18}$ (Millipore, Billerica, MA, USA) before MS analysis. Each sample was separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific, Waltham, MA, USA). Buffer A was 0.1% vol/vol formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 lm ID, 3 lm resin, Dionex). The gradient was as follows: 5% buffer B (10 minutes), 5% to 40% B (60 minutes), 40% to 50% B (10 minutes), 95% B (5 minutes) at a flow rate of 0.3 μL/min.

Mass spectrometry was performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific) equipped with a

nanospray source (Proxeon Biosystems, now Thermo Fisher Scientific). Eluted peptides were directly electrosprayed into the mass spectrometer through a standard noncoated silica tip (New Objective, Woburn, MA, USA) using a spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Three replicate analysis of each sample were performed.

Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

2.6. Protein identification and computational analysis

For identification of ubiquitinated proteins, raw data files were processed and analyzed using MaxQuant 1.3.0.5. [25]. Parent ion and MS2 spectra were searched against the human and *Felis catus* Uniprot/SwissProt database (release: 04/10/2013) using the Andromeda search engine. Search parameters allowed for two missed tryptic cleavages, a mass tolerance of 6 ppm in MS mode, and 20 ppm in CID MS/MS mode, a static modification carbamidomethylation (Cys), and up to three total dynamic modifications [(*N*-acetylation (protein), oxidization (Met), and ubiquitination (Lys))]. To achieve highly reliable identifications, the following criteria were used: maximal protein, peptide and site false discovery rate (FDR) of 0.01, and minimal peptide length of 6. The default setting of the maximal peptide posterior error probability of 1 was used. The presence of ubiquitinated amino acids on each peptide was confirmed by visual inspection of the corresponding MS/MS spectrum by Perseus, discharging all the spectra presenting a modification at the C-terminal lysine.

3. Results and discussion

The presence of ubiquitinated proteins in cat epididymal semen was evaluated in experiment 1 by anti-ubiquitin antibodies upon separation on 1D electrophoresis of the whole cell extract (Fig. 1A). The pattern of ubiquitination is very similar to the one described by Thompson et al. [26] in bovine seminal plasma for the proteins at higher molecular weight (more than 50 kDa) (see Fig. 5C in Thompson et al. [26] for comparison). The major differences can be observed at lower molecular weight (37–20 kDa) where few bands, clearly detectable in cat semen, are absent in the bovine sample.

The semen was analyzed by 2D electrophoresis to better separate all the proteins and evaluate the ubiquitination pattern. Figure 1B shows all the proteins detected by silver staining, whereas Figure 1C reports the corresponding Western blot analysis using anti-ubiquitin antibodies, which clearly shows that the proteins ubiquitinated are mainly present in the 70- to 40-kDa region, except for few spots at lower molecular weight. This observation was further confirmed in the proteomic analysis described in the section below.

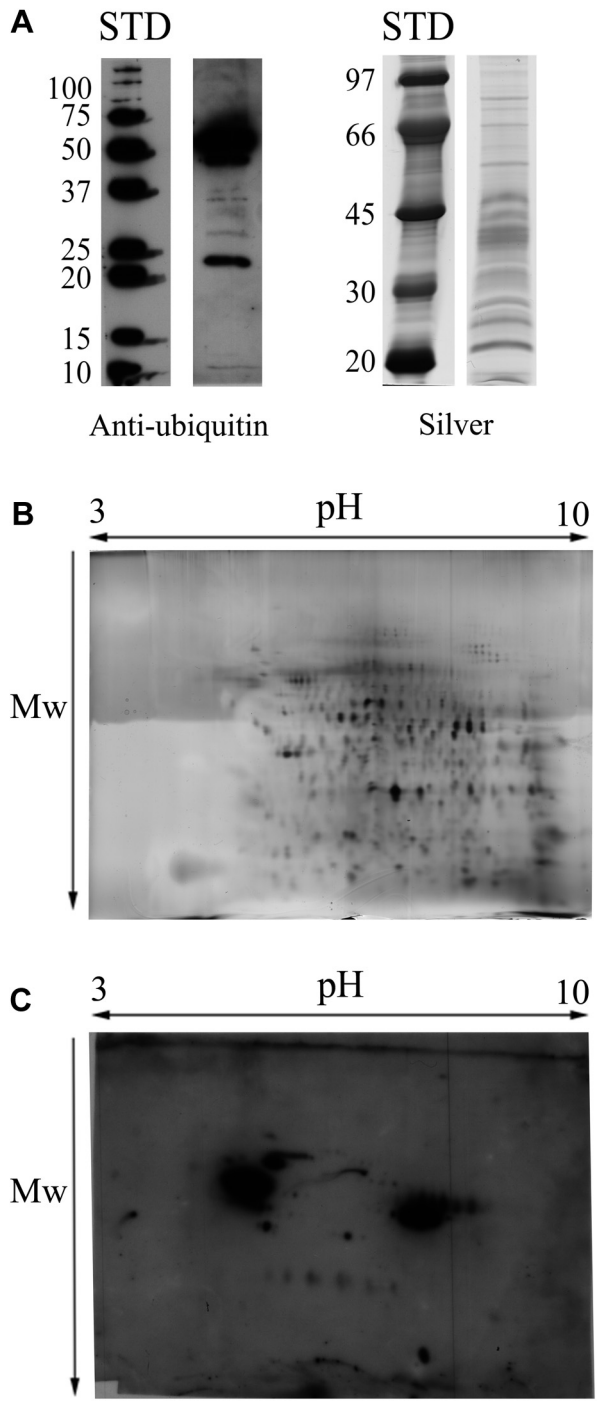

Fig. 1. One- and two-dimensional electrophoresis of whole cat epididymal semen and Western blot analysis by anti-ubiquitin antibodies. (A) One-dimensional electrophoresis: proteins were separated on two homemade 11% polyacrylamide gels. One was blotted on a PVDF membrane to detect ubiquitinated proteins by anti-ubiquitin antibodies, whereas the other was stained by silver to detect all the proteins present in the sample. (B, C) Semen proteins were separated by two-dimensional electrophoresis and either stained by silver (B) or blotted and immunodecorated with anti-ubiquitin antibodies (C). PVDF, polyvinylidene difluoride; STD, molecular weight standard.

Table 1
Sperm parameters in cat epididymal semen untreated or treated with magnetic ubiquitin beads.

Samples	Sperm concentration (sp/mL) × 10^6	Sperm count (sp/sample) × 10^6	Normal morphology (%)	Motility (%)	Acrosome integrity (%)
Untreated control	148.9 ± 102.8[a]	12.0 ± 7.5[a]	44.5 ± 16.4	51.0 ± 16.0	76.5 ± 15.1
Treated with beads	47.1 ± 31.6[b]	6.7 ± 5.2[b]	52.9 ± 11.9	49.0 ± 21.7	81.3 ± 13.0

[a,b] Different superscripts within columns indicate significant differences ($P < 0.01$).

After treatment of epididymal samples with magnetic ubiquitin beads (Table 1), a significant decrease of sperm concentration and sperm count was observed in the fraction of spermatozoa unbound to the beads. This finding demonstrated the efficacy of beads in binding ubiquitinated spermatozoa. However, the unbound fraction did not present higher sperm motility, better morphology, or increase in acrosomal integrity in comparison with the whole sample, suggesting that the magnetic beads did not select a population of cat epididymal spermatozoa with better semen quality. In accordance with our finding, Mota et al. [6] using immunofluorescence observed that besides abnormal spermatozoa, cells that were apparently normal also tested positive in this assay. These data reveal a lack of correlation between ubiquitinated and abnormal spermatozoa.

The analysis by a proteomic approach of the ubiquitinated spermatozoa bound to the beads contributed to extensively characterize all the proteins ubiquitinated in the three regions of the epididymis. The so-called shot-gun proteomic approach, which relies on tandem mass spectroscopy for protein identification and characterization of posttranslational modifications, has been applied here to characterize ubiquitination of the proteins in the cat spermatozoa collected from different epididymal regions.

Each fraction (caput, corpus, and cauda) enriched in ubiquitinated proteins was analyzed by liquid chromatography-tandem mass spectrometry to determine the identities and the ubiquitination sites of the proteins present in the samples. The proteomic analysis, carried out combining the results of three replicates, allowed us to identify 766, 708, and 646 proteins in caput (A), corpus (B), and cauda (C), respectively, (see Table S1, S2, and S3 in Supplementary Materials) among which 212 proteins are common in the three epididymal tracts (A, B, and C) and are reported in Table S4 (Supplementary Materials). No one of the 212 common proteins is ubiquitinated. Focusing only on the ubiquitinated proteins expressed in each epididymal tract, it was possible to identify 7 proteins in A, 17 in B, and 6 in C: a total of 30 proteins reported in Table 2. For each of them, one representative MS/MS spectrum of an ubiquitinated peptide is reported as Supplementary Materials in Figures S1–S30. As indicated in Table 2, ubiquitination of

Table 2
Ubiquitinated proteins found in the three epididymal regions: caput (A), corpus (B), and cauda (C).

Protein ID	Protein names	Gene names	Localization	PhosphoSite PLUS
P231412	Liver carboxylesterase 1	CES1; CES1	A	X
H0Y8G3		*IL7R*	A	
H0YL13	Uncharacterized protein	*UNC13 C*	A	
O60290	Zinc finger protein 862	*ZNF862*	A	
Q8WWQ0	PH-interacting protein	*PHIP*	A	X
Q9UQ13	Leucine-rich repeat protein SHOC-2	*SHOC2*	A	X
Q96DR7	Rho guanine nucleotide exchange factor 26	*ARHGEF26*	A	
Q8NF91	Nesprin-1	*SYNE1; SYNE1; SYNE1*	B	X
B3KSY9	E3 SUMO-protein ligase PIAS1	*PIAS1*	B	
Q68DE3	Basic helix-loop-helix domain-containing protein KIAA2018	*KIAA2018; KIAA2018*	B	X
O95069	Potassium channel subfamily K member 2	*KCNK2*	B	
O95989	Diphosphoinositol polyphosphate phosphohydrolase 1	*NUDT3*	B	
A2A2Y4	FERM domain-containing protein 3	*FRMD3*	B	
E7EUH9	Condensin-2 complex subunit G2	*NCAPG2*	B	X
G3V5U4	Paired box protein Pax-2	*PAX2*	B	
Q8WXH0-2	Nesprin-2	*SYNE2; SYNE2*	B	X
H7C3W3	AF4/FMR2 family member 3	*AFF3*	B	
Q7Z7G2	Complexin-4	*CPLX4*	B	
O96028	Probable histone-lysine N-methyltransferase NSD2	*WHSC1*	B	X
P45983	Mitogen-activated protein kinase 8	*MAPK8; MAPK8*	B	X
P51813	Cytoplasmic tyrosine-protein kinase BMX	*BMX*	B	
Q8N8A8	Protein FAM169B	*FAM169 B*	B	
Q8N9L7	Putative uncharacterized protein FLJ36925		B	
Q92908	Transcription factor GATA-6	*GATA6*	B	
O95257	Inducible protein GADD45 gamma	*GADD45 G; GADD45 G*	C	
Q7Z4L5	Tetratricopeptide repeat protein 21B	*TTC21 B; TTC21 B*	C	X
Q16585	Beta-sarcoglycan	*SGCB*	C	X
Q5THR3-4	EF-hand calcium-binding domain-containing protein 6		C	
Q68DH5	LMBR1 domain-containing protein 2	*LMBRD2*	C	X
Q92620	Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16	*DHX38*	C	

The X in the PSPLUS column indicates proteins previously described as ubiquitinated in human or mouse according to the PhosphoSite Plus Data Base (http://www.phosphosite.org) [27].

many of these proteins have been previously observed in human or mouse, according to the PhosphoSite Plus Data Base (http://www.phosphosite.org) [27].

The distribution of the 30 ubiquitinated proteins in terms of molecular function (GOMF), cellular components (GOCC), and biological processes (GOBP) are reported in the corresponding histograms of Figure 2. Among the ubiquitinated proteins, there are cytoskeletal proteins or proteins interacting with the cytoskeleton that are key players in the cytoskeleton organization, in cellular differentiation, and in the morphogenesis processes. Overall, more than 30% of the ubiquitinated proteins are involved in the anatomical structure development in keeping with the possible role of this modification as a way to remove defective spermatozoa in the epididymal tract. Most are proteins important for the regulation of gene expression or are components of signal transduction pathways, suggesting that ubiquitin, by playing a major role in the modulation of their homeostasis, may affect the expression level and the activation of other proteins present in the epididymal environment.

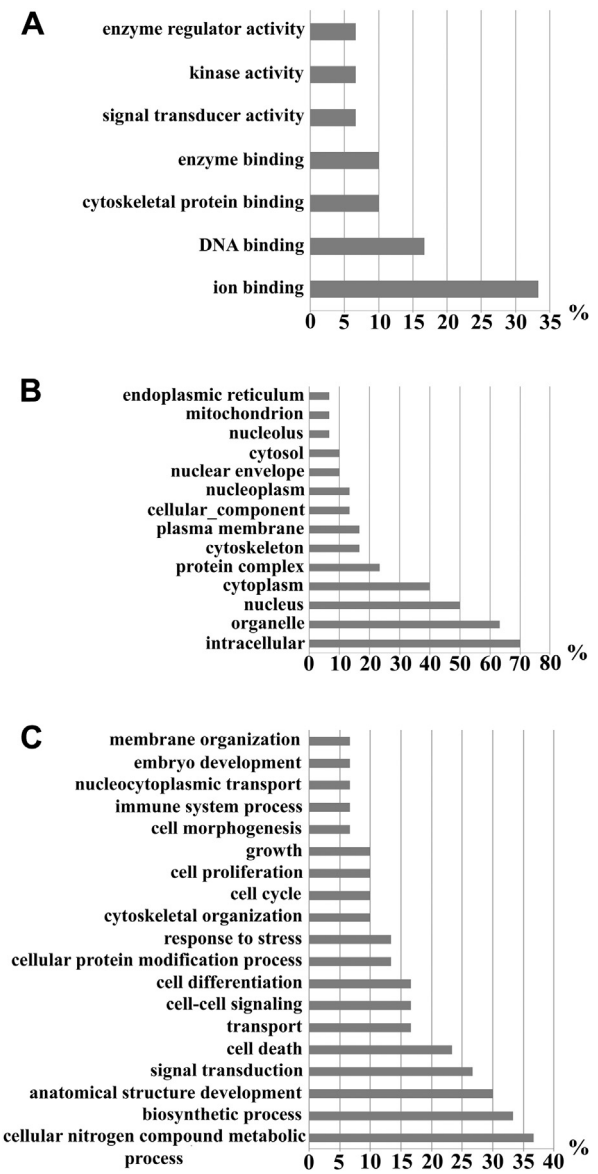

Fig. 2. Histograms of the distribution of the ubiquitinated proteins in the cat epididymal spermatozoa. The epididymal sample was enriched in ubiquitinated proteins by using magnetic beads, and bound spermatozoa were analyzed by a shot-gun proteomics approach. The distribution of the ubiquitinated proteins in terms of molecular function (GOMF), cellular components (GOCC), and biological processes (GOBP) are reported in (A, B, and C), respectively.

In this environment, substantial maturational changes of spermatozoa occur. During epididymal transit, spermatozoa acquire the capability to be motile, the cytoplasmic droplet migrates from a proximal to a distal position, the composition of the plasma membrane changes, acrosomal modifications occur, and sperm fertilizing ability develops [28]. The presence of different ubiquitinated proteins in the three regions of the epididymis suggests a role of ubiquitin in sperm maturation, probably due to a protein turnover typical of this process.

3.1. Conclusions

The present data demonstrated the presence of ubiquitinated proteins in cat epididymal semen. However, a correlation between abnormal and ubiquitinated spermatozoa has not been found, and ubiquitin cannot be considered as a biomarker of quality of epididymal feline spermatozoa.

To author's knowledge, this is the first identification of all the ubiquitinated proteins of cat spermatozoa collected from different epididymal regions. The proteomic pattern allows a further characterization of cat epididymal semen and represents a contribute to a better understanding of the ubiquitin role in feline sperm maturation.

Acknowledgments

Author contributions: GCL, VV, and GT contributed to design the study, analyze the data, and draft the article. Laboratory work was carried out by VV, SV, MGM, SN, EM, and AN. All authors have approved the final article.

Competing interests

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2014.06.002.

References

[1] Nandi D, Tahiliani P, Kumar A, Chandu D. The ubiquitin-proteasome system. J Biosci 2006;31:137–55.
[2] Baska KM, Manandhar G, Feng D, Agca Y, Tengowski MW, Sutovsky M, et al. Mechanism of extracellular ubiquitination in the mammalian epididymis. J Cell Physiol 2008;215:684–96.

[3] Sutovsky P, Hauser R, Sutovsky M. Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population. Hum Reprod 2004;19:628–38.
[4] Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 2001;114:1665–75.
[5] Muratori M, Marchiani S, Forti G, Baldi E. Sperm ubiquitination positively correlates to normal morphology in human semen. Hum Reprod 2005;20:1035–43.
[6] Mota PC, Ramalho-Santos J. Comparison between different markers for sperm quality in the cat: Diff-Quik as a simple optical technique to assess changes in the DNA of feline epididymal sperm. Theriogenology 2006;65:1360–75.
[7] Axnér E, Holst BS, Linde-Forsberg C. Morphology of spermatozoa in the cauda epididymidis before and after electroejaculation and a comparison with ejaculated spermatozoa in the domestic cat. Theriogenology 1998;50:973–9.
[8] Axnér E. Sperm maturation in the domestic cat. Theriogenology 2006;66:14–24.
[9] Kuster CE, Hess RA, Althouse GC. Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplets on ejaculated porcine spermatozoa. J Androl 2004;25:340–7.
[10] Suryawanshi AR, Khan SA, Gajbhiye RK, Gurav MY, Khole VV. Differential proteomics leads to identification of domain-specific epididymal sperm proteins. J Androl 2011;32:240–59.
[11] Souza CE, Rego JP, Lobo CH, Oliveira JT, Nogueira FC, Domont GB, et al. Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams. J Proteomics 2012;75:4436–56.
[12] Yuan S, Zheng H, Zheng Z, Yan W. Proteomic analyses reveal a role of cytoplasmic droplets as an energy source during epididymal sperm maturation. PLoS One 2013;8:e77466.
[13] Oliva R, de Mateo S, Estanyol JM. Sperm cell proteomics. Proteomics 2009;9:1004–17.
[14] Oliva R, Castillo J. Proteomics and the genetics of sperm chromatin condensation. Asian J Androl 2011;13:24–30.
[15] Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. Hum Reprod Update 2014;20:40–62.
[16] Peng J. Evaluation of proteomic strategies for analyzing ubiquitinated proteins. BMB Rep 2008;41:177–83.
[17] Schimming BC, Vicentini CA, Orsi AM, Franceschini-Vicentini IB, Abreu Rays MA. Regional histology of the ductus epididymidis in the dog (*Canis familiaris*, L.). Rev Chil Anat 1997;5:5–12.
[18] Schimming BC, Vicentini CA. Ultrastructural features in the epididymis of the dog (*Canis familiaris*, L.). Anat Histol Embryol 2001;30:327–32.
[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[20] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[21] Tedeschi G1, Cappelletti G, Negri A, Pagliato L, Maggioni MG, Maci R, et al. Characterization of nitroproteome in neuron-like PC12 cells differentiated with nerve growth factor: identification of two nitration sites in a-tubulin. Proteomics 2005;5:2422–32.
[22] Mortimer D. Sperm recovery techniques to maximize fertilizing capacity. Reprod Fertil Dev 1994;6:25–31.
[23] Christiansen IJ. Andrology of the normal male. In: Christiansen IJ, editor. Reproduction in the dog and cat. London: Baillière Tindall; 1984. p. 80–109.
[24] Cheng FP, Fazeli A, Voorhout W, Marks A, Bevers M, Colenbrander B. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J Androl 1996;17:674–82.
[25] Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 2008;26:1367–72.
[26] Thompson WE, Ramalho-Santos J, Sutovsky P. Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. Biol Reprod 2003;69:254–60.
[27] Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, et al. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res 2012;40:D261–70.
[28] Amann RP, Hammerstedt RH, Veeramachaneni DN. The epididymis and sperm maturation: a perspective. Reprod Fertil Dev 1993;55:361–81.

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Original Research Article

DNA integrity of fresh and frozen canine epididymal spermatozoa

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ABSTRACT

The aims of this study were to evaluate: (1) the effect of cryopreservation on DNA fragmentation of canine epididymal spermatozoa, and (2) the potential protective effect of melatonin on post-thaw sperm quality (motility, morphology, acrosomal and DNA integrity). Epididymal spermatozoa were collected after orchiectomy of ten dogs. Sperm samples were frozen in the presence or absence of melatonin (1 mM). DNA fragmentation index (percentage of spermatozoa with fragmented DNA) was similar in fresh samples (3.3 ± 3.6) and samples frozen with (4.2 ± 3.8) or without (3.6 ± 3.7) melatonin. Sperm motility was significantly ($p < 0.0001$) higher in fresh compared to frozen samples. The presence of melatonin in the freezing extender did not affect the sperm motility. Proportions of spermatozoa with normal morphology were similar in fresh and frozen samples, irrespective of the presence of melatonin in the extender. Acrosome integrity was significantly decreased ($p < 0.01$) by cryopreservation, and melatonin did not exert any beneficial effects. In conclusion, DNA fragmentation of canine epididymal spermatozoa was not affected by the freezing procedure, and the presence of melatonin did not preserve motility and acrosome integrity which were adversely affected by cryopreservation. The evaluation of DNA status of thawed gametes is particularly relevant for epididymal spermatozoa since these spermatozoa are usually stored and used in assisted reproductive techniques.

1. Introduction

The cryopreservation of epididymal spermatozoa is aimed at maintaining long-term availability of male germplasm for future use. This is particularly crucial for the conservation of endangered species and for the generation of offspring from individuals of high genetic value that die accidentally or undergo orchiectomy for medical purposes. In dogs, artificial insemination with frozen epididymal spermatozoa resulted in

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the birth of offspring with a low conception rate [1–3]. The effects of cryopreservation on motility, as well as membrane and acrosomal integrity of canine epididymal spermatozoa have been previously investigated [4–6], but no information is available on its potential effect on DNA integrity.

Sperm DNA integrity has been evaluated in fresh ejaculated [7–10] and epididymal canine semen [11]. Few reports have compared fresh and post-thaw chromatin integrity of canine ejaculated spermatozoa, obtaining variable results [12–16], but the post-thaw DNA stability of canine epididymal spermatozoa has not been investigated. The integrity of the paternal DNA is of crucial importance for embryo development [17], and a relationship between DNA damage and infertility has been demonstrated in humans. Spermatozoa with severe DNA damage remain functionally intact, with normal fertilizing ability, but a high index of DNA fragmentation results in a significant decrease in pregnancy rates [18,19]. Nevertheless, there is no agreement neither on whether cryopreservation induces DNA fragmentation, nor on the mechanism which actually induces this damage [20,21]. It has been hypothesized that the increase of reactive oxygen species (ROS) during cryopreservation and the decrease of antioxidant activity of the spermatozoa cause the peroxidative damage to the sperm plasma membrane and affect DNA integrity [13,14,20].

The role of antioxidant supplementation in protecting the sperm DNA from oxidative damage is still under investigation. Among antioxidants, it has been shown that melatonin (1–2 mM), has an effective action in protecting ram and bull spermatozoa from the freezing injuries as evidenced by post-thaw DNA integrity, viability, motility, morphology and fertilizing ability [22,23]. The aims of this study were to evaluate the effect of cryopreservation on DNA fragmentation of canine epididymal spermatozoa and the potential protective effect of melatonin on post-thaw sperm quality (motility, morphology, acrosomal and DNA integrity).

2. Materials and methods

2.1. Animals and epididymal spermatozoa retrieval

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Ten healthy and pubertal, privately owned stud dogs, aged between 1 and 10 years (6–30 kg body weight) presented to the Department for routine orchiectomy were included in this study. Canine gonads were transported to the laboratory within 10 min after surgical removal. Each epididymis was dissected from the testis and pampiniform plexus using a scalpel blade. The small vessels were removed with scissors to reduce blood contamination, and each cauda epididymis was isolated and placed in a Petri dish containing 4 mL of Ham's F-10 medium supplemented with 2 mM glutamine, 100 IU/mL Na-benzyl penicillin, 0.1 mg/mL streptomycin sulphate, and 5% fetal bovine serum (mOsm 285). The caudae were minced with a scalpel blade, and after 30 min of incubation (37 °C), the suspension was collected from each dish and divided into three aliquots.

2.2. Semen freezing procedure

One aliquot was used as fresh control, and the others were frozen with or without melatonin (1 mM) in a freezing extender. After centrifugation (700 × *g* for 5 min) and removal of the supernatant, the second aliquot was diluted (200×10^6 sperm/mL) with the following freezing extender: TRIS buffer with 5% glycerol, 1% Equex and 20% egg yolk, and the third aliquot was diluted with the extender supplemented with melatonin. Both aliquots were frozen according to the Uppsala system [24]. The 0.5 mL straws placed in a styrofoam box were submerged in liquid nitrogen vapors (10 min, 4.5 cm above liquid nitrogen) and subsequently immersed into liquid nitrogen. The straws were thawed in a water bath at 37 °C for 30 s.

2.3. Spermatozoa evaluation

Sperm concentration in fresh semen was determined with a Bürker chamber. Sperm motility, morphology and acrosomal integrity were evaluated in fresh and thawed samples. Motility was subjectively assessed under a light microscope with a heated stage (38 °C). The spermatozoa were considered to be motile only if they exhibited progressive motility of a score of at least 3 or 4 on a scale of 0–4 (0, absent; 1, weak or sluggish; 2, definite; 3, good; 4, vigorous) [25]. Morphology of spermatozoa was assessed following smear staining with Bengal Rose and Victoria Blue B. At least 100 spermatozoa were evaluated under light microscopy with oil immersion objective (1000× magnification). Normal spermatozoa and defect sites (head, neck/midpiece, tail) in abnormal spermatozoa were recorded. Abnormal sperm heads included those that were pear-shaped, narrow at the base or detached. Alterations of the neck/midpiece included bent neck and proximal or distal cytoplasmic droplet, and abnormal tail included single bent, coiled or broken tail.

The acrosome integrity was evaluated by staining spermatozoa with Peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI). Acrosome status was evaluated under fluorescent microscope (Axiovert 100, Zeiss, Oberkochen, Germany) in at least 100 spermatozoa per group by FITC-PNA/PI staining according to the procedure described for stallion spermatozoa [26]. The observed fluorescence images of the stained spermatozoa were classified as: (1) intact acrosome – spermatozoa displaying intensively bright fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane; (2) vesiculated acrosome – spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane; and (3) acrosome residues or loss – spermatozoa displaying a fluorescent band at the equatorial segment indicated residues of the outer acrosomal membrane or displaying no fluorescence indicated a complete loss of the outer acrosomal membrane [26].

2.4. Assessment of sperm DNA fragmentation

The sperm DNA fragmentation was assessed using the Sperm-Halomax® commercial kit specifically developed for canine

semen (Halotech DNA SL, Madrid, Spain) and based on the sperm chromatin dispersion (SCD) test. Values of sperm DNA fragmentation were evaluated in fresh and frozen samples at the concentration of 50×10^6 sperm/mL and processed following the manufacturer's instructions. Briefly, 25 μL of diluted samples were added to a vial with 50 μL of low melting agarose, and mixed. Pre-treated slides were placed onto a metallic plate which was previously cooled at 4 °C. A drop of the cell suspension (2 μL) was spread onto the treated face of the cooled slide, covered with a glass coverslip and maintained at 4 °C for 5 min. The coverslip was smoothly removed, and the layered sample was covered with the lysing solution provided in the kit. Finally, slides were washed for 5 min, dehydrated in sequential 70 and 100% ethanol baths and stained for 35 min in 1:1 Wright solution (Merck, Whitehouse Station, NJ, USA) and phosphate buffer (pH 6.88, Merck). When the slides were perfectly dried, they were mounted with Eukitt® and observed under bright-field microscopy (400× magnification). A minimum of 500 spermatozoa was evaluated in each sample. Intact sperm showed a small and compact halo, intensely colored, around the spermatozoa head. Spermatozoa with fragmented DNA presented a widespread and soft halo of chromatin dispersion. Spermatozoa showing a halo of dispersion were considered to have high DNA fragmentation index (percentage of spermatozoa with fragmented DNA over the total number of sperm counted per sample) [27].

2.5. *Statistical analysis*

Values are presented as mean ± standard deviation (SD). Arcsine transformation for percentage data was performed before the use of the one-way analysis of variance (ANOVA), followed by the post hoc Tukey test. Statistical significance was set at $p < 0.05$.

3. Results

Spermatozoa concentration averaged $252.7 \pm 161.8 \times 10^6$ sperm/mL. Cryopreservation did not affect DNA status of canine epididymal spermatozoa since similar DNA fragmentation indices were demonstrated in fresh sperm and sperm frozen with or without melatonin. Motility was significantly higher ($p < 0.0001$) in fresh compared to frozen spermatozoa. The presence of melatonin in the freezing extender did not affect sperm motility (Table 1). Morphological features of epididymal spermatozoa before and after freezing are summarized in Table 2. Proportions of spermatozoa with normal morphology as well as with abnormal heads, necks/midpieces or tails were similar in fresh and frozen samples irrespective of the presence of melatonin in the extender. Acrosome integrity was significantly affected ($p < 0.01$) by cryopreservation (Table 3). Proportions of spermatozoa showing vesiculated acrosome or acrosomal residues or loss were significantly higher in frozen samples compared to those of fresh samples. Melatonin did not affect the integrity of acrosome.

Table 1 – DNA fragmentation index and motility of canine epididymal spermatozoa (mean ± SD), fresh and frozen with or without melatonin (1 mM).

	Fresh spermatozoa	Frozen spermatozoa without melatonin	Frozen spermatozoa with melatonin
DNA fragmentation index (%)	3.3 ± 3.6	3.6 ± 3.7	4.2 ± 3.8
Motility (%)	74.5 ± 9.6[a]	37.5 ± 15.3[b]	36 ± 9.7[b]

Data derived from 10 replicates; different superscripts within a row indicate significant differences ($p < 0.0001$).

4. Discussion

In the current study, the DNA fragmentation index of canine epididymal spermatozoa was not affected by the freezing procedure, and the presence of melatonin did not preserve motility and acrosome integrity which were adversely affected by cryopreservation. Because the sperm morphology and DNA integrity were not compromised by the freezing procedure in the examined samples (both with or without melatonin), the potential protective effect of melatonin could not be proved.

The impact of cryopreservation on sperm DNA integrity is still a controversial matter in mammals including dogs. Some authors showed that the freezing/thawing procedure did not produce significant adverse effects on chromatin status in canine ejaculated spermatozoa [12,15,16]. In contrast, Kim et al. [14] found a higher DNA fragmentation in thawed compared to fresh spermatozoa. In the present study, DNA fragmentation was examined with the use of a commercial kit Halomax® based on the sperm chromatin dispersion (SCD) test – previously employed for ejaculated canine semen [9,16], whereas the cited authors used the sperm chromatin structure

Table 2 – Abnormal morphological features (%; mean ± SD) of canine epididymal spermatozoa, fresh or frozen with and without melatonin (1 mM).

Sperm morphology	Fresh spermatozoa	Spermatozoa frozen without melatonin	Spermatozoa frozen with melatonin
Normal spermatozoa	47.9 ± 24.9	49.9 ± 17.3	52.7 ± 12.9
Abnormal heads	13.5 ± 20.4	11.8 ± 25.0	11.3 ± 20.7
Abnormal necks/midpieces	19.7 ± 16	8.2 ± 8.8	10.3 ± 6.9
Abnormal tails	18.8 ± 11.3	30.2 ± 11.7	25.7 ± 10.0

Data derived from 10 replicates; no significant differences were observed.

Table 3 – Different acrosomal patterns (mean ± SD) of canine epididymal spermatozoa, fresh and frozen with or without melatonin (1 mM).

Acrosome pattern	Fresh spermatozoa	Spermatozoa frozen without melatonin	Spermatozoa frozen with melatonin
Intact (%)	66.6 ± 25.5^{a}	37.2 ± 11.6^{b}	36.3 ± 15.0^{b}
Vesiculated (%)	29.3 ± 23.7^{a}	52.1 ± 12.1^{b}	46.7 ± 16.6^{b}
Residues/loss (%)	4.2 ± 5.5^{a}	10.7 ± 3.5^{b}	17.0 ± 7.5^{b}

Data derived from 10 replicates, different superscripts (ab) within row indicate significant differences ($p < 0.01$).

assay (SCSA) and acridine orange assay. The SCD test was reported to be simple, highly reproducible and inexpensive technique, and the SCD results correlated with the SCSA results [28]. In the current paper, the DNA fragmentation indices were similar in fresh and frozen canine epididymal spermatozoa, which confirms the resilience of canine ejaculated sperm DNA to cold stress [12,15,16]. A progressive deterioration of DNA has been observed after exposure of fresh or thawed spermatozoa to different stressors (e.g., incubation time and temperature, toxicants, ROS) [21,29], indicating that thefreezing/thawing procedure facilitates the destabilization of the chromatin structure of spermatozoa [21]. As DNA fragmentation appears to be a dynamic process that depends on how quickly the iatrogenic DNA damage occurs, further investigations on canine epididymal sperm DNA damage over incubation time would be advisable.

The factors that determine sperm DNA status during cryopreservation are not known and various hypotheses have been suggested; this characteristic seems to be species-specific [30]. In a comparative study performed on eleven different mammalian species, a correlation was found between the presence of protamines 1 (P1) and 2 (P2) in the sperm head and the DNA status after thawing. The spermatozoa of species lacking P2 resisted fragmentation during the freezing/thawing procedure more effectively than species that contained both protamines. The dog was not included in this study, but the lack of P2 in canine spermatozoa [31] might explain the resilience of its DNA to the freezing/thawing procedure.

Another factor that may preserve DNA stability during the freezing/thawing procedure is the presence of seminal plasma in the sample. The post-thaw DNA integrity was improved when human spermatozoa were frozen with seminal plasma [20]. In dogs, the removal of plasma from the second fraction of the ejaculate before cryopreservation is not recommended, because the samples frozen with prostatic fluid showed higher DNA stability [13]. The beneficial effect of seminal plasma on DNA may be related to the presence of antioxidants in the plasma [13] since DNA fragmentation in cryopreserved spermatozoa may be caused by oxidative damage resulting from the imbalance between ROS and antioxidants [13,14,20]. Epididymal semen, which does not benefit from the antioxidant effect of seminal plasma, might be more vulnerable to the oxidative stress occurring during cryopreservation. Hence, in the present study, melatonin supplementation was used in order to compensate for the lack of antioxidants. However, due to the lack of cryopreservation impact on DNA fragmentation, the potentially protective effects of melatonin on sperm DNA could not be shown. Sperm morphology was also not affected by cryopreservation. Kim et al. [14] found an increased DNA fragmentation index and a higher proportion of head abnormalities in thawed compared to fresh spermatozoa. As a spermatozoon head consists almost entirely of DNA, it would have been interesting if the cited study had evaluated the correlation between head anomalies and DNA fragmentation, as demonstrated in fresh ejaculated canine semen [7,8,10].

A potential protective effect of melatonin was also examined in the context of other sperm parameters. Sperm motility and acrosomal integrity were significantly affected by cryopreservation, but no effect of melatonin was demonstrated. In ram and bull ejaculated semen, the presence of 1 or 2 mM melatonin in the freezing extender preserved post-thaw sperm DNA integrity and motility [22,23]. In red deer epididymal spermatozoa, melatonin gave only limited protection to sperm motility and acrosomal integrity after thawing [32]. It remains to be elucidated whether the lack of melatonin antioxidant effect in canine cryopreserved semen was due to an inappropriate melatonin concentration or to iatrogenic non-oxidative damage.

In conclusion, DNA fragmentation of canine epididymal spermatozoa was not affected by the freezing procedure, and the presence of melatonin did not preserve motility and acrosome integrity which were adversely affected by cryopreservation. The evaluation of DNA status of thawed gametes is particularly relevant for epididymal spermatozoa since these spermatozoa are usually stored and used in assisted reproductive techniques.

Authors contributions

GCL and SV contributed to design the study, analyze the data and draft the paper. Laboratory work was carried out by SV, MGM and VV. All authors have approved the final article.

Conflict of interest

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

REFERENCES

[1] Marks SL, Dupuis J, Mickelsen WD, Memon MA, Platz Jr CC. Conception by use of postmortem epididymal semen extraction in a dog. JAVMA 1994;204:1639–40.

[2] Hori T, Ichikawa M, Kawakami E, Tsutsui T. Artificial insemination of frozen epididymal sperm in beagle dogs. J Vet Med Sci 2004;66:37–41.
[3] Hori T, Matsuda Y, Kobayashi M, Kawakami E, Tsutsui T. Comparison of fertility on intrauterine insemination between cryopreserved ejaculated and cauda epididymal sperm in dogs. J Vet Med Sci 2011;73:1685–8.
[4] Hewitt DA, Leahy R, Sheldon IM, England GC. Cryopreservation of epididymal dog sperm. Anim Reprod Sci 2001;67:101–11.
[5] Ponglowhapan S, Chatdarong K, Sirivaidyapong S, Lohachit C. Freezing of epididymal spermatozoa from dogs after cool storage for 2 or 4 days. Theriogenology 2006;66:1633–6.
[6] Hori T, Uehara Y, Kawakami E, Tsutsui T. Influence of the time between removal and cooling of the canine epididymis on post-thaw caudal epididymal sperm quality. J Vet Med Sci 2009;71:811–5.
[7] Núñez-Martinez I, Moran JM, Peña FJ. Do computer-assisted, morphometric-derived sperm characteristics reflect DNA status in canine spermatozoa? Reprod Domest Anim 2005;40:537–43.
[8] Núñez-Martinez I, Moran JM, Peña FJ. Identification of sperm morphometric subpopulations in the canine ejaculate: do they reflect different subpopulations in sperm chromatin integrity? Zygote 2007;15:257–66.
[9] Hidalgo M, Murabito MR, Galvez MJ, Demyda S, De Luca LJ, Moreno M, et al. Assessment of sperm DNA fragmentation in canine ejaculates using the Sperm-Halomax® kit: preliminary results. Reprod Fertil Dev 2010;22:312–3.
[10] Lange-Consiglio A, Antonucci N, Manes S, Corradetti B, Cremonesi F, Bizzaro D. Morphometric characteristics and chromatin integrity of spermatozoa in three Italian dog breeds. JSAP 2010;51:624–7.
[11] Garcia-Macias V, Martinez-Pastor F, Alvarez M, Garde JJ, Anel E, Anel L, et al. Assessment of chromatin status (SCSA) in epididymal and ejaculated sperm in Iberian red deer, ram and domestic dog. Theriogenology 2006;66:1921–30.
[12] Rota A, Rota A, Martini M, Milani C, Romagnoli S. Evaluation of dog semen quality after slow (biological freezer) or rapid (nitrogen vapours) freezing. Reprod Nutr Dev 2005;45:29–37.
[13] Koderle M, Aurich C, Schäfer-Somi S. The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology 2009;72:1215–20.
[14] Kim SH, Yu DH, Kim YJ. Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. Theriogenology 2010;73:282–92.
[15] Prinosilova P, Rybar R, Zajicova A, Hlavicova J. DNA integrity in fresh, chilled and frozen–thawed canine spermatozoa. Vet Med (Praha) 2012;57:133–42.
[16] Urbano M, Dorado J, Ortiz I, Morrell JM, Demyda-Peyrás S, Gálvez MJ, et al. Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test. Anim Reprod Sci 2013;143:118–25.
[17] Andrabi SM. Mammalian sperm chromatin structure and assessment of DNA fragmentation. J Assist Reprod Genet 2007;24:561–9.
[18] Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril 2004;81:1289–95.
[19] Silva PF, Gadella BM. Detection of damage in mammalian sperm cells. Theriogenology 2006;65:958–78.
[20] Di Santo M, Tarozzi N, Nadalini M, Borini A. Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. Adv Urol 2012;2012:854837.
[21] Alkmin DV, Martinez-Alborcia MJ, Parrilla I, Vazquez JM, Martinez EA, Roca J. The nuclear DNA longevity in cryopreserved boar spermatozoa assessed using the Sperm-Sus-Halomax. Theriogenology 2013;79:1294–300.
[22] Succu S, Berlinguer F, Pasciu V, Satta V, Leoni GG, Naitana S. Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. J Pineal Res 2011;50:310–8.
[23] Ashrafi I, Kohram H, Ardabili FF. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci 2013;139:25–30.
[24] Linde-Forsberg C. Hints on dog semen freezing, cryoextenders and frozen semen artificial insemination. Proc. 2002 society for theriogenology meeting; 2002. pp. 303–20.
[25] Mortimer D. Sperm recovery techniques to maximize fertilizing capacity. Reprod Fertil Dev 1994;6:25–31.
[26] Cheng FP, Fazeli A, Voorhout W, Marks A, Bevers M, Colenbrander B. Use of Peanut agglutinin to assess the acrosomal status and the Zona Pellucida-induced acrosome reaction in stallion spermatozoa. J Androl 1996;17:674–82.
[27] De Ambrogi M, Spinaci M, Galeati G, Tamanini C. Viability and DNA fragmentation in differently sorted boar spermatozoa. Theriogenology 2006;66:1994–2000.
[28] Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59–66.
[29] Santiso R, Tamayo M, Gosálvez J, Johnston S, Mariño A, Fernández C, et al. DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. Mutat Res 2012;734:41–9.
[30] Gosálvez J, López-Fernández C, Fernández JL, Gouraud A, Holt WV. Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. Mol Reprod Dev 2011;78:951–61.
[31] Lee CH, Cho YH. Aspects of mammalian spermatogenesis: electrophoretical analysis of protamines in mammalian species. Mol Cells 1999;9:556–9.
[32] Domínguez-Rebolledo AE, Fernández-Santos MR, Bisbal A, Ros-Santaella JL, Ramón M, Carmona M, et al. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reprod Fertil Dev 2010;22:856–70.

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DNA fragmentation and sperm head morphometry in cat epididymal spermatozoa

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ABSTRACT

Sperm DNA fragmentation is an important parameter to assess sperm quality and can be a putative fertility predictor. Because the sperm head consists almost entirely of DNA, subtle differences in sperm head morphometry might be related to DNA status. Several techniques are available to analyze sperm DNA fragmentation, but they are labor-intensive and require expensive instrumentations. Recently, a kit (Sperm-Halomax) based on the sperm chromatin dispersion test and developed for spermatozoa of different species, but not for cat spermatozoa, became commercially available. The first aim of the present study was to verify the suitability of Sperm-Halomax assay, specifically developed for canine semen, for the evaluation of DNA fragmentation of epididymal cat spermatozoa. For this purpose, DNA fragmentation indexes (DFIs) obtained with Sperm-Halomax and terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) were compared. The second aim was to investigate whether a correlation between DNA status, sperm head morphology, and morphometry assessed by computer-assisted semen analysis exists in cat epididymal spermatozoa. No differences were observed in DFIs obtained with Sperm-Halomax and TUNEL. This result indicates that Sperm-Halomax assay provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. The DFI seems to be independent from all the measured variables of sperm head morphology and morphometry. Thus, the evaluation of the DNA status of spermatozoa could effectively contribute to the completion of the standard analysis of fresh or frozen semen used in assisted reproductive technologies.

1. Introduction

The evaluation of DNA status is not included in the standard semen analysis, but the frequency of spermatozoa containing fragmented DNA may be an important parameter of semen quality and a useful index of fertility potential.

Spermatozoa with severe DNA damage remain functionally intact, with normal fertilizing ability, but a high incidence of DNA fragmentation results in a significant decrease in pregnancy rates [1]. The exact mechanism of sperm DNA damage has not yet been clarified, but environmental stresses, gene mutations, chromosomal abnormalities, or oxidative damages might be involved [2].

Several methods have been developed to assess sperm DNA fragmentation such as *in situ* nick translation,

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terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL), comet assay, acridine orange test, and the sperm chromatin structure assay [3,4]. However, these techniques are labor-intensive and require expensive instrumentations.

Recently, a kit (Sperm-Halomax) based on the sperm chromatin dispersion (SCD) test [5] and specifically developed for boar [6], bull [7], stallion [8], and dog semen [9,10], but not for cat semen, became commercially available. Spermatozoa are immersed in an agarose matrix on a slide and briefly incubated in a lysing solution to remove membranes and proteins. DNA fragmentation produces large halos, whereas sperm with low levels of fragmentation show circumscribed halos. The evaluation can be performed by fluorescence or light microscopy. This kit would allow the routine assessment of sperm DNA fragmentation in laboratories dealing with andrological examinations and assisted reproductive technologies (ARTs).

Among different methods for the evaluation of sperm DNA fragmentation, TUNEL presents a good correlation with the SCD test [4,11] and has been previously used for cat semen [12].

In cats, significant advances in ART have been achieved, thanks to the embryo production by intracytoplasmic sperm injection (ICSI) of mature oocytes [13]. Sperm selection for ICSI is based on motility and morphology patterns, and the evaluation of the DNA status is not generally performed [14]. Because the sperm head consists almost entirely of DNA, subtle differences in sperm head morphometry might be related to DNA content and organization, as demonstrated in dogs [15] and humans [16]. To the authors' knowledge, similar studies have not been performed in cats.

The first aim of the present study was to verify the suitability of Sperm-Halomax assay, specifically developed for canine semen, for the evaluation of DNA status of epididymal cat spermatozoa. For this purpose, values of DNA fragmentation obtained with Sperm-Halomax and TUNEL were compared. The second aim was to investigate whether a correlation between DNA status, sperm head morphology, and morphometry assessed by computer-assisted semen analysis (CASA) exists in cat epididymal spermatozoa.

These data could contribute to achieve a better diagnosis in case of infertility due to male factors, to obtain a better evaluation of spermatozoa used in ARTs, and to refine the epididymal sperm selection criteria for ICSI.

The use of epididymal cat spermatozoa is currently a subject of interest with the purpose of establishing an efficient gene banking model for threatened and endangered wild felids and contributing to the preservation of genetic material from valuable males that die unexpectedly or undergo orchiectomy for medical reasons.

2. Materials and methods

2.1. Semen collection

Epididymal spermatozoa were collected from 28 tomcats subjected to routine castration at the Department of Health, Animal Science and Food Safety, University of Milan, Italy. Informed owner consent was obtained.

Epididymides and vasa deferentia were dissected and squeezed to collect epididymal fluid in a warmed (37 °C) PBS without calcium and magnesium.

2.2. Experimental design

2.2.1. Experiment 1: Sperm-Halomax assay versus TUNEL test

Epididymal fluid collected from 10 cats was divided into two aliquots for the evaluation of DNA status by Sperm-Halomax for canine spermatozoa (Halotech DNA SL, Madrid, Spain) and TUNEL test (Calbiochem FragEL DNA fragmentation detection kit, fluorescent–terminal deoxynucleotidyl transferase (TdT) enzyme; EMD Millipore Billerica, MA, USA).

2.2.2. Experiment 2: Correlation between sperm DNA status, head morphology, and morphometry

Epididymal fluid collected from the remaining 18 cats was used for the evaluation of DNA status by Sperm-Halomax, for the conventional sperm head morphology evaluation, and for the sperm head morphometry by CASA.

2.3. Sperm-Halomax assay

Sperm DNA fragmentation indexes (DFIs) were evaluated in semen samples diluted in PBS at the concentration of 5×10^6 to 10×10^6 sperm/mL and processed following the manufacturer's instructions. Briefly, 25 μL of diluted samples were added to a vial with 50 μL of low melting agarose and mixed. Pretreated slides were placed onto a metallic plate that was previously cooled at 4 °C. A drop of the cell suspension (5 μL) was spread onto the treated face of the cooled slide, covered with a glass cover slip, and maintained at 4 °C for 5 minutes. The cover slip was smoothly removed, and the layered sample was covered with the lysing solution provided in the kit for 5 minutes. Finally, the slides were washed for 5 minutes with distilled water, dehydrated in sequential 70% and 100% ethanol baths, and stained for 35 minutes in 1:1 Wright solution (Merck, Whitehouse Station, NJ, USA) and phosphate buffer (pH 6.88, Merck). When the slides were perfectly dried, they were mounted with Eukitt and observed under bright-field microscopy with ×40 magnification lens. A minimum of 500 spermatozoa was evaluated in each sample. Intact sperm showed a small and compact halo, intensely colored, around the spermatozoa head. Spermatozoa with fragmented DNA presented a widespread and soft halo of chromatin dispersion. Spermatozoa showing a halo of dispersion were considered to have high DFI (percentage of spermatozoa with fragmented DNA over the total number of spermatozoa counted per sample) [17].

2.4. TUNEL test

Sperm DFI was evaluated using a detection kit (Calbiochem FragEL DNA fragmentation detection kit, fluorescent–TdT enzyme; EMD Millipore Billerica). The principle of Fluorescein-FragEL is that TdT catalyzes the addition of fluorescein-labeled and -unlabeled deoxynucleotides to the

3′-OH ends generated by endonucleases during apoptotic degeneration.

Sperm samples were smeared on a slide and air-dried. Then, the smears were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature and washed twice in Tris-buffered saline (TBS) for 15 minutes. The slides were then covered with the permeabilization solution (protein kinase 2 diluted 1:100 in Tris solution 10 mM) and incubated for 6 minutes in a moist chamber. The slides were washed twice in TBS solution and maintained in a moist chamber. In the dark, an aliquot of the equilibration solution (TdT equilibration buffer diluted 1:5 in sterile water (Sigma Chemical Co., St. Louis, MO, USA)) was added to each slide that was incubated for 30 minutes in a moist chamber at room temperature. After the removal of the equilibration solution, an aliquot of the labeling solution (Fluorescein-FragEL TdT labeling reaction mix diluted 1:20 in TdT enzyme) was added to each slide that was incubated for 90 minutes in a moist chamber at 37 °C. The slides were washed three times in TBS, and a drop of an antifade reagent (Gel Mount; BiØmeda Corporation, Foster City, CA, USA) was added. The slides covered with a cover slip were examined under fluorescent microscope (Eclipse E600, Nikon Corporation, Tokyo, Japan) with ×40 magnification lens and oil immersion. A 4′,6-diamidino-2-phenylindole filter (330–380 nm) was used to visualize the total cell population (blue). Using a fluorescein filter (465–495 nm), a bright green signal indicated positive staining (DNA-fragmented sperm cells), whereas dull green or hard to visualize cells signified a nonreactive cell [18]. At least 500 spermatozoa of each sample were analyzed randomly to evaluate the percentage of TUNEL-positive sperm cells (DFI).

2.5. Conventional sperm head morphology

Undiluted samples were stained with a rapid Giemsa-Wright stain (Diff-Quick, Merck), and in each sample, a total of 200 spermatozoa was evaluated under light microscope (Diaplan Leitz, Wetzlar, Germany) with ×100 magnification lens and oil immersion. Abnormal sperm heads, included those that were pear-shaped, large, small, or amorphous, were recorded.

2.6. CASA sperm head morphometry

The same slides stained for the conventional morphology were examined for the evaluation of the sperm head morphometry using a light microscope (Olympus BX51, Olympus America Inc., Melville, NY, USA) with ×100 magnification lens and oil immersion equipped with a video camera (Scion Corporation 1394, Frederick, MD, USA) interfaced to a computer. The software used for image acquisition and analysis was Image-Pro Plus 5.1; Media Cybernetics (Immagini & Computer, Bareggio, Italy).

Each sperm head was measured for different parameters: area (μm^2), aspect (ratio between major and minor axes of the ellipse), perimeter (μm), maximum diameter (dmax, μm), minimum diameter (dmax, μm), maximum radius (radmax, μm), minimum radius (radmin, μm), radius ratio, and roundness [19].

2.7. Statistical analysis

Results of experiment 1 (Sperm-Halomax assay vs. TUNEL test) have been evaluated by the Bland–Altman plot technique [20] to assess the agreement between tests, considering the TUNEL procedure as reference method.

In experiment 2, to establish the reference values for the morphological sperm variables (area, aspect, perimeter, dmax, dmin, radmax, radmin, radius ratio, roundness), a nonparametric approach (2.5–97.5 percentile of the distribution) was followed on 2425 spermatozoa.

Variables not determined on a single spermatozoon (i.e., DFI and head anomalies) were submitted to the calculation of the 95% confidence interval as indicative reference values.

Aiming to evaluate the multivariate relations between DFI and the morphological variables, a principal component analysis (PCA) was applied: data were submitted to PCA after normalization and the *varimax* rotation. The number of retained components was calculated when at least the 90% of the total variability was explained. Moreover, the Pearson univariate correlation between DFI and the morphological variables was calculated ($P < 0.05$).

3. Results

3.1. Experiment 1: Sperm-Halomax assay versus TUNEL test

Epididymal cat spermatozoa processed with an SCD test as Sperm-Halomax developed for dogs produce similar patterns than those described in dog spermatozoa [9]. Spermatozoa with unfragmented DNA do not show or show very small halos of dispersion of DNA loops, whereas those with DNA fragmentation release peripheral halos from the central core (Fig. 1).

No differences were observed in DFI values obtained with Sperm-Halomax and TUNEL (4.34 ± 0.93% vs. 4.26 ± 0.83%; $P = 0.84$).

The Bland–Altman test was applied to evaluate the level of agreement between the TUNEL test and Sperm-Halomax. The results showed that there was a good agreement between the considered tests, because all points lay within the boundaries (Fig. 2).

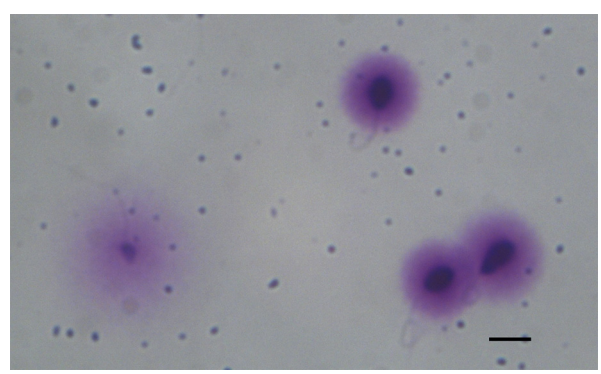

Fig. 1. Spermatozoa processed with Sperm-Halomax kit and stained with Wright solution. Those with a small halo have normal status of DNA and the spermatozoon with a large halo contains fragmented DNA. Bar represents 10 μm.

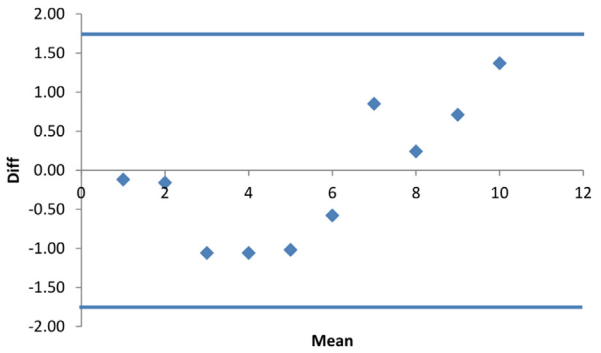

Fig. 2. Bland–Altman plot for Sperm-Halomax/TUNEL DNA fragmentation index results agreement. The line boundaries indicate the 95% CI of the difference between variables. Diff: difference between DFIs obtained by TUNEL and Halomax. Mean: mean of DFIs obtained by TUNEL and Halomax.

3.2. Experiment 2: Correlation between sperm DNA status, conventional head morphology, and CASA morphometry

The calculated reference values for the morphological variables of the sperm head were as follows: area 7.34–15.59 mm^2; aspect 1.69–2.86; perimeter 10.44–14.87 mm; dmax 4.09–6.19 mm; dmin 1.90–2.96 mm; radmax 2.12–3.20 mm; radmin 0.90–1.44 mm; radius ratio 1.83–3.24; and roundness 1.11–1.44.

The 95% confidence intervals for DFI and head anomalies evaluated with conventional analysis were 0.037% to 0.044% and 0.034% to 0.047%, respectively.

The results for PCA analysis are reported in Table 1; the first three components account for the 96.62% of the total variability. In particular, the morphological variables are mainly expressed in the first two principal components (PC) with high correlations. The third component is represented by the DFI only, accounting for the 7.6% of the total variability. Being the PC orthogonal vectors, DFI seems to be independent from the other measured variables. The multivariate results are confirmed by the calculation of the Pearson correlation coefficients: none of the r coefficients resulted significant.

Table 1
Principal component analysis (PCA) loadings for morphological variables and DNA fragmentation index (DFI) on the first three components.

Attribute	PC 1 (51.91%)	PC 2 (37.29%)	PC 3 (7.63%)
Roundness	**−0.93238**	−0.34192	−0.05891
Radius ratio	**−0.91686**	−0.37916	−0.06608
Aspect	**−0.90646**	−0.39293	−0.09087
Radius maximum (mm)	**−0.88915**	0.45212	0.01863
Diameter maximum (mm)	**−0.88565**	0.45974	0.00636
Head anomalies (%)	**−0.83809**	−0.14168	0.01752
Perimeter (mm)	**−0.74541**	0.66100	0.03964
Diameter minimum (mm)	0.23555	**0.96457**	0.10689
Radius minimum (mm)	0.26324	**0.95855**	0.08731
Area (mm^2)	−0.43677	**0.89489**	0.07500
DFI (%)	−0.17188	−0.41807	**0.89131**

The loadings represent the correlation with the corresponding PC. Loadings with values greater than 0.7 are bold typed. In brackets on the headers: variability explained by the PC.

4. Discussion

Present data show that Sperm-Halomax assay, specifically developed for canine semen and based on SCD test, provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. Most of the differences between the DFIs obtained with the Sperm-Halomax assay and TUNEL test were within the 95% confidence interval limits, suggesting that the level of agreement between the two methods of analysis is satisfactory.

The conditions for sperm DNA fragmentation may not be the same in different animals, mainly because protamine residues, which form an important part of sperm chromatin, differ between species [21]. It has been found that canine and feline spermatozoa are characterized by only protamine 1 [22,23], and this could be the reason that the SCD test protocol designed for dogs has resulted equally efficient in analyzing DFI in cats.

Cat spermatozoa processed with Sperm-Halomax produce images of similar characteristics to those obtained in dogs [9,10]. Discrimination of the size of the halos was easy to establish in cat sperm samples because the size of the halos of DNA dispersion was large as those obtained in dogs.

In the present work, DFI of cat epididymal spermatozoa ranged from 2.4% to 5.7%. These values are in agreement with those reported in the literature and obtained with different methods [12,24].

In humans, semen with 30% of spermatozoa with fragmented DNA is considered of low or poor quality to be used in assisted reproduction [25]. In feline sperm samples, additional data are necessary to establish a solid threshold value of this parameter.

To the authors' knowledge, this is the first time that the relationship between conventional sperm head morphology, CASA morphometry, and DNA status has been assessed in cat spermatozoa.

With CASA system, the post-acquisition processing of digitalized data offers an objective and detailed characterization of several sperm morphometric parameters that cannot be detected by conventional visual evaluation. In the present study, the analysis of more than 2400 spermatozoa representing 18 mature tomcats would also contribute to the definition of normal values of morphometric measurements that can be used as a background for further extended studies aimed at better investigating the phenomenon of teratozoospermia in this species.

Present data indicate that DFI is independent from sperm head morphology and morphometry. This finding confirms what has been demonstrated in boar [26], but it is in contrast with the general assumption that head shape is mainly related to the status of sperm DNA due to the fact that most of the sperm head is compacted chromatin. Significant relationships among sperm morphometry and the percentage of denatured DNA have been described in dogs [15,27], bulls [28], brown bears [29], and humans [30].

In feline epididymal spermatozoa, it has been previously shown that head abnormalities are strongly correlated with, and could accurately predict, sperm DNA defects revealed by TUNEL test [12]. However, the conventional evaluation of sperm head morphology by diff-quick

staining was only performed, and no information on head morphometry was reported.

Morphometry provides a more objective evaluation of the sperm head shape compared with conventional examination of head morphology, and the results of the present study show that head shape is not a reliable predictor of DNA fragmentation in cat spermatozoa. Thus, different factors other than chromatin compaction might affect the sperm head shape [26].

In felids, there are large individual variations in semen quality, and many wild and domestic cats have a low percentage of normal spermatozoa [31,32]. However, teratozoospermic cats may be fertile [31], and this further supports that sperm morphology alone should be interpreted with caution.

For this reason, a sperm selection for ICSI, typically based on motility and morphology attributes, might not ensure the use of a high-quality spermatozoon. Sperm DNA integrity is of crucial importance for the embryo development and concerns have been raised regarding possible use of spermatozoa with DNA damage during ICSI [33]. Although a technique for DNA evaluation in viable sperm cells that can be subsequently used for ICSI is not available, the percentage of fragmented spermatozoa in the sample might have a potential role in predicting ART outcome.

4.1. Conclusions

In conclusion, Sperm-Halomax assay, specifically developed for canine semen and based on SCD test, provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. The availability of this simple technique could be useful to improve the feline semen evaluation in clinical practice, and it could contribute to better select semen samples for biotechnological procedures. In fact, DFI is independent from sperm head morphology and morphometry, and the evaluation of the DNA status of spermatozoa would be of great interest in the completion of the standard analysis of fresh or frozen semen used for ICSI or other ARTs.

Competing interests

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

References

[1] Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril 2004;81:1289–95.
[2] Aitken RJ, Koppers AJ. Apoptosis and DNA damage in human spermatozoa. Asian J Androl 2011;13:36–42.
[3] Schulte RT, Ohl DA, Sigman M, Smith GD. Sperm DNA damage in male infertility: etiologies, assays, and outcomes. J Assist Reprod Genet 2010;27:3–12.
[4] Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, Abad C, Amengual MJ, Prada E, et al. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology 2013;1:715–22.
[5] Fernández JL, Muriel L, Rivero MT, Goyanes V, Vázquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59–66.
[6] Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez A, Gosálvez J. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology 2006;65:308–16.
[7] García-Macías V, de Paz P, Martinez-Pastor F, Alvarez M, Gomes-Alves S, Bernardo J, et al. DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. Int J Androl 2007;30:88–98.
[8] Cortés-Gutiérrez EI, Crespo F, Serres-Dalmau C, Gutiérrez de las Rozas AL, Dávila-Rodríguez MI, López-Fernández C, et al. Assessment of sperm DNA fragmentation in stallion (*Equus caballus*) and donkey (*Equus asinus*) using the sperm chromatin dispersion test. Reprod Domest Anim 2009;44:823–8.
[9] Hidalgo M, Murabito MR, Galvez MJ, Demyda S, De Luca LJ, Moreno M, et al. Assessment of sperm DNA fragmentation in canine ejaculates using the Sperm-Halomax® Kit: preliminary results. Reprod Fertil Dev 2010;22:312–3.
[10] Urbano M, Dorado J, Ortiz I, Morrell JM, Demyda-Peyrás S, Gálvez MJ, et al. Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test. Anim Reprod Sci 2013;143:118–25.
[11] Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl 2006;27:53–9.
[12] Mota PC, Ramalho-Santos J. Comparison between different markers for sperm quality in the cat: Diff-Quik as a simple optical technique to assess changes in the DNA of feline epididymal sperm. Theriogenology 2006;65:1360–75.
[13] Pope CE, Gómez MC, Dresser BL. In vitro production and transfer of cat embryos in the 21st century. Theriogenology 2006;66:59–71.
[14] Celik-Ozenci C, Jakab A, Kovacs T, Catalanotti J, Demir R, Bray-Ward P, et al. Sperm selection for ICSI: shape properties do not predict the absence or presence of numerical chromosomal aberrations. Hum Reprod 2004;19:2052–9.
[15] Lange-Consiglio A, Antonucci N, Manes S, Corradetti B, Cremonesi F, Bizzaro D. Morphometric characteristics and chromatin integrity of spermatozoa in three Italian dog breeds. J Small Anim Pract 2010;51:624–7.
[16] Mangiarini A, Paffoni A, Restelli L, Ferrari S, Guarneri C, Ragni G, et al. Specific sperm defects are differentially correlated with DNA fragmentation in both normozoospermic and teratozoospermic subjects. Andrology 2013;1:838–44.
[17] De Ambrogi M, Spinaci M, Galeati G, Tamanini C. Viability and DNA fragmentation in differently sorted boar spermatozoa. Theriogenology 2006;66:1994–2000.
[18] De Pauw IM, Van Soom A, Mintiens K, Verberckmoes S, de Kruif A. In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. Theriogenology 2003;59:1093–107.
[19] Núñez-Martinez I, Moran JM, Peña FJ. Identification of sperm morphometric subpopulations in the canine ejaculate: do they reflect different subpopulations in sperm chromatin integrity? Zygote 2007;15:257–66.
[20] Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307–10.
[21] Ausió J, Eirín-López JM, Frehlick LJ. Evolution of vertebrate chromosomal sperm proteins: implications for fertility and sperm competition. Soc Reprod Fertil Suppl 2007;65:63–79.
[22] Lee CH, Cho YH. Aspects of mammalian spermatogenesis: electrophoretical analysis of protamines in mammalian species. Mol Cells 1999;31:556–9.
[23] Corzett M, Mazrimas J, Balhorn R. Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 2002;61:519–27.
[24] Thuwanut P, Chatdarong K, Techakumphu M, Axnér E. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology 2008;70:233–40.
[25] Evenson DP, Larson KJ, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002;23:25–43.
[26] Saravia F, Núñez-Martínez I, Morán JM, Soler C, Muriel A, Rodríguez-Martínez H, et al. Differences in boar sperm head shape and dimensions recorded by computer-assisted sperm morphometry

are not related to chromatin integrity. Theriogenology 2007;68:196–203.
[27] Núñez-Martinez I, Moran JM, Peña FJ. Do computer-assisted, morphometric-derived sperm characteristics reflect DNA status in canine spermatozoa? Reprod Domest Anim 2005;40:537–43.
[28] Sailer BL, Jost LK, Evenson DP. Bull sperm head morphometry related to abnormal chromatin structure and fertility. Cytometry 1996;24:167–73.
[29] Alvarez M, García-Macías V, Martínez-Pastor F, Martínez F, Borragán S, Mata M, et al. Effects of cryopreservation on head morphometry and its relation with chromatin status in brown bear (*Ursus arctos*) spermatozoa. Theriogenology 2008;70:1498–506.
[30] Aziz N, Fear S, Taylor C, Kingsland CR, Lewis-Jones DI. Human sperm head morphometric distribution and its influence on human fertility. Fertil Steril 1998;70:883–91.
[31] Axnér E, Linde Forsberg C. Sperm morphology in the domestic cat, and its relation with fertility: a retrospective study. Reprod Domest Anim 2007;42:282–91.
[32] Swanson WF, Magarey GM, Herrick JR. Sperm cryopreservation in endangered felids: developing linkage of in situ-ex situ populations. Soc Reprod Fertil Suppl 2007;65:417–32.
[33] Seli E, Sakkas D. Spermatozoal nuclear determinants of reproductive outcome: implications for ART. Hum Reprod Update 2005;11:337–49.