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Microenvironment factors promoting the quality of immature vitrified oocytes in the domestic cat model

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Table of contents

Summary

Domestic cat immature vitrified oocytes (VOs) are a useful research model to develop gamete cryopreservation protocols for fertility and biodiversity preservation in wild, endangered felids. Although vitrification of immature oocytes is convenient for its practical advantages (availability of gametes, speed and field-feasibility of the technique), VOs often degenerate after warming, and their *in vitro* maturation and embryo development rates are poor compared to fresh oocytes. Therefore, improvements are necessary to fully exploit their potential. Physico-chemical enrichments to the vitrification protocol or to the post-warming culture conditions could be useful to improve VOs developmental competence.

In this project, minimum volume vitrification of immature oocytes with the Cryotop method resulted in high (90.8%), consistent and reproducible post-warming viability rates. To improve VOs maturation and development outcomes, two strategies were then employed. The creation of 3D follicle-like structures (i.e. granulosa cells cultured in alginate microcapsules) to be used as enriched culture condition for the *in vitro* maturation of VOs resulted in maintenance of steroidogenic activity of granulosa cells and in meiosis resumption of co-cultured VOs (11.8% maturation). Instead, after the assessment of apoptotic activation in VOs, the addition of a chemical pan-caspase inhibitor resulted in maturation rates (53.1%) similar to those of fresh control oocytes and in good embryonic developmental rates (34.4%).

Embryo development to late embryo stages remained scarce, and further investigation to enhance VOs developmental competence are needed. Molecular investigation on the mechanisms of cryopreservation-induced damages and on the peculiarities of the embryos derived from cryopreserved oocytes could improve VOs *in vitro* outcomes and therefore foster the creation of feline gamete biobanks.

Riassunto

Gli oociti immaturi vitrificati (VOs) di gatto domestico sono un modello utile per sviluppare protocolli di crioconservazione dei gameti per la salvaguardia della fertilità e della biodiversità nei felini selvatici a rischio estinzione. Sebbene la vitrificazione di oociti immaturi sia conveniente per i suoi vantaggi pratici (disponibilità di gameti, velocità e possibilità di esecuzione in campo della tecnica), i VOs spesso degenerano dopo il *warming* (i.e. il ripristino della temperatura fisiologica dopo vitrificazione), e i loro tassi di maturazione e sviluppo embrionale *in vitro* sono scarsi se comparati a quelli degli oociti freschi. Pertanto, sono necessari miglioramenti per sfruttare appieno il loro potenziale, come degli arricchimenti fisico-chimici al protocollo di vitrificazione o alle condizioni di coltura *post-warming*.

In questo progetto, la vitrificazione di oociti immaturi con il metodo Cryotop ha prodotto tassi di vitalità elevati (90,8%), ripetibili e riproducibili. Per migliorare la maturazione dei VOs e il loro sviluppo, sono state poi impiegate due strategie. La prima ha previsto la creazione di strutture tridimensionali simil-follicolari (i.e. cellule della granulosa coltivate in microcapsule di alginato), utilizzate come condizione di coltura arricchita per la maturazione *in vitro* dei VOs, e ha portato al mantenimento dell'attività steroidogenica delle cellule della granulosa e alla ripresa della meiosi dei VOs co-coltivati (11,8% di maturazione). La seconda strategia invece, dopo la valutazione dell'attivazione apoptotica nei VOs, ha previsto l'aggiunta di un inibitore chimico delle caspasi che ha prodotto tassi di maturazione (53,1%) simili a quelli degli oociti freschi di controllo e buoni tassi di sviluppo embrionale (34,4%).

Lo sviluppo embrionale a stadi avanzati è rimasto scarso, e sono necessarie ulteriori ricerche per ottimizzare la competenza di sviluppo dei VOs. Indagini molecolari sui danni indotti dalla crioconservazione e sulle peculiarità degli embrioni derivati da oociti

crioconservati potrebbero migliorare i risultati dei VOs *in vitro*, e quindi favorire la creazione di biobanche per i gameti felini.

1. Introduction

In veterinary sciences, assisted reproductive technologies (ARTs) have acquired a pivotal role in biodiversity conservation programs. Handling of gametes and embryos, as in artificial insemination, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), *in vitro* embryo culture, embryo transfer and germplasm cryopreservation, is aimed to improve the reproductive performances with the generation of offspring or the preservation of fertility. This can help maintaining and increasing the population of wild, endangered animals [1] and contribute to genome resource banking, which is the systematic collection, storage, and redistribution of biomaterials [2] including reproductive cells, which serve as repository of genetic material for future use.

Although with some drawbacks (e.g. monetary and energetic cost, risks associated with manipulation and storage of liquid nitrogen), cryopreservation is currently the only method to store biological material indefinitely. Both somatic and reproductive cells can be cryopreserved and employed for the generation of new individuals. With the former, methods such as cloning by (interspecies) somatic cell nuclear transfer or generation of artificial gametes through induced pluripotent stem cells have to be employed [3], but these techniques have a low efficiency or are still at an experimental level [4]. Instead, cryopreserved gametes and embryos are the easiest and most direct sources to be used for population management strategies [5], if combined with specific ARTs such as *in vitro* fertilization, *in vitro* embryo culture and embryo transfer.

However, ARTs protocols are not efficient for most endangered species [6] and the use of closely related domestic species as models should be implemented to design specific procedures. In the Felidae family, most species are classified as near threatened, vulnerable or endangered by the International Union for Conservation of Nature (IUCN red list of threatened species™, 2020), even if artificial insemination, embryo production and

embryo transfer have already been developed in 24 wild cat species [1] and some successes have been achieved (e.g. the *in situ* and *ex situ* conservation program for the Iberian lynx, which reverted the decline in the population and supported its growth [7,8]). The domestic cat (*Felis catus*) is an excellent model for the development and optimization of ARTs in exotic felids [9], and gonads discarded from routine surgeries provide a source of gametes for experimental studies which also allows researchers to increase their knowledge of carnivore reproductive biology.

Furthermore, the domestic cat can be considered a biomedical model for human reproductive medicine, since there are some similarities in reproductive features and physiology between felids and humans (e.g. ovarian tunica albuginea, oocyte and germinal vesicle characteristics, timing of oocyte *in vitro* maturation [24 hours], asynchronous oocyte cytoplasmic and nuclear maturation, ovarian hypersensitivity and luteal dysfunction after gonadotropin therapy, male teratospermia [10,11]).

Therefore, the increasing demand for human and wildlife ARTs supports the choice of the domestic cat as an animal model in reproduction biotechnology research.

1.1. Gamete biobanking in felids

Oocytes, spermatozoa, ovarian and testicular tissues have peculiar features which influence their cryosensitivity and have to be addressed carefully. Different techniques have been applied in domestic and wild felids for germplasm preservation. The most used are slow-freezing, which relies on a slow, controlled decrease of temperature, and vitrification, which occurs with the solidification in a glass-like state of small volumes of highly viscous solutions with a fast decrease of temperature [12,13]. Innovative “Drying” techniques, which are based on conservation of dehydrated cells at non-cryogenic temperatures, are also being increasingly investigated due to the interest in liquid

nitrogen-free storage and shipment [14,15]. However, each long-term preservation method has advantages and disadvantages, but preserved cells typically undergo several cryoinjuries which affect their functionality, therefore they need to be handled accordingly (e.g. artificial insemination closer to the fertilization site for cryopreserved spermatozoa, addition of angiogenic substances to transplanted cryopreserved tissues) [16]. An overview of the cryopreservation milestones reached with felid gametes and gonadal tissues is presented below. Oocyte vitrification, which is the focal point of this thesis, will be also analyzed in depth in the following paragraphs.

1.1.1. Oocytes

Oocytes are large cells, characterized by a low surface/volume ratio and a high content of water. These features, as well as the presence of the zona pellucida and, sometimes, of the cumulus cells which surround the oocyte, hamper the permeability and the movement of water and cryoprotectants across the cellular membrane, making cryopreservation of female gametes challenging and prone to the formation of intracellular ice crystals that can damage the membranes and the intracellular organelles [13]. In addition, compared to other species, the cytoplasm of domestic animal oocytes is rich in lipids, especially in carnivores [17,18], and this may increase cellular cryosensitivity [19].

However, oocyte cryopreservation is a hot topic in biomedical research thanks to its applications in fertility and biodiversity preservation, and an increasing number of records has been published over the years (Figure 1). Both slow freezing and vitrification are now considered standard techniques, although vitrification is currently the most popular for its practical advantages, including its speed of execution and the fact it can be performed without specific equipment. Indeed, slow freezing of oocytes (and embryos) requires the

use of a programmable freezer. After the oocytes are loaded into straws, they are cooled to equilibrate in the programmable freezer. After equilibration, “seeding” (i.e. the induction of ice nucleation to avoid that the freezing point lowers too much due to the increasing concentration of solutes) is performed, followed by the slow cooling, which lasts until the final immersion into liquid nitrogen [12].

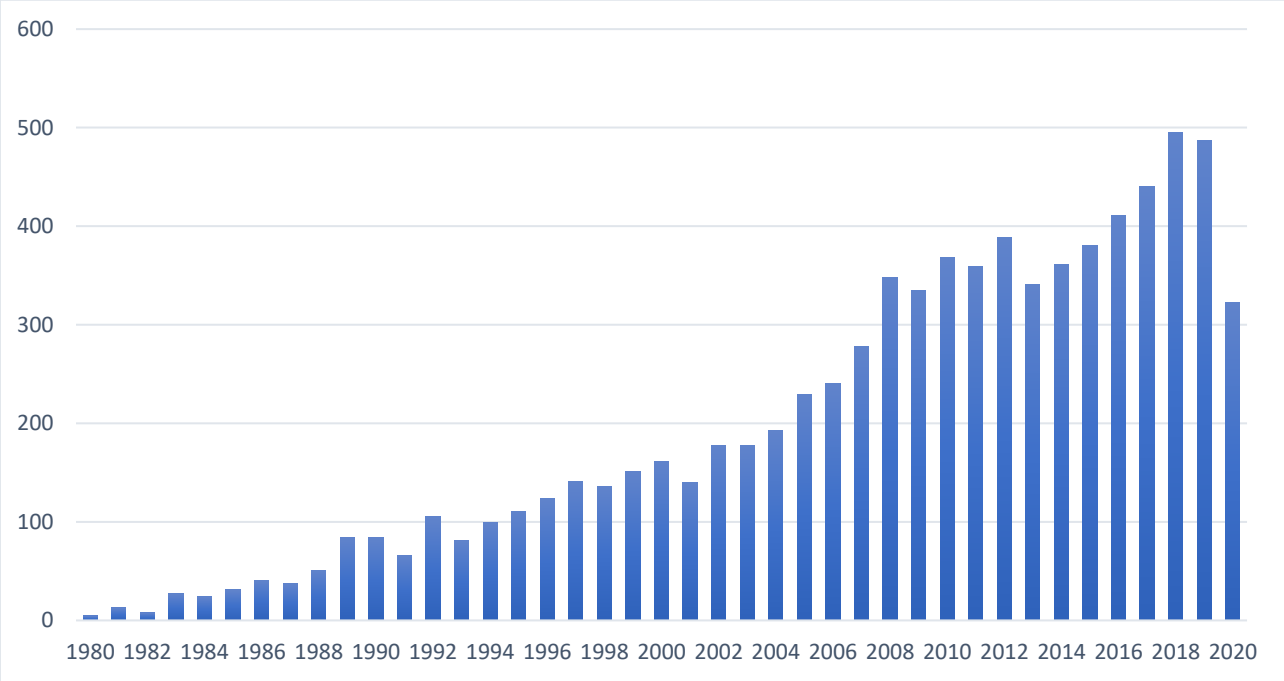

***Figure 1**. Number of PubMed (https://pubmed.ncbi.nlm.nih.gov/) records related to oocyte cryopreservation in the last forty years.*

The query "oocyt AND (cryopreserv* OR freez* OR frozen OR vitrif*)" was searched on August 10th, 2020.*

Many studies have focused on the cryopreservation of domestic cat oocytes since it was first reported in 1997 [20], more as a model for the preservation of female germplasm in wild felids than *per se*. Yet, the interest is kept alive by the poor outcomes of cryopreserved feline oocytes, which often degenerate or develop *in vitro* at low rates, and so by the need of finding new strategies. Although the vast majority of gametes usually

survives to the cryoprocedure (> 90%, [16]), post-warming embryo development is poor and very few embryos reach advanced developmental stages *in vitro* [21–26], probably due to the several cryoinjuries affecting oocyte subcellular structures (which will be reviewed in paragraph 1.2.3.). The biggest success so far was the achievement of a pregnancy from vitrified immature oocytes [27] and of live births from vitrified mature oocytes [28,29], but optimizations in cryopreservation protocols, especially for immature oocytes, are still strongly needed to improve the efficiency of the technique.

Drying techniques are also being experimented. Air-desiccation, storage at 4°C and rehydration allowed the recovery of the nucleus of immature oocytes (i.e. the germinal vesicle), its transfer to recipient cytoplasts (i.e. a nucleus-deprived oocyte), and the resumption of meiosis of the reconstructed oocyte [30]. Moreover, the DNA integrity of microwave-dried germinal vesicles was determined after rehydration [31], and more recently the same method was proved to be superior to vitrification in the maintenance of nuclear envelope conformation and chromatin configuration, and it also led to similar epigenetic modifications [32].

In wild cats, whose gonads are mostly retrieved post-mortem, few data are available. Pallas cat and serval *in vitro* matured oocytes were vitrified by commercial device and media, to evaluate whether the method was suitable to preserve oocyte viability. Ten oocytes for each species were warmed, and the viability was 60% for Pallas cat and 70% for serval oocytes. In addition, cryopreservation of immature oocytes from jaguars and tigers and of *in vitro* matured oocytes from a sand cat were reported within a gamete rescue project promoted by the European Association of Zoos and Aquaria (EAZA) [5]. No reports were published concerning *in vitro* embryo production (IVEP) from this source of oocytes, yet, but in our most recent paper immature African lion oocytes were vitrified, *in vitro* matured and fertilized by ICSI leading to the generation of cleaved embryos [33].

1.1.2. Spermatozoa

Due to their small dimension and high surface/volume ratio, spermatozoa are probably the simplest cells to cryopreserve. For conservation purposes, two sources of male mature gametes can be exploited: ejaculated spermatozoa from live animals (obtained by electroejaculation or by urethral catheterization after pharmacological induction with medetomidine [34]) or epididymal spermatozoa retrieved from isolated organs of dead or neutered animals. Fresh or cryopreserved, these spermatozoa can be employed for artificial insemination (even if transcervical insemination is very complex in felids, due to their anatomical features [35,36]), surgical insemination, or *in vitro* techniques (IVF or ICSI).

Cryopreservation protocols for cat spermatozoa have been thoroughly reviewed in [37]. Most commonly, TRIS-based extenders are used, and egg yolk is usually added to the mixture for its membrane-protective effects, while one of the most used cryoprotectants is glycerol. Additives such as sodium dodecyl (lauryl) sulfate, which is an anionic surfactant, can be included to improve cryotolerance and post-warming quality through the modification of egg yolk lipoproteins. To limit cryoprotectant toxicity and to avoid the use of programmable freezers to control the temperature decrease during freezing, a two-step system, derived from the Uppsala method for dog semen, is often used [38,39]. Spermatozoa are firstly diluted at room temperature with an extender containing a low concentration of glycerol, equilibrated at 4-5°C and then further diluted at this temperature to reach the final glycerol concentration. Straw-packaged spermatozoa are finally frozen in liquid nitrogen vapors, then plunged in the liquid phase of the nitrogen and stored.

In the domestic cat, several studies investigated semen cryopreservation and its outcomes. No differences in motility, morphology and membrane integrity were reported between cryopreserved electroejaculated and epididymal spermatozoa [40], and motility, membrane and acrosome integrity, mitochondrial potential and chromatin status did also not differ between spermatozoa collected by urethral catheterization and epididymal spermatozoa [41]. Fresh and cryopreserved ejaculated spermatozoa showed differences in their fertilizing abilities, since after laparoscopic oviductal insemination more kittens were born from fresh than from frozen-thawed spermatozoa [42], probably due to the cryoinjuries which might affect spermatozoa motility and viability after cryopreservation. Encouragingly, also cryopreserved epididymal spermatozoa maintained their fertilizing ability, and kittens were born after they were used for artificial insemination [43], confirming that if the number of spermatozoa is sufficient there is no need for surgical or *in vitro* approaches. When the quality of an ejaculate is poor, or when few spermatozoa can be retrieved from epididymides or testis, *in vitro*-ARTs have to be used for embryo production. During IVF, frozen epididymal spermatozoa performed as well as frozen spermatozoa collected by urethral catheterization in terms of embryo development [44].

Freezing apart, there is an increasing interest for innovative preservation techniques, and some reports were already published in the cat, although these spermatozoa can just be employed by IVF or ICSI. Although ultra-rapid-frozen epididymal spermatozoa maintained good viability, morphology and acrosome integrity [45], vitrified spermatozoa gave lower fertilization rates than standard frozen controls after IVF and were less likely to bind to oocytes [46], underlining the necessity for further developments in spermatozoa vitrification techniques. Moreover, drying techniques are another interesting alternative to preserve cells such as spermatozoa. Dehydrated sperm cells lose their motility, but they can be used by ICSI to obtain embryos, and different strategies

have already been experimented. Microwaved-dried spermatozoa were inferior to fresh controls in terms of fertilization rates [47], while contrasting results were reported for freeze-dried spermatozoa [48,49]. Finally, following an alternative preservation method, less recently, alcohol-stored spermatozoa were also able to produce embryos which developed until the 16 cells stage [50].

In wild felids, semen freezing is probably the most used and studied cryo-ART. For collection, the same methods mentioned before can be used (i.e. electroejaculation, urethral catheterization after pharmacological induction, retrieval from isolated testis [51]), and semen freezing was attempted for different wild species (ocelot [51–54], lion [51,55], tigrina [54], flat-headed cat [56], black-footed cat and sand cat [57], Iberian lynx [58], bobcat [59], Far-Eastern wildcat [60], Amur leopard [61], clouded leopard [62], tigers [63–65], Pallas cat [66], cheetahs [67–69], jaguar [70], fishing cat [71], margay [72] and some others, including golden cats and rusty-spotted cats [5]). Freeze-drying was also proposed for wild animals, and jaguar spermatozoa remained viable and were able to form pronuclei in mouse oocytes after ICSI [73].

1.1.3. Gonadal tissues

Banking of gonadal tissues allows the storage of a wide pool of gametes at different developmental stages. Despite this advantage, the difficulties in the method and the lack of efficient protocols to exploit immature gametes after warming are its challenges. Indeed, the presence of many cells of different types, sizes, features and cryosensitivity, as well as the *in vitro* growth of gamete precursor cells, makes the design of efficient protocols arduous.

For ovarian tissue, both slow freezing and vitrification can be used, even if the former seemed to give the best results [74]. However, morphofunctional damages can be

found in the cryopreserved tissue. Abnormal shapes of follicles and oocyte degeneration with nuclear pyknosis, cytoplasmic and nuclear swelling are common findings [75], especially in preantral follicles [76] which are often empty upon post-warming observation [77]. Granulosa cells might be more resistant, even if the loss of intercellular communication with the oocyte may happen [76,77]. Instead, primordial follicles seem to be the least cryosensitive, and they can get through some of the cryoinjuries also during their further growth [76].

In cats, many investigations have been performed since the pioneer cryopreservation of isolated follicles [78]. The main focus of several recent studies is the optimization of protocols, including cryoprotectant type and exposure, tissue fragment size and preservation system, but some milestones have already been reached, such as the maintenance of follicle viability and of the ability of the enclosed oocytes to resume meiosis following tissue slow freezing or vitrification [79]. In addition, following ovarian tissue freezing and xenotransplantation to severe combined immunodeficient (SCID) mice, follicles survived and could grow to later stages [80]. More recently, ultrastructure, viability and apoptotic gene expression in vitrified ovarian cortex after transplantation were evaluated [75], as well as survival and development of follicles to the antral stage in transplanted slow-frozen tissue [81]. Viability, morphology and RNA synthesis of vitrified tissue during post-warming *in vitro* culture [82,83] were also assessed, and after solid surface vitrification of ovarian tissue, follicles developed and granulosa cell proliferated [84]. Microwave-assisted dry storage of ovarian tissue has also been recently proposed in the domestic cat, allowing the maintenance of morphology and DNA integrity of follicles and stromal cells and, partially, of follicle viability and transcriptional ability [85].

However, post-warming protocols still need to be optimized. If the preserved tissue is grafted in a recipient animal, follicle loss or cell apoptosis are likely to occur due to ischemic injury and delayed revascularization [74]. If it is cultured *in vitro*, follicular growth and especially production of mature oocytes are challenging to achieve from preserved early stage follicles, and in domestic animals nothing similar has been reported.

In wild animals, ovarian tissue samples, including those of cheetahs, are routinely preserved in some zoological institutions [10]. Ovarian cortex from African lions was slow-frozen and either xenotransplanted into immunodeficient mice, obtaining follicular growth [86], or submitted to *in vitro* culture, showing no differences in histological follicular survival compared to fresh controls [87]. Ovarian cortex from other species, such as Northern Chinese leopard, rusty-spotted cat, Geoffroy's cat, Amur leopard, oncilla, serval and Sumatran tiger, was also preserved and cultured [87], giving hope for future developments of ovarian tissue cryopreservation.

Concerning male gonads, testicular tissue or cell suspensions can be preserved to later retrieve spermatozoa or spermatogonial stem cells (SSCs) to be used in ARTs, or to transplant or culture the whole tissue. Such retrieved spermatozoa, just like fresh testicular spermatozoa, are immotile, and ICSI is the only way to produce embryos [88,89]. Therefore, tissue preservation does not influence the usability, and spermatozoa from fresh and frozen testicular tissue had comparable fertilizing ability in the domestic cat [89]. Remarkably, the birth of two kittens from spermatozoa derived from frozen-thawed testicular tissue was reported [88], and this is probably the biggest accomplishment in male gonadal tissue preservation in felids, so far. However, cryopreservation of cell suspension better maintained spermatozoa membrane integrity compared to frozen tissue [90], but these studies are not very common.

Tissue cryopreservation might affect its integrity and ability to restore physiological functions after grafting. Damages to seminiferous tubules, including the detachment from the basement membrane, and to single cells, such as Sertoli cells, were reported in cat vitrified testicular tissue [91], even if the preservation of seminiferous tubule structure, cell viability and mitochondrial activity was later shown in vitrified tissue by the same authors [92]. Studies on tissue preservation are more common than those on cell suspensions, and they investigated different methods and cryoprotectants (e.g. [91,93]), also combined with xenografting, which did not allow to find germ cells in the seminiferous tubules of cryopreserved tissue from either prepubertal or pubertal cats after transplantation [94], probably due a lack of angiogenesis in the graft.

Post-warming transplantation of testicular tissue would theoretically allow the development of germ cells into spermatozoa: the physiological niche of SSCs would be preserved, and they could receive signals from the body, making spermatogenesis occur and potentially allowing the retrieval of mature spermatozoa. However, grafts could sclerotize or arrest meiotic divisions [95] and more studies in felids are needed. Similarly, *in vitro* culture of preserved tissue might allow spermatogenesis, but trials are focusing on the mouse model, as well as for SSCs preservation, whose success is still limited because of the difficulties in their culture and in the colonization of the *rete testis* of the recipient animals after transplantation [95]. More recently, microwave-drying of testicular tissue allowed the maintenance, to some extent, of DNA integrity, viability and morphology (especially in prepubertal cats compared to adults) [96], but more investigations should be performed.

In wild felids, slow and fast freezing of testicular tissue from jungle cat, lion and leopard were reported, revealing that fast freezing better maintained sperm membrane and DNA integrity, that detachment and shrinkage of the seminiferous tubules were similar

between the two methods, and that intra-tubular cells of slow frozen samples had lower apoptotic changes [96]. More recently, cryopreservation of testicular cell suspension of a cheetah and an Asiatic golden cat testis was attempted, and more than 30% cell viability was observed after thawing [97].

1.2. Female gamete rescue

Female germplasm might be more challenging to preserve than its male counterpart because of the poor availability of gametes. In males, spermatogenesis is a long-lasting process which produces millions of gametes, which can even be collected from isolated organs after a valuable animal died. In females, as discussed before, gamete reserve is limited, and most of the techniques to retrieve and exploit all the differentially developed gametes inside the ovary are still at an experimental level. For fertility preservation purposes (Figure 2), the most readily available source of gametes for IVEP, and the one which already demonstrated some effectiveness, is represented by the fully-grown, germinal vesicle (GV) oocytes.

1.2.1. Folliculogenesis, oogenesis and sources of preservable oocytes

Feline ovaries contain hundreds to thousands of follicles [98,99], but less than 1% of them is at the antral (tertiary) stage [100], which contains fully-grown GV oocytes, and most are at less advanced stages (i.e. primordial, primary, secondary or preantral). To understand differences and growth between the different follicle types, so the whole oogenesis, it is necessary to start from the beginning of life. During gestation, primordial germ cells (i.e. oogonia), migrate from the yolk sac to the primitive immature ovaries (i.e. genital ridges) where they reach their maximum number after several cellular divisions [101]. Surrounded by a single layer of follicular cells, oogonia form the primordial follicles

in the ovarian cortex: their development involves an increase in germ cell size and changes in the morphology and structure of follicular cells, whose shape goes from flat to cubical. In this phase, the secretion of glycoproteins that will form the zona pellucida also begins. During the development, some of these primary follicles are stimulated to grow into a secondary follicular stage, characterized by a higher number of more specialized follicular cells surrounding the oocyte, i.e. granulosa cells (GCs). Then, the secretion and the accumulation of fluid in an antrum define the tertiary, or antral, follicular stage [102], in which the oocyte is typically displaced to one side, and together with the highly specialized GCs surrounding it (i.e. cumulus oophorus cells) forms the fully-grown cumulus-oocyte complex (COC).

From a genetic perspective, oogonia undergo meiotic divisions during the follicular development until a meiotic arrest at prophase I (i.e. GV), which is characterized by diffuse chromatin surrounded by an intact nuclear membrane and occurs shortly before or soon after birth [103]. With the onset of puberty, the resumption of oocyte meiosis in the dominant follicles takes place with the germinal vesicle break-down (GVBD). During GVBD, the nuclear membrane fades and the chromatin starts condensing into bivalents, that align on the meiotic spindle at metaphase I (MI). Segregation of the homologues starts during anaphase I (AI), continues through telophase I (TI), and meiosis I is completed with the extrusion of the first polar body, containing one set of chromosomes. The other set of chromosomes is retained into the oocyte, which enters the second meiotic division until metaphase II (MII). At this point, ovulation occurs in most species, while the nuclear progression arrests again, bound to resume meiosis only with fertilization [103,104].

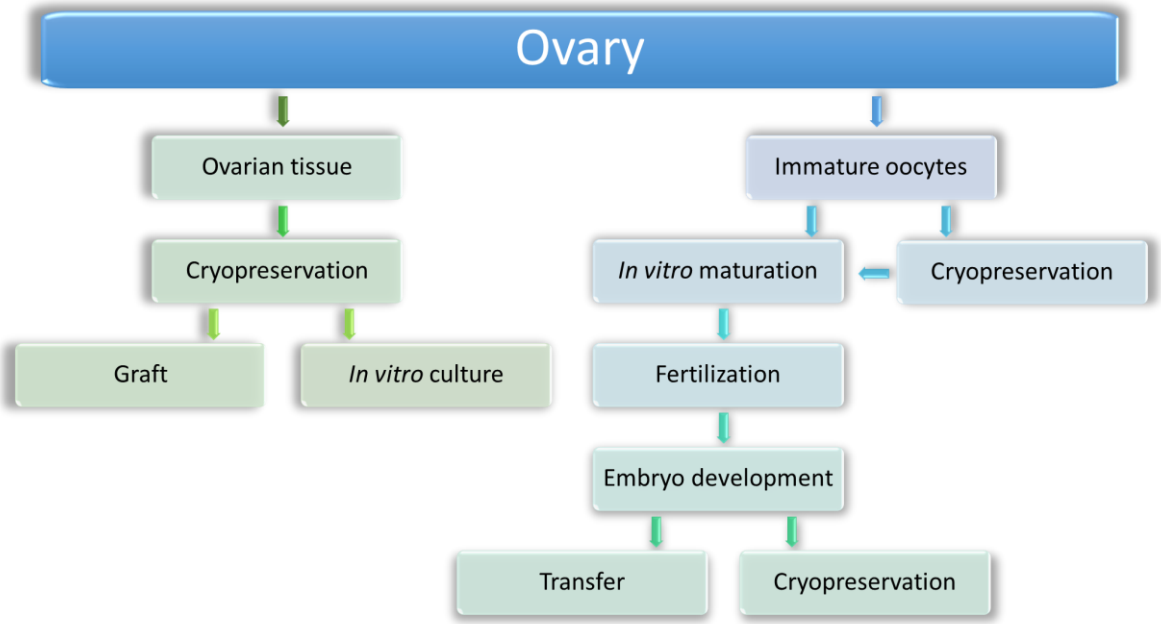

***Figure 2**. Schematic overview of fertility preservation strategies for female animals whose gonads can be retrieved post-mortem or after spaying.*

The use of follicles at early stages is still challenging, and the development of combined isolation, culture and cryopreservation protocols is needed [105]. Although smaller follicles better resist to cryopreservation [105,106], probably thanks to the small size of the oocyte, its low metabolic rate, its cell cycle arrest, its lower lipid content, the presence of fewer support cells and the absence (or small development) of the zona pellucida [107], protocols for the *in vitro* culture of preserved tissue fragments, as before mentioned, are still at an experimental level, and we are currently not able to obtain fully-grown oocytes (intended for *in vitro* maturation) from this germplasm source. Isolated follicles, freed from the surrounding tissue, might be another option, but their *in vitro* culture would be applicable only to more advanced follicular stages (from secondary onwards) and culture strategies are also inadequate, so far [106].

Instead, *in vitro* maturation (IVM) of fully-grown, GV oocytes retrieved by slicing the ovarian surface or by directly puncturing the antral follicles gives acceptable results in

the domestic cat (60-70% MII rate in fresh oocytes, with 60% cleavage after *in vitro* fertilization and 50% cleaved embryos forming blastocysts [4]). Even better, *in vivo*-matured oocytes can be retrieved after hormonal stimulation with gonadotropins and laparoscopic aspiration of preovulatory follicles, and the collected oocytes can be cryobanked or *in vitro* fertilized [108]. When fertilized, *in vivo*-matured oocytes show an higher developmental competence (i.e. oocyte ability to develop into normal, viable and fertile offspring after fertilization, or to develop to the blastocyst stage *in vitro* [109]) than their *in vitro*-matured counterparts, resulting in higher *in vitro* blastocyst rates [108].

For biodiversity preservation purposes in wild felids, both mature and immature oocytes could theoretically be retrieved. However, hormonal treatments and oocyte retrieval to collect mature oocytes are not very common because of the ethical issues (e.g. animal suffering from injections and surgery, related health risks) and the species- and individual-specific response to treatments [110]. Therefore, collection of immature oocytes after death or ovariectomy is the most frequent scenario [5], and specific protocols for the preservation of this valuable material should be designed.

1.2.2. Vitrification of immature oocytes: the field-friendly cryopreservation method

As mentioned above, vitrification has few material requirements, compared to slow freezing, therefore it is more feasible in field conditions. These requirements are linked to the three physical principles which allow the vitrification itself, i.e. the solidification of a liquid without precipitation of water as ice [12]:

- High viscosity of the medium, correlated with cryoprotectant concentration and activity;

- Cooling rates of hundreds to tens of thousands Celsius degrees per minute, obtained by direct immersion of the sample into liquid nitrogen;
- Small volumes of liquid, to improve heat transfer and facilitate higher cooling rates.

When an animal dies, its ovaries and its fully-grown immature COCs can be retrieved, and if cryoprotectant-rich vitrification solutions (commercial or laboratory-made) and liquid nitrogen are available, they can be vitrified *in loco*, without the need for a specialized, well-equipped lab. In addition, to improve vitrification efficiency, several supports have been developed to reach the "Minimum volume" goal, some custom-made and some commercially available. Apart from the so-called tubing techniques, which actually allow only a relative reduction of the vitrification volume (e.g. plastic straw, open pulled straw [OPS], closed pulled straw [CPS], superfine OPS, CryoTip, sealed pulled straw), surface techniques include a variety of supports that allow major reductions in volume, until fractions of microliters (e.g. electron microscope grid, Cryotop, Cryoloop, hemi-straw, solid surface, vitrification spatula, plastic blade, Vitri-Inga) [12]. All these devices are small and easy to transport to the field, and one of them (i.e. Cryotop), in particular, is easy to handle (consisting of a small film strip attached to a hard plastic holder, protected by a plastic tube cap during storage [111]), allows an extreme reduction in vitrification volume (as little as 0.1 µL), which also leads to extremely rapid cooling and warming rates, and is probably the current elective vitrification system for human and animal oocytes, including feline's [24,28,29,112,113].

Finally, as well as for its practical advantages, vitrification was suggested to be more effective than slow freezing for the conservation of lipid-rich mammalian oocytes and embryos [17], such as those from domestic and wild felids.

In this practical scenario, where only field vitrification of immature oocytes is possible due to the lack of a well-equipped laboratory, there is a drawback to be noted. The efficiency of cryopreservation is usually considered better, or preferable, for mature than immature oocytes (human: [114]; cat: [115,116]), so wherever a specialized laboratory and IVM protocols are available, the best choice could be to mature the retrieved GV oocytes first, and then to cryopreserve them at the MII stage.

The cryosensitivity of GV oocytes is likely due to their morphology and to its differences with MII oocytes. Although mature oocytes are believed to be vulnerable to cryoinjuries because the meiotic spindle can temporarily depolymerize when the temperature decreases [117] and immature oocytes do not have this issue because their chromosomes are surrounded by a nuclear membrane, GV oocytes struggle to mature *in vitro* after cryopreservation [118,119]. While MII-stage oocytes do not rely on support from their cumulus cells, immature COCs need it to accomplish a proper maturation process [120], but the cumulus often functionally detaches from the oocyte during cryopreservation and this might be the reason for the poor maturation outcomes. Furthermore, the oolemma changes its permeability during maturation, being more favorable to cryopreservation in MII oocytes [121]. Lastly, it should also be taken into consideration that immature oocytes are only morphologically selected for cryo-ARTs, and no information on their inner competence is available, whereas mature gametes have already demonstrated their intrinsic developmental ability, at least for what concerns nuclear maturation, and this could be another logical reason for the poor *in vitro* development of cryopreserved GV gametes [115].

However, the number of ARTs laboratories close to wildlife habitats, parks or zoos is scarce, and preservation of immature oocytes is often the only option to safeguard the

precious germplasm of valuable animals. Although some attempts have been and are being made, strategies to improve the efficiency of this method remain to be investigated.

1.2.3. Challenges of immature oocyte vitrification

Although cryopreservation is a fundamental tool in ARTs, different negative effects, known as cold-induced damages or cryoinjuries, can easily occur in cells [13,122]. Cryodamages are mainly caused by the exposure of cells to non-physiological conditions, as sub-zero temperatures and osmotic stress, and consequently by the difficulties in the restoration of membrane integrity [122].

Cryoinjuries affecting cryopreserved immature oocytes are reviewed in depth in [13] and [119]. In general, both the germinal (i.e. the gamete itself) and the somatic compartment (i.e. the surrounding cumulus cells) of the COCs can be affected. When the temperature decreases, membranes are significantly altered. Although more common during conventional freezing, chilling injury causes irreversible changes in lipid droplets and lipid-rich membranes (including the oolemma, where phospholipids switch from a liquid-crystalline to a gel phase, resulting in an increased rigidity [123]) and in microtubules of the mitotic or meiotic spindle [124]. Moreover, due to the high concentration of cryoprotectants used during vitrification, oocytes are exposed to a strong osmotic stress. Hence, shrinkage and change in cellular shape might occur, also leading to cytoskeleton damages. Due to the solidification of the solution, a fracture damage might arise. It is a mechanical damage which affects the integrity of the zona pellucida [125] and of other biological membranes, including the oolemma. In addition, the zona pellucida could be further affected by a phenomenon known as zona pellucida hardening. Due to a transient calcium increase caused by cryoprotectants [126], there might be a

precocious exocytosis of cortical granules that might later compromise sperm penetration and fertilization.

Inside the ooplasm, apart from the aforementioned cytoskeleton damage, DNA fragmentation and chromosomal abnormalities might occur, as well as epigenetic modifications, especially during vitrification [127], and modifications in the expression of genes related to oxidative stress, apoptosis, cell cycle and sperm-oocyte interaction [128]. Among the organelles, mitochondria function and distribution (due to microtubule depolymerization and alterations of the microfilaments functions) might vary, and this could also be linked to the activation of apoptotic pathways. Furthermore, multiple aster formation, which leads to disruption of migration and development of pronuclei, delay of the first cleavage and reduced potential for blastocyst formation, has been detected after oocyte vitrification [129].

Concerning the somatic compartment, cumulus cell loss might occur due to the sensitivity of transzonal projections (i.e. the connections between the oocyte and its cumulus) to low temperatures and cryoprotectants. Indeed, cryopreservation protocols are designed for the oocytes, which are very different in size, shape and features from the cumulus cells [130]. The physical or functional disconnection of cumulus cells from the oocyte during the cooling/warming process (Figure 3) is a direct consequence of the negative effects on the cytoskeleton components of the transzonal projections [131]. The lack of functionally connected cumulus cells is detrimental for the performances of oocytes after the restoration of physiological conditions, as it impairs maturation and fertilization, particularly for immature gametes [119].

In cats, few studies investigated what happens to immature vitrified oocytes (VOs) after cryopreservation. While the distribution of mitochondria did not change upon vitrification, their function (evaluated as MitoTracker® staining intensity) and aggregation

were affected [132]. In addition, it was hypothesized that modifications in the permeability of gap junctions hemichannels occur, causing the loss of small essential metabolites, ionic imbalance and the penetration of small, potentially toxic, molecules [133]. A proteomic study also highlighted that, after IVM of immature VOs, membrane and nuclear proteins were downregulated, while apoptosis and DNA repair proteins were overexpressed compared to fresh control oocytes [134]. Apoptosis, indeed, was also proposed as a possible detrimental effect of cryopreservation, but the exact causes of cell injury and the mechanisms leading to poor *in vitro* survival and development of VOs are still unknown [135].

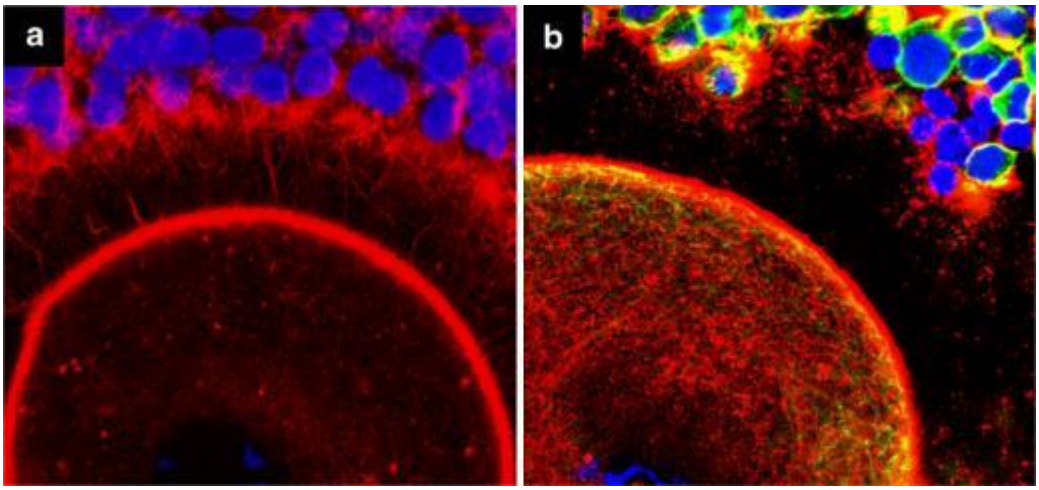

Figure 3*. Representative confocal images of human immature cumulus-oocyte complexes before (a) or after (b) vitrification (from Brambillasca et al., 2013* [119]*). Cell nuclei are labeled in blue, while f-actin (i.e. a component of cytoskeleton microfilaments) is labeled in red. Cryopreservation damages the layers of cumulus cells due to the disaggregation of actin filaments, which are the main components of transzonal projections.*

All these vitrification-induced alterations are likely to be accountable for the poor post-warming *in vitro* outcomes of cat VOs. *In vitro* maturation is the first obstacle, and the results are highly variable. Oocyte maturation is a delicate process which implies the

acquisition of nuclear and cytoplasmic competence [136]. The resumption of meiosis from GV to MII requires a good coordination of nuclear and cytoplasmic events, with chromosomes segregation and formation of the meiotic spindle thanks to the cytoskeleton. At the same time, the acquisition of the ability to be fertilized and develop into embryos involves changes in the whole cell: organelles reorganize in the cytoplasm, transcription slows down, stored mRNAs are partially used, and the pattern of protein phosphorylation changes [137,138].

Depending on the system, 15-55% of cat VOs are able to achieve full maturation [16,21,23,24,27,139], but this is not a guarantee for subsequent embryo development. As recently reviewed, after fertilization cellular divisions are impaired, and cleavage takes place only in around 25% of the preserved oocytes [105]. In other species, such as humans and bovines, cleavage happens in 60-90% of the oocytes [140], indicating a strong cryosensitivity of cat gametes. In addition, development to late embryo stages still occurs at low rates (Table 1). After cleavage, further cellular divisions are probably affected in VOs-derived embryos, whose specific features are still scarcely characterized. Currently, most studies are focused on practical outcomes, and researchers are attempting several approaches to enhance VOs developmental competence.

***Table 1**. Overview of in vitro embryo production results achieved with domestic cat immature vitrified oocytes.*

Vitrification support	Embryo production method	Embryo development			Reference
		Cleavage %	Morulae %	Blastocysts %	
OPS-like	IVF	17.7 ± 2.5	0	0	Comizzoli et al., 2009 [21]
OPS	IVF	18.6	10.4	4.3	Cocchia et al., 2010 [22]
OPS-like	IVF	31.0 ± 7.9 (out of matured oocytes)	0	0	Comizzoli et al., 2011 [23]
OPS	IVF	24.8	47.6 (out of cleaved embryos)	30.2 (out of cleaved embryos)	Tharasanit et al., 2011 [27]
Cryotop	ICSI	28.6	30	N.A.	Fernandez-Gonzalez & Jewgenow, 2017 [24]
OPS	IVF	33.2 ± 7.5	22.3 ± 3.8 (out of cleaved embryos)	16.2 ± 5.2 (out of cleaved embryos)	Arayatham et al., 2017 [135]
Hemi-straw-like	PA	9	N.A.	2	Snoeck et al., 2018 [133]
Cryotop	IVF	18.39 ± 16.67	1.79 ± 3.07	0.95 ± 2.52	Colombo et al., 2019 [112]

OPS = open pulled straw; IVF = in vitro fertilization; ICSI = intracytoplasmic sperm injection; PA = parthenogenic activation; N.A.: Data not available.

Note: For the studies where more treatments were compared, the one which gave the best results was hereby reported.

1.3. Culture strategies for the improvement of oocyte developmental competence

In vivo, cells live and grow in specific conditions and are exposed to various signals, depending on their location, function and host organism. During culture, essential nutrients (e.g. amino acids, carbohydrates, vitamins, minerals), growth factors, hormones and gases (O_2, CO_2), as well as a controlled environment (pH, osmotic pressure, temperature), are provided to guarantee cell survival *in vitro*.

However, *in vivo*, cells are immersed in the extracellular matrix (ECM) and connected to each other through specific surface receptors, such as integrins, forming three-dimensional (3D) structures or tissues [141]. The ECM is a complex environment composed by structural proteins, proteoglycans, glycoproteins and other molecules, which allows the spatial organization of cells and tissues. The mechanical properties of ECM are mainly determined by different proteins, such as collagen, elastin and laminin, which organize the communication network between the enclosed cells [141]. Moreover, the ECM is able to influence several fundamental cellular behaviors, including adhesion, migration, proliferation and differentiation [142]. Yet, *in vitro*, cells are traditionally cultured in two-dimensional (2D) environments, as Petri dishes, slides and multi-well plates. These surfaces, although allowing cell growth, fail to mimic properly the *in vivo* conditions, because they lack the structural conformation of living tissue and the gradients of signalling molecules, oxygen, nutrients and catabolic products [143]. These 2D conditions are detected by the cells, which respond by flattening on the culture surfaces and remodelling their cytoskeleton conformation in an attempt to adapt to the flat environment [144] (Figure 4). This also leads to changes in the nuclear shape and to altered gene expression and protein synthesis of cultured cells [145,146]. Only lately, biologists started to apply the 3D structural properties of ECM to the culture systems to

overcome the limits of 2D surfaces. Innovative 3D environments, named scaffolds, were created to provide a suitable environment for cell growth, differentiation and function, where cell morphology, structure and biological activities are maintained and closely resemble the *in vivo* conditions, increasing the physiological relevance of *in vitro* experiments [141,144].

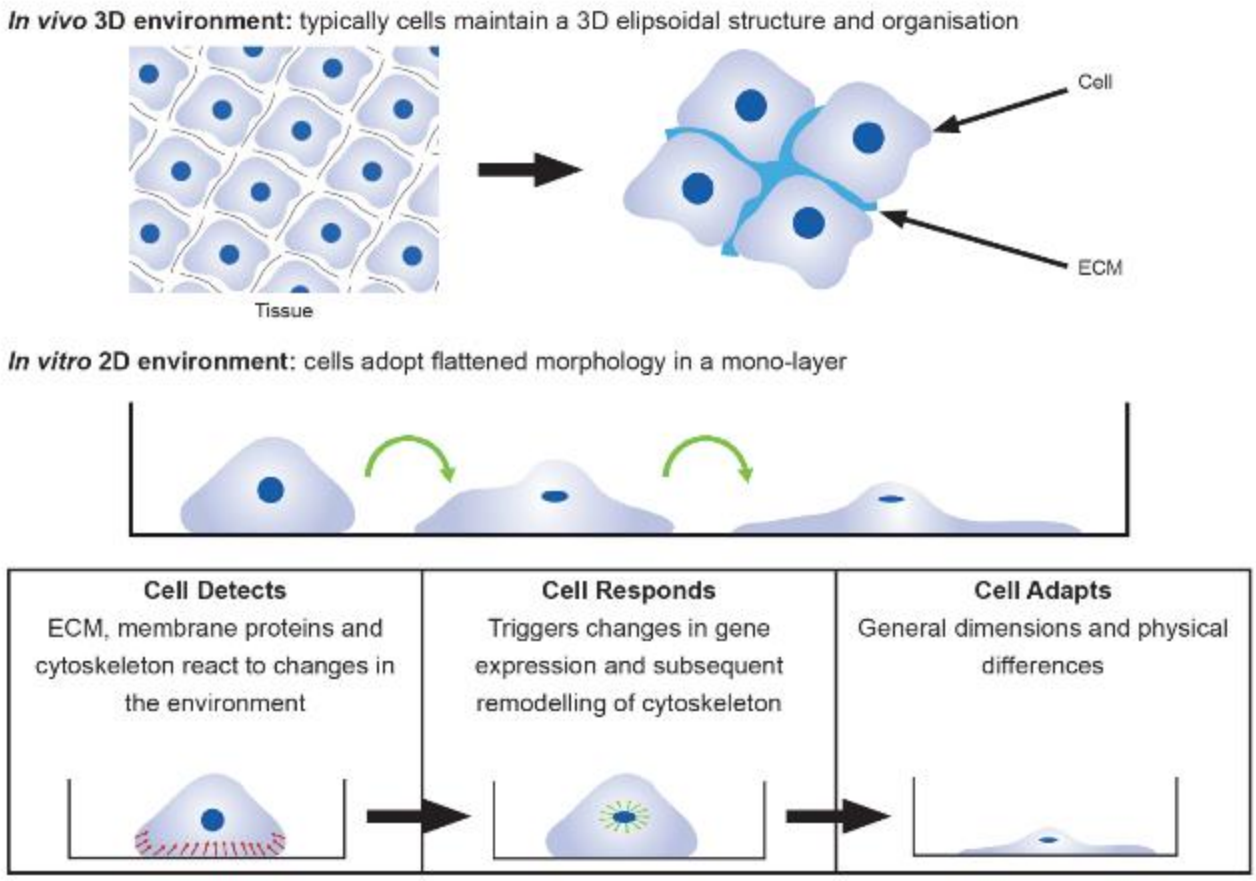

***Figure 4**. Cellular effects after a change in the growth environment from 3D-in vivo to 2D-in vitro (modified from www.reprocell.com).*

ECM = extracellular matrix.

Standard *in vitro* culture conditions of domestic animal oocytes are microdrops of medium in Petri dishes or multi-well plates, incubated at 38-39°C with 5% CO_2, in air or with 5% O_2. In general, IVM media try to chemically mimic the follicular niche, where

maturation occurs *in vivo* [147] and usually contain follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and sometimes estradiol, growth hormones and insulin [148]. The medium is changed for IVF to support sperm capacitation and fertilization, and then again for embryo *in vitro* culture, to promote embryo development until the blastocyst stage [148], trying to mimic the microenvironmental conditions found in the oviducts and in the uterus, respectively [147].

For the IVM of domestic cat oocytes, conventional media contain hormones as FSH and LH, which promote the preservation of functional gap junctions (i.e., transmembrane communications) between the oocyte and its cumulus cells and improve maturation rates [149]. The use of FSH from different sources (porcine and human) and at different concentrations led to the conclusion that optimal hormonal supplementations (i.e. human urinary FSH at 0.02 IU/mL) could benefit maturation outcomes and gene expression profiles [150]. In addition, FSH is useful to enhance the *in vitro* outcomes of cat oocytes during the non-breeding season, when results are usually poorer [151]. Speaking of protein supplementations, bovine serum albumin (BSA) is commonly employed during IVM, since fetal bovine or calf serum (FBS or FCS) could inhibit the maturation process [152–155]. Novel protein supplementations could also be investigated based on the physiological composition of follicular and tubal fluids, since high-performance liquid chromatography (HPLC) revealed some peculiarities in cats [156]. Indeed, similarly to other species, alanine and glycine were the most abundant amino acids in follicular and tubal fluid, respectively, but surprisingly hypotaurine, which is a major constituent of the amino acid pool in other mammals, was not detected [156]. Finally, growth factors are often added to the culture medium for their functions in cell differentiation, cell proliferation and apoptosis regulation [157,158]. The presence of epidermal growth factor (EGF) during IVM

improved embryo development at late stages [159,160], while insulin-like growth factor-I (IGF-I) alone [161] or together with EGF [162] improved maturation results.

With the same rationale of mimicking the *in vivo* physiological environment, synthetic oviduct fluid (SOF; [163]), designed taking inspiration from sheep oviducts, Earle's salt solution [164,165] or Tyrode's salt solution [166], are commonly used for fertilization and *in vitro* culture of cat embryos [167,168]. To satisfy the fast-evolving needs of growing embryos, Pope and co-workers created, from their one-step system [169], two- and three-steps culture systems [28,29,170–174]. In this case, media composition varies during *in vitro* culture, beginning with BSA and non-essential amino acids, then also including essential amino acids, and finally substituting BSA with FBS [166] to stimulate embryo development [175]. Furthermore, a reduced oxygen concentration in the gas atmosphere (5%) can also be employed, since it was found to improve embryo development [176] especially at late stages, when *in vivo* there is a low uterine oxygen content [177]. Other studies analyzed the gene expression of *in vitro*-derived embryos [178–180] and the protein distribution in the oviducts with the aim of determining factors, and consequently *in vitro* requirements, involved in early embryo development [181] and hopefully further developing suitable *in vitro* culture systems.

Additional strategies, from the most basic to the most advanced, have been tested to improve IVEP outcomes [182], and these could be especially useful in the species whose results of *in vitro* embryo production could still be improved, such as the cat [183].

The simplest approach might be the addition of antioxidants, since oxidative stress is one of the main reasons for poor *in vitro* developmental competence [184]. Oxidative stress results from an imbalance between the production and elimination of the intracellular reactive oxygen species (ROS), which are normally produced by cell

metabolism, but increase during *in vitro* culture, as the oocytes have been removed from the antioxidant protection of the ovarian follicle and are exposed to an oxidative environment (e.g. oxygen tension in the incubator, culture media) [184]. Several chemical supplementations have been successfully tested in different species, including vitamins, coenzyme Q10, thiol compounds, melatonin and plant-derived antioxidants. In the domestic cat, cysteine (i.e. a thiol) is usually added to the culture medium [149] since it is a precursor of glutathione (GSH), and GSH is an antioxidant peptide [185] which is accumulated during maturation and promotes sperm decondensation and pronuclear formation [186–192]. The use of other thiols, such as cysteamine and ß-mercaptoethanol, contributes to the maintenance of GSH levels during oocyte culture, since these compounds keep reduced cysteine available as a precursor for GSH cellular synthesis [193]. With a somehow similar principle, the addition of mitochondria to the *in vitro* maturing oocytes was also experimented. Indeed, mitochondria are involved in oocyte protection from oxidative damage, as well as in energy production, necessary for early embryo development [194]. Ooplasmic transfer through injection of ooplasm of a donor oocyte resulted in enhanced developmental competence of human oocytes [195], and mitochondrial supplementation was also beneficial in some animal species (swine [196] and murine models [197]), but not in others (bovine [198]). Yet, its effects remain to be investigated in cats.

Other strategies to improve IVEP outcomes involve physical modifications to the culture environment. As before mentioned, 3D systems have started to be applied to cell cultures to better maintain cell morphofunctionality, and they could also be interesting for reproductive cells. The 3D *in vitro* environments provide better conditions for oocyte culture, avoiding the unnatural adhesion of cumulus cells to the substrate. The flat arrangement of the traditional 2D culture system (i.e. microdrops of medium) leads to a

disruption of communications between somatic and germinal compartments, a distorted cell-to-cell orientation and an abnormal diffusion of paracrine signals, modifying the physiological polarity and secretions of COCs and cumulus cells [199–202].

The first application of 3D systems for oocyte culture was for murine COCs maturation [203], and satisfactory results were obtained. *In vitro* 3D culture of ovarian follicles and embryos were also attempted, resulting in follicular and oocyte development, as well as GCs proliferation [204], and in embryo survival, growth and differentiation [205]. Besides maintaining the physiological oocyte conformation, 3D systems support nuclear and cytoplasmic maturation, allowing competent gametes to develop into viable progeny after IVF, *in vitro* culture and embryo transfer into recipients [200,206]. Moreover, 3D scaffolds preserve cell-to-cell contacts between embryo blastomeres and stimulate a genetic expression similar to that observed in *in vivo* conditions [205,207,208]. Different biomaterials proved their suitability as scaffold constituents for reproductive cells, and among all, alginate is often the choice to guarantee follicle growth, oocyte survival and maturation, and embryo development [205,209,210]. In the domestic cat, 3D alginate microcapsules were used for follicle [211,212], oocyte and embryo culture [213,214], allowing their survival and development.

Other 3D environments, i.e. liquid marbles microbioreactors, which are drops of liquid encapsulated by hydrophobic powder particles, were employed for COCs in the sheep, and oocyte maturation and embryo development occurred at a similar extent to control 2D culture in multi-well plates [215]. Our group attempted to use liquid marbles for feline COCs also obtaining no differences between liquid marbles and 2D microdrops in terms of meiosis resumption and maturation capacity (unpublished data).

Similarly, the use of microfluidic systems, also known as organ-on-a-chip or lab-on-a-chip, which combine 3D architectures, different types of cells and fluid flow, would

allow creating a dynamic culture environment [216], very different from traditional 2D systems for what concerns the exchange of nutrients, gases and metabolites [217]. In cats microfluidic systems have only been applied for the culture of ovarian follicles, and they were useful to maintain follicular viability [218]. However, optimization is needed, and further applications of these systems should be explored for the IVM of feline oocytes or also as an "All-in-one" system, where the whole IVEP could be performed without unnecessary oocyte and embryo manipulation [219,220].

Representative pictures of non-traditional culture systems described in the text are depicted in Figure 5.

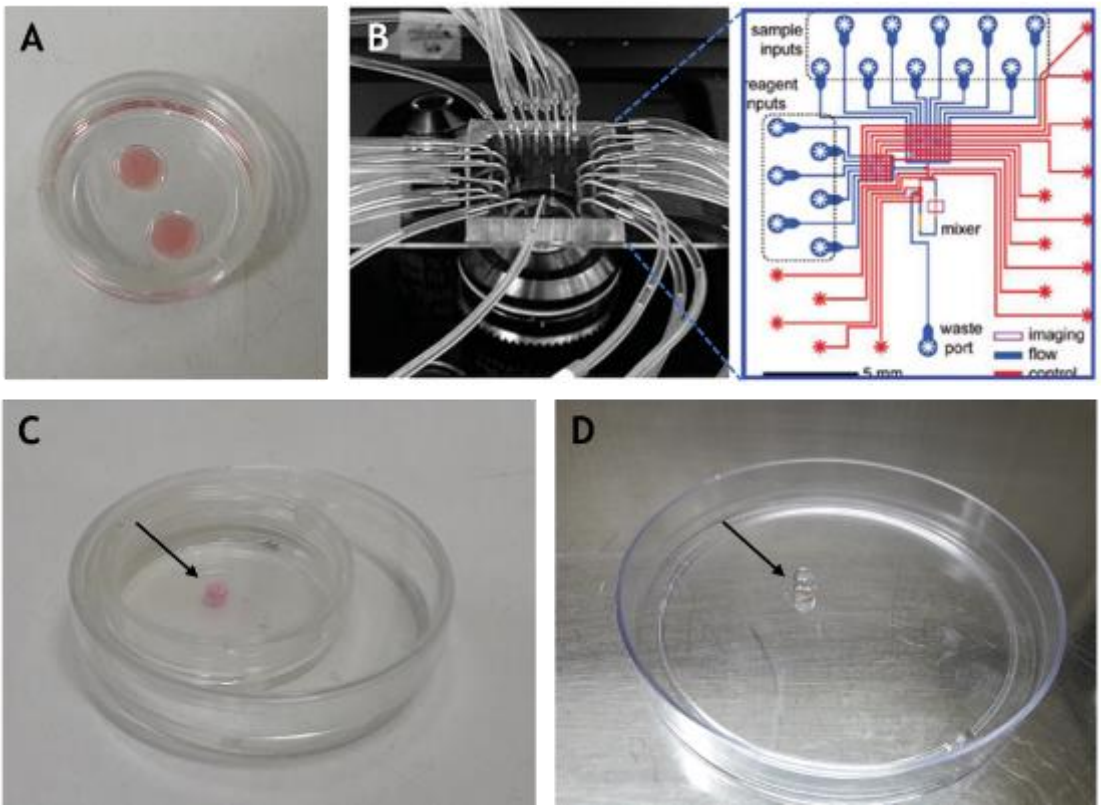

***Figure 5**. Culture systems with different physical characteristics currently employed for oocyte in vitro culture. (A) Traditional two-dimensional system consisting of medium microdrops covered by mineral oil in a Petri dish; (B) microfluidic system, picture and scheme (modified from Swain et al., 2013* [219]*); (C) three-dimensional liquid marble microbioreactor (black arrow); (D) three-dimensional alginate microcapsule (black arrow).*

Other attempts to improve the *in vitro* developmental competence of cultured oocytes include physico-chemical modifications to the microenvironment, since they combine the advantages of physical enrichments with those derived from the presence of chemical signals. Historically, the most used systems are co-cultures. The idea of *in vitro* co-culture system has been developed to recreate the natural intercellular communications, since in *in vivo* conditions cells interact with each other in complex systems. The co-existence of different cell types stimulates signalling and cross-talking through soluble factors (chemical cues) or direct cell-to-cell contacts (physical cues) [221], and could also be beneficial for *in vitro* culture of female gametes.

For mammalian oocytes, oviductal cells in monolayers have been able to improve full maturation and embryo development in different species (human [222,223], dog [224–228] and mouse [223]), whereas co-cultures with GCs were aimed at mimicking the follicular environment both in 2D (mouse [229–231], human [232,233], pig [234] and dog [235]) and 3D systems (human [236] and pig [237]). In particular, the culture of isolated GCs in 3D conditions revealed that these cells are able to self-organize into clusters, proliferate and carry out their endocrine functions, supporting the oocytes growth and development [238,239]. In the domestic cat, gamete and oviductal cells co-cultures [240,241] were tested, giving no improvements.

In addition, these co-culture systems could be useful for oocytes which lost their somatic support (e.g. denuded oocytes, which are deprived of their own cumulus cells). Co-culture of denuded oocytes with isolated cumulus or granulosa cells or with whole COCs was tested, and generally had a beneficial role in terms of cytoplasmic and nuclear maturation in 2D (cow [242,243], rabbit [244], goat [245] and pig [246]) and 3D conditions (human [247,248] and mouse [249]). In cats, co-culture of denuded oocytes with granulosa

or COCs in 2D systems gave conflicting results [250–252]. Co-culture with COCs in 3D alginate microcapsules improved the viability of denuded oocytes [214], but not their meiosis resumption [213,214], even if the presence of denuded oocytes benefitted the embryo development of co-cultured COCs [213]. The same strategy could also be useful for other gametes which lost the support of their cumulus cells, such as cryopreserved oocytes, whose cumulus is often functionally detached after warming.

The presence of co-cultured oocytes is likely to be beneficial thanks to the exchange of oocyte-secreted factors (OSFs), which are produced by the oocytes and regulate their metabolism, cumulus proliferation, expansion and differentiation [243,253]. In more detail, OSFs can exert their functions on both the gamete and its cumulus cells, and when they are secreted in the culture medium some can promote oocyte competence [253–255], while others, including the most known growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), can regulate the function, differentiation and gene expression of cumulus cells [243,253,256,257]. Indeed, the addition of synthetic OSFs was also proposed as an ameliorative strategy for oocyte IVM (reviewed in [182]).

Finally, it is worth to mention that, even considering all these strategies and their diffuse application, in wild felids, due to the scarcity of biological material, there is a lack of experimental studies on variations of culture conditions. A method to improve maturation efficiency in some species is to prolong IVM for up to 34 hours [5], as in the African lion [258], but more investigations are needed.

1.3.1. Latest approaches for immature vitrified oocytes

Inspired by the most recent strategies applied on fresh oocytes, outlined above, or by the cellular and molecular consequences of cryopreservation, researchers tested different

methods to assess whether the developmental competence of cryopreserved oocytes could be improved. Considering the aforementioned convenience of immature oocyte vitrification, this overview will focus on GV VOs, which are the subject of this thesis.

Similarly to what happens to fresh oocytes, cryopreserved oocytes undergo oxidative stress. Indeed, not only the culture conditions, but also the cryoprocedures themselves may cause the generation of ROS through different mechanisms (e.g. osmotic stress, increased oxidative metabolism, alterations to electron transport chain in the mitochondria), especially during warming [259]. This can result in DNA fragmentation, as well as in damages to proteins and lipids. In particular, lipid-rich mammalian gametes and embryos are highly sensitive to oxidative stress [260]. In addition, oxidative stress might stimulate the release of cytochrome c and other apoptogenic factors from the mitochondria, inducing programmed cell death [261]. Apoptosis involves the activation of specific enzymes, known as caspases, which are proteases activated by other caspases in a cascade [262] and, in turn, it causes nuclear condensation, DNA fragmentation in multiples of 180–200 base-pairs, and phosphatidylserine exposure [263,264], which are broadly recognized as hallmarks of this type of cell death [264].

For these reasons, the use of antioxidant and antiapoptotic molecules has been a popular strategy for cryopreserved oocytes. Different compounds, including vitamins or plant-derived molecules, have been tested. Ascorbic acid (i.e. vitamin C) and N-acetyl-cysteine improved the developmental competence of ovine VOs when added to the IVM medium [265]. The same substances were useful for mice VOs, since ascorbic acid improved their survival, maturation, and embryo development [266] while N-acetyl-cysteine enhanced their developmental competence [267]. The antioxidant cysteamine, instead, was experimented on buffalo VOs without beneficial effects [268]. Supplementation with vegetal compounds also gave promising results. Rosmarinic acid,

alone or combined with ascorbic acid, improved VOs survival, maturation and fertilization in mice [266]. Resveratrol, a natural phenol produced by plants in response to injuries, improved viability, maturation, and mitochondrial membrane potential [269] of porcine VOs, as well as their development to the blastocyst stage when it was added to the IVM medium [270].

In addition, melatonin, thanks to the combination of its antioxidant and antiapoptotic effects, has also been applied in an attempt to improve VOs *in vitro* outcomes. Melatonin is a hormone secreted by the pineal gland, involved in the regulation of circadian rhythms and seasonal reproduction. Its role as an antioxidant and antiapoptotic factor is due to its ability to scavenge toxic oxygen derivatives and reduce the formation of ROS [260]. In mice, melatonin protected VOs from mitochondrial, chromosomal and oxidative alterations induced by vitrification [271] and contributed to increase their maturation rate [272]. The same beneficial effect in maturation outcomes was observed for human VOs [273].

Other synthetic antiapoptotic compounds, specifically acting against apoptosis effector enzymes, i.e. caspases, also exist. Accumulating evidence suggests that a partial inhibition of apoptosis can lead to increased cryosurvival in mammalian cells, and that it can be accomplished by the supplementation of (pan)-caspase inhibitors during vitrification and warming or during post-warming incubation [274–277]. No reports were published concerning GV oocytes, but the use of two different caspase inhibitors, known as Z-IETD-FMK or Z-LEHD-FMK, lowered apoptotic markers and improved mitochondrial activity, survival, cleavage and blastocyst rates in porcine MII VOs [278].

Due to the high lipid content of domestic animal oocytes, which contributes to gamete cryosensitivity [19], other chemicals, such as cholesterol loaded cyclodextrins, were used to compensate for lipid abundance. Cholesterol, transported by cyclodextrins,

can be incorporated into the oocyte plasma membrane and might render the gamete more resilient to cryopreservation [279]. In pigs, this treatment resulted in increased survival and maturation rates of VOs, whose mitochondria and zona pellucida were protected from cryodamage, but it was not useful for embryo development [280]. Instead, for bovine VOs contrasting results regarding developmental competence were obtained [279,281]. Furthermore, based on the same rationale, partial removal of lipids through centrifugation and delipidation was experimented in pig oocytes before vitrification, and it was beneficial for maturation rates [282,283].

Last among chemical supplements, antifreeze proteins are a group of polypeptides produced by certain animals, plants, fungi and bacteria to survive at sub-freezing temperatures. Thanks to the ability of these proteins to control ice growth and lower the freezing point of water, they are useful in stabilizing ice crystals and avoid re-crystalization [284], thus they have been used to limit cryopreservation-induced cellular damages. In mice VOs, the addition of antifreeze protein III in vitrification solutions led to an improvement in survival, cleavage and blastocyst rates, as well as to a better blastocyst quality (i.e. higher number of blastomeres, higher proportion of inner cell mass, lower apoptotic activity) [285].

On the other side, to give to VOs the best possible microenvironment for their post-warming development, variations in physical conditions were also tested. The application of enriched culture conditions was limited, but co-cultures were employed. An IVM co-culture system with GCs improved maturation outcomes of porcine denuded VOs [286], and higher cleavage and blastocyst rates were obtained [287]. More recently, in the same animal model, co-culture with fresh COCs during IVM was performed, and improvements in VOs quality (in terms of oxidative status and gene expression), meiotic progression and blastocyst formation were observed [288].

Furthermore, a semi-*in vivo* approach was also attempted to supply VOs with their physiological maturation conditions. With a technique called intrafollicular transfer, bovine VOs were injected into preovulatory follicles to achieve maturation, and recipient animals were then inseminated to obtain embryos. Unfortunately, no improvements in the developmental competence of VOs were obtained compared to the standard *in vitro* system [289].

In the domestic cat, some of these strategies have been experimented since the *in vitro* development of cat GV VOs is still poor [16] and improvements are strongly needed.

Some research groups decided to act on the vitrification procedure itself to check whether a different method could improve VOs outcomes. Considering the big size of cat GV, which is a challenge for cryopreservation, its compaction through the exposure to resveratrol (as a histone deacetylase enhancer) before vitrification was tested [21]. It allowed the accomplishment of better maturation, cleavage rates and embryo development to the 8-16 cells stage compared to the non-compacted control, although no morulae were obtained [21]. Instead, to improve cooling rates during vitrification, the application of slush nitrogen for oocyte vitrification was performed, but no improvements in maturation were observed, and the treatment almost seemed detrimental for subsequent embryo development [24]. Most recently, cat follicular fluid extracellular vesicles were characterized and, since they can be taken up by the immature COCs which can exploit their content (i.e. regulatory molecules including proteins, RNAs, lipids, DNA fragments and microRNAs), they were supplemented to vitrification media of immature oocytes, resulting in an enhanced ability of VOs to resume meiosis after warming [113].

Moreover, modifications to the IVM microenvironment were proposed. Besides an extension of the IVM length to 48 hours, that improved meiosis resumption of oocytes

vitrified with the Cryotop method [290], physico-chemical enrichments to the culture environment were also experimented. In our previous study, 3D barium alginate microcapsules were used, alone or combined with a fresh COCs co-culture, and although the non-flat environment was not enough to enhance VOs developmental competence, the co-culture seemed beneficial for blastocyst formation [112]. Following that, we applied 3D liquid marble microbioreactors for the IVM of VOs, but we did not observe any difference in meiosis resumption or full maturation compared to VOs cultured in control 2D microdrops [291].

On a parallel track, other groups tried to specifically target the molecular alterations induced by vitrification. To oppose cryopreservation-induced degeneration, targeting of apoptotic pathways was attempted. The addition of an inhibitor of an apoptotic signalling molecule known as ROCK (Rho-associated coiled-coil containing protein kinase) to the IVM medium brought to an enhancement in fertilization and cleavage rates [135]. In addition, since vitrification can damage the functional coupling between the oocyte and its cumulus cells (which include hemichannels and gap junctions), a block of these communication channels might be beneficial to avoid their unsolicited opening and the consequent loss of essential molecules and the penetration of potentially harmful compounds. The use of a molecule known as Gap26, added to vitrification media, led to higher maturation and embryo development rates, probably because it better allowed the preservation of channel functionality [133].

Even if some progresses have been made and some innovative approaches have been experimented, the ability of cat VOs to resume meiosis and develop into embryos remains impaired, and *in vitro* outcomes are still unsatisfactory. The mechanisms involved and

new culture strategies remain to be investigated to improve VOs developmental competence and apply new and efficient protocols in felids conservation programs.

2. Aim of the project

The domestic cat is the model of choice for the development of ARTs in wild, endangered felids. In particular, the development of gamete cryopreservation protocols is crucial for long-term biodiversity conservation and for breeding and reintroduction programs of threatened species. For female germplasm, immature oocyte vitrification is the best technique in terms of cost-effectiveness, speed, and feasibility in field conditions. However, cat VOs often degenerate or struggle to mature and develop into embryos *in vitro* after warming, and improvements in vitrification protocols and post-warming culture conditions are needed.

While the available literature mainly focused on the modification of the vitrification procedure itself [21,23,24,113], the overarching goal of this PhD thesis was to assess whether the *in vitro* developmental competence of domestic cat VOs could be improved through physical and/or chemical enrichments to the culture microenvironment.

Specifically, the project aimed to:

(a) describe a vitrification protocol for cat immature oocytes and define its efficiency based on VOs post-warming viability rates (**Paper I**);

(b) verify the *in vitro* functionality of domestic cat GCs and create a follicle-like structure (i.e. a 3D barium alginate microcapsule with encapsulated GCs) for the IVM of VOs in enriched culture conditions (**Paper II**);

(c) determine whether vitrification might induce apoptosis in cat VOs and the effects of a chemical inhibitor on VOs apoptotic activation and *in vitro* embryo development (**Paper III**).

3. Materials and Methods

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

3.1. Experimental design

In order to find an efficient vitrification strategy for cat immature oocytes and to test possible improvements in VOs microenvironment, this PhD project included different studies, currently published in international peer-reviewed journals as three different manuscripts.

In **Paper I** [292], a vitrification-warming protocol for cat immature oocytes was described and its efficiency was defined based on the evaluation of VOs post-warming viability.

In **Paper II** [293], two experiments were performed to investigate the steroidogenic activity of cat GCs and their ability to support VOs meiosis resumption during IVM.

In Experiment I, follicle-like structures (FLS) of GCs encapsulated in 3D barium alginate microcapsules were created, and estradiol (E2) and progesterone (P4) concentrations along 6 days of culture were assessed to confirm GCs functionality. These were compared to E2 and P4 concentrations obtained from GCs cultured in 2D monolayers (MONO).

In Experiment II, GCs cultured in 3D FLS or 2D MONO for different time spans [2 days (FLS-2d and MONO-2d) or 6 days (FLS-6d and MONO-6d)] were used as artificial co-culture environments for the IVM of VOs. After 24 hours, oocyte chromatin configurations were evaluated as final endpoint.

In **Paper III** [294], three experiments were performed to investigate the effect of vitrification of immature domestic cat oocytes on some apoptosis markers and whether cat VOs cryotolerance and *in vitro* development can be improved with the use of a pan-caspase inhibitor (i.e. Z-VAD-FMK).

In Experiment I, the goal was to check whether vitrification induces the activation of apoptotic pathways in cat VOs. Fresh (FOs, negative control), hydrogen peroxide-exposed (HPOs, positive control) and vitrified-warmed oocytes were analyzed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, to detect DNA fragmentation, and by a caspase activity assay. Since degeneration might start slowly after warming, VOs were analyzed after 2 (2hVOs, based on [295]) or 24 hours (24hVOs, standard length of cat oocytes IVM) incubation.

Experiment II was aimed to investigate the influence of a pan-caspase inhibitor on the same apoptosis markers (i.e. DNA fragmentation, caspase activity) analyzed in Experiment I; Z-VAD-FMK was added to vitrification-warming media only [VOs(+/-)] or to both vitrification-warming media and post-warming incubation medium [VOs(+/+)]. The length of the post-warming incubation (24 hours) was chosen based on the results of Experiment I. Untreated VOs [VOs(-/-)],which were vitrified-warmed and then incubated without Z-VAD-FMK, were used as control.

In Experiment III, the aim was to check the influence of the pan-caspase inhibitor Z-VAD-FMK on the *in vitro* development of cat VOs; the treatment that gave the best results in Experiment II [VOs(+/+)] was used for *in vitro* embryo production to assess maturation and embryo development compared to untreated VOs [VOs(-/-)] and fresh oocytes (FOs). In this Experiment, post-warming incubation corresponded to IVM.

A scheme of the complete experimental design is depicted in Figure 6.

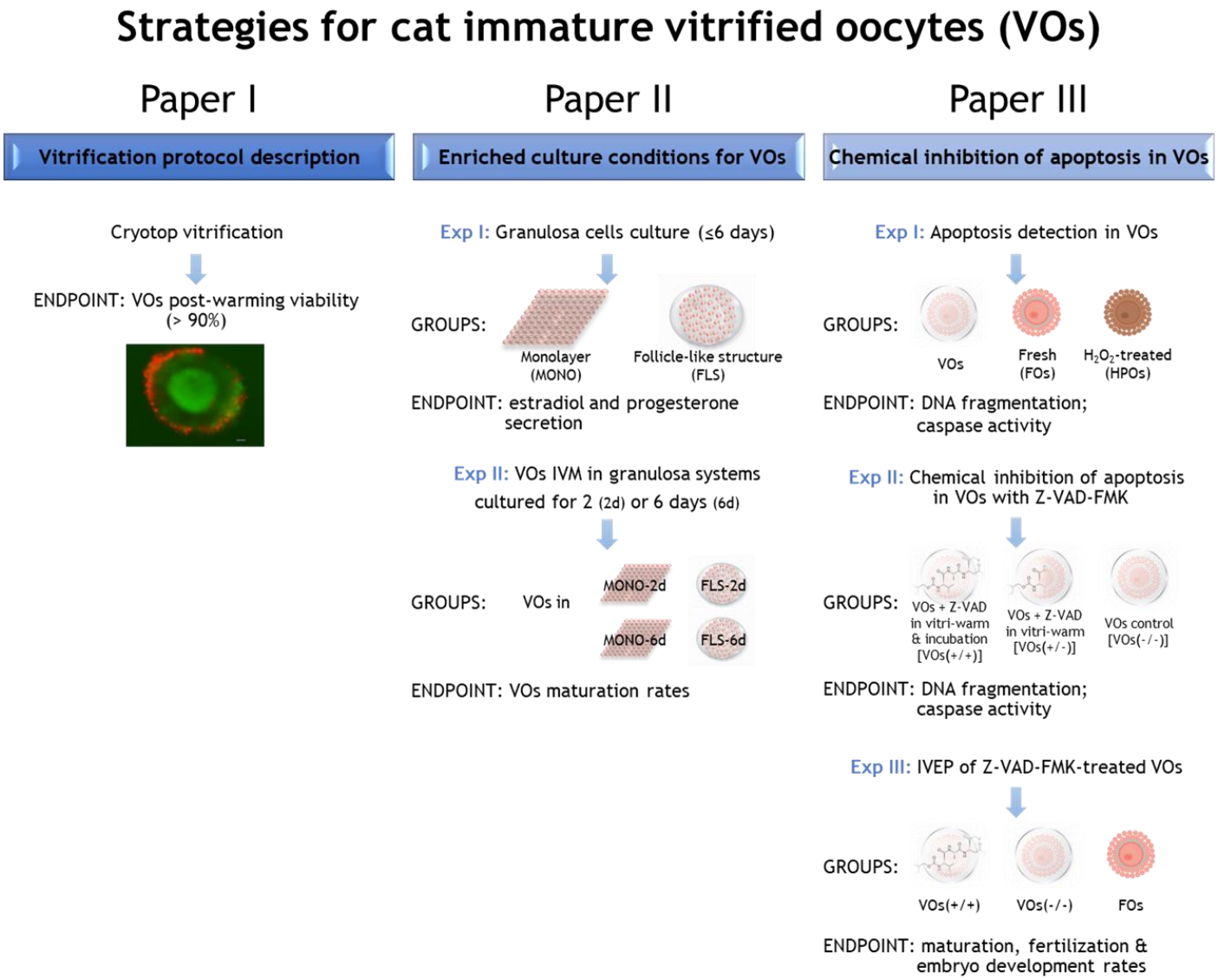

***Figure 6**. Schematic overview of experimental design and experimental groups.*

IVM = in vitro maturation; IVEP = in vitro embryo production.

3.2. Ovaries and oocyte retrieval

Ovaries (n = 566) from healthy queens (*Felis catus*) were harvested at random stages of the estrous cycle during routine ovariectomies or ovariohysterectomies. The project did not require an ethical approval because cat ovaries were collected as byproducts from routine surgeries.

After surgery, ovaries were immediately placed in a phosphate buffered saline (PBS) supplemented with a mixture of antibiotics and antimycotics (100 IU/mL of penicillin G sodium, 0.1 mg/mL of streptomycin sulfate, 0.25 µg/mL of amphotericin B), and transported to the laboratory within few hours.

Ovaries were minced with a scalpel blade in PBS with 0.1% (w/v) polyvinyl alcohol at room temperature to release the oocytes, and only immature COCs with darkly pigmented ooplasm completely surrounded by several layers of cumulus cells (Grade I [296]) were selected for the experiments.

3.3. Collection and culture of granulosa cells (Paper II)

For GCs isolation, retrieved COCs were denuded by incubation in hyaluronidase (80 IU) for 7 minutes at 38.5°C and repeated pipetting by a small bore glass pipette. Cumulus-deprived oocytes were discarded, and GCs were processed as described in [297] before culture. Briefly, GCs were pooled and centrifuged at 400 g for 10 minutes; then, the supernatant was discarded and GCs pellet was resuspended in PBS (1 mL). Double-distilled water (1 mL) was added, and the cells were agitated for 10 seconds to lyse red blood cells derived from collection. Isotonicity was quickly restored by the addition of PBS (4 mL) and GCs were pelleted again by centrifugation, followed by removal of the supernatant (twice). Finally, GCs pellet was resuspended in culture medium and cell concentration was determined by a manual count with a Bürker chamber.

Culture of GCs was performed in 2D MONO or 3D FLS. Monolayers were cultured in 24-wells plates. Follicle-like structures were obtained by one-step encapsulation in barium alginate [238]. Briefly, GCs were resuspended in Medium 199 (concentration: 1–2 × 10^6 cells/mL) and $BaCl_2$, and aliquots were dropped in stirred Na-alginate solution (0.5%, 3,500 centipoise) at room temperature; after 35–40 minutes, the microcapsules were collected and washed twice with PBS before culture.

Granulosa cells in microcapsules (3D FLS) or in MONO were cultured for 6 days in a controlled atmosphere (38.5°C and 5% CO_2 in air) in Medium 199 (as a modification of [297]) supplemented with: 0.1% BSA, 5% FBS, 0.5 IU FSH + 0.5 IU LH (Pluset®, Calier, Spain), 10 µg/mL 22(R)-hydroxycholesterol, 25 ng/mL EGF, 0.1% ITS premix (1.0 µg/mL insulin; 0.55 µg/mL transferrin; 0.5 ng/mL sodium selenite) and 10^{-7} M androstenedione, as estradiol precursor. On days 2, 4 and 6, half of the culture medium was changed with fresh one and stored (days 2 and 6) at −20°C for hormonal determination. In Experiment II, culture medium for GCs at days 2 and 6 was supplemented with 0.6 mM cysteine to perform IVM of VOs.

3.4. Estradiol and progesterone determination (Paper II, Experiment I)

Concentrations of E2 and P4 were assessed in conditioned medium with an enzyme-linked fluorescent assay (ELFA, MiniVIDAS®, Biomerieux). Day 2 and day 6 samples of the 2D MONO that exceeded the instrument maximum detection range were diluted (in Medium 199) 1:100 for E2 determination and 1:10 for P4 determination.

3.5. Vitrification and warming of immature cumulus-oocyte complexes

Oocytes were vitrified by the Cryotop method [298,299], as we described for cat oocytes (Figure 7; [292]). Briefly, groups of 2-8 oocytes were equilibrated at room temperature in

an equilibration solution containing 7.5% (v/v) ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in Medium 199, with 20% FBS for 15 minutes. Then, they were transferred into a vitrification solution [15% (v/v) EG, 15% DMSO and 0.5 M sucrose in Medium 199 with 20% FBS], placed on Cryotop polypropylene strip, removing the excess of liquid to reduce the volume as much as possible, and directly immersed into liquid nitrogen in less than 90 seconds.

At warming, the Cryotop strip was immersed for 1 minute in a thawing solution at 37ºC containing 1 M sucrose in Medium 199, with 20% FBS. Vitrified oocytes were retrieved and transferred for 3 minutes in a solution containing 0.5 M sucrose in Medium 199, with 20% FBS and then for 5 minutes in a solution without sucrose. Finally, they were washed again in the same solution (Medium 199 with 20% FBS) and then stained for viability assessment (Paper I) or moved to the appropriate medium:

- IVM medium (Paper II, Experiment II; Paper III, Experiment III)
- Medium 199 for incubation (Paper III, Experiments I and II).

3.5.1 Notes for vitrification-warming of VOs treated with the apoptosis inhibitor Z-VAD-FMK (Paper III, Experiments II and III)

For Z-VAD-FMK-treated oocytes, the final concentration of Z-VAD-FMK (Z-VAD(OMe)-FMK, MedChemExpress, Monmouth Junction, NJ, USA) in all vitrification-warming solutions and IVM media was 20 μM [274,275,278,300]. The stock solution (20 mM) was prepared in DMSO. This concentration was chosen so that DMSO would have been diluted 1:1000 in the media, with a final concentration (0.1% v/v) which was advised by the manufacturer to avoid cell toxicity. Since the addition of Z-VAD-FMK meant an addition of DMSO, the volume of inhibitor was subtracted from the total amount of DMSO for the preparation of equilibration and vitrification solutions for Z-VAD-FMK-treated VOs. Likewise, an amount

of DMSO corresponding to the amount of inhibitor (1:1000) was added to the solutions for stepwise warming of control VOs. For the same reason, in incubation and IVM media of control VOs (i.e. without Z-VAD-FMK), a corresponding volume of DMSO (1:1000) was added.

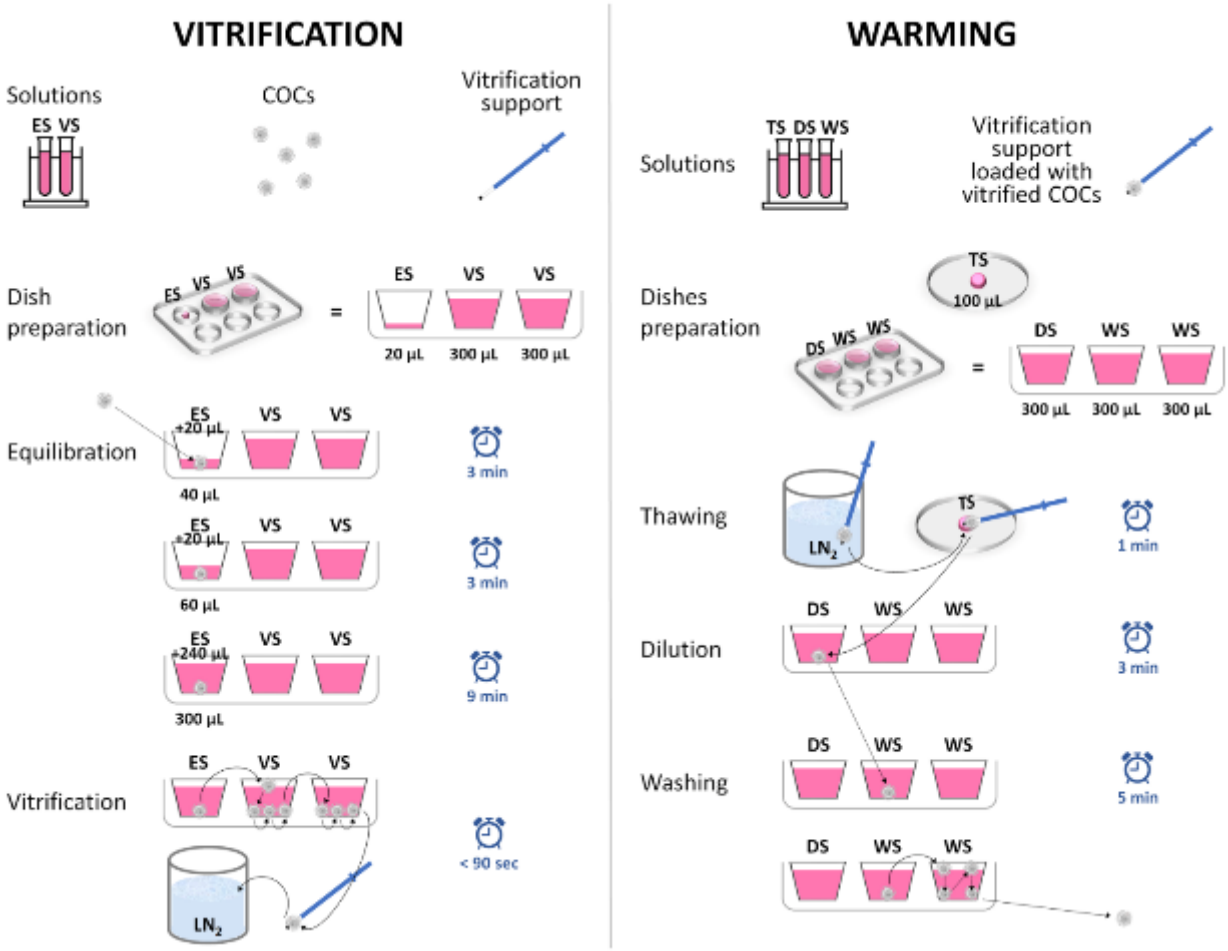

***Figure 7**. Schematic depiction of cat oocyte vitrification-warming protocol with the Cryotop® method (from Colombo & Luvoni, 2020* [292]*).*

COCs = cumulus-oocyte complexes; ES = equilibration solution; VS = vitrification solution; TS = thawing solution; DS = dilution solution; WS = washing solution; LN_2 = liquid nitrogen.

3.6. Vitrified oocyte viability assessment (Paper I; Paper II, Experiment II)

After warming, VOs viability was evaluated by fluorescein diacetate/propidium iodide (FDA/PI) staining to assess post-warming survival rate (Paper I). Briefly, VOs were maintained in the dark in the staining solution (PI:10 mg/mL; FDA: 5 mg/mL) for 3 minutes and then rapidly evaluated under a fluorescent microscope (Axiovert 100, Zeiss). Dead VOs showed red fluorescence (PI excitation/emission wavelengths: 535 nm/617 nm), whereas viable VOs showed bright green fluorescence (FDA excitation/emission wavelengths: 495 nm/517 nm). In Paper II, Experiment II, dead VOs were discarded, and only viable VOs were washed in Medium 199 and used for IVM.

3.7. *In vitro* maturation in enriched culture conditions (Paper II, Experiment II)

In Paper II, vitrified–warmed cat oocytes were *in vitro* matured for 24 hours in a controlled atmosphere (38.5°C and 5% CO_2 in air) in the following conditions:

- Follicle-like structures cultured for 2 days (FLS-2d);
- Follicle-like structures cultured for 6 days (FLS-6d);
- Monolayers cultured for 2 days (MONO-2d);
- Monolayers cultured for 6 days (MONO-6d).

Vitrified–warmed oocytes were injected into the inner core of 3D FLS or placed on MONO by a small bore glass pipette in the same well where GCs were cultured. Other VOs were cultured separately as control group in traditional microdrops (100 µL) of maturation medium without GCs (Medium 199 supplemented with 3 mg/mL BSA, 10 ng/mL EGF, 0.6 mM cysteine and 0.5 IU/mL FSH + 0.5 IU/mL LH), placed in a Petri dish and covered by mineral oil.

3.7.1. Assessment of maturation rates

After 24 hours of IVM, chromatin configuration of VOs was evaluated by Hoechst 33342 staining. Oocytes 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 with the working solution (Hoechst, 0.01 mg/mL). After 5 minutes of incubation in the dark, the Hoechst solution was removed, the slides were covered with an antifade reagent (Fluoromount™ Aqueous Mounting Medium) and nuclear evaluation under a fluorescence microscope (Axiovert 100, Zeiss) at 400× magnification was performed. Chromatin configurations were classified as follows [224,301]:

- Germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- Germinal vesicle breakdown–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.

3.8. Staining for apoptotic signal detection (Paper III, Experiments I and II)

Cell Meter™ TUNEL Apoptosis Assay Kit *Red Fluorescence* (AAT Bioquest, Sunnyvale, CA, USA) and CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific – Invitrogen, Monza, Italy) were used to stain the oocytes for DNA fragmentation and caspase activation, respectively, following the manufacturers' instructions. Briefly, oocytes were incubated for 1 hour at 38.5°C in TUNEL and caspase dyes. Then, they were denuded and counterstained with Hoechst 33342 (0.01 mg/mL) to identify the nucleus.

Decumulation was performed after incubation in the dyes to avoid a possible influence of pipetting-derived shear stress on cell apoptosis [302]. Finally, the slides were covered with an antifade reagent (Fluoromount™ Aqueous Mounting Medium). Dyes maximum excitation/emission wavelengths were as follows:

- TUNEL Assay: 556 nm/579 nm;
- Caspase Detection Reagent: 502 nm/530 nm;
- Hoechst: 352 nm/461 nm.

Slides were evaluated under a fluorescence microscope (Axiovert 100, Zeiss) equipped with a digital camera (AxioCam MRc 5, Zeiss) at 400× magnification to count the number of TUNEL-positive oocytes (showing a bright red fluorescence in nucleus area) and to esteem caspase activity (i.e. green fluorescence) with the fluorescence intensity mean value of ooplasm, measured with the Imaging Software ZEN 2.5 blue edition (Carl Zeiss Microscopy). Fluorescence values were corrected for background fluorescence. Oocytes whose nucleus could not be identified by Hoechst staining were excluded by the analysis (for Experiment I: one oocyte in 24hVOs and one in FOs were discarded; for Experiment II: three oocytes in VOs(+/+), one in VOs(+/-) and one in VOs(-/-) were discarded; no significant differences among groups – Fisher's $p=0.358$). Images were captured in black and white and under the same settings. Figures shown in the results section were pseudo-colored after image acquisition.

In Paper III, Experiment I, four experimental groups were compared. Fresh oocytes (FOs, negative control) were stained right after collection. In positive control oocytes (HPOs), apoptosis was induced with a three- hours incubation in 100 μM H_2O_2 in Medium 199 [303,304] before staining. Vitrified oocytes were incubated after warming for 2 hours (2hVOs) [295] or 24 hours (24hVOs) in Medium 199 to assess whether the activation of

apoptotic pathways might occur soon after warming or slowly during the following incubation time.

In Paper III, Experiment II, three experimental groups of VOs were compared. Based on the results of Experiment I, warmed oocytes were incubated for 24 hours before staining. Control VOs [VOs(-/-)], which were vitrified-warmed without Z-VAD-FMK, were incubated in plain Medium 199. Oocytes vitrified-warmed with the addition of Z-VAD-FMK, instead, were either incubated in plain Medium 199 [VOs(+/-)] or in Medium 199 with the addition of 20 μM Z-VAD-FMK [VOs(+/+)].

3.9. *In vitro* embryo production (Paper III, Experiment III)

3.9.1. *In vitro* maturation

Based on the results of Paper III, Experiment II, three experimental groups underwent IVM to assess the developmental competence of VOs treated with a pan-caspase inhibitor:

- Treated VOs [VOs(+/+)], i.e. oocytes vitrified-warmed and *in vitro* matured with the addition of Z-VAD-FMK.
- Control VOs [VOs(-/-)], i.e. oocytes which were vitrified-warmed and matured in standard conditions (without Z-VAD-FMK).
- Fresh oocytes (FOs), as system control.

Oocytes were singly *in vitro* matured for 24 hours in a controlled atmosphere (39ºC and 5% CO_2 in air) in 20 μL microdrops of IVM medium [305] (Medium 199 supplemented with 3 mg/mL BSA, 0.1 mg/mL cysteine, 1.4 mg/mL HEPES, 0.25 mg/mL sodium pyruvate, 0.6 mg/mL sodium lactate, 0.15 mg/mL L-glutamine, 0.055 mg/mL gentamicin, 0.2 IU/mL human LH and 0.5 IU/mL human pituitary FSH) covered by mineral oil in Petri dishes.

3.9.2. Epididymal sperm recovery and *in vitro* fertilization (IVF)

In vitro fertilization was performed with fresh feline epididymal spermatozoa obtained after routine orchiectomy of adult tomcats (n = 10). Testes were maintained at 4°C without any medium for up to 24 hours before processing. The epididymides were dissected from isolated testicles and placed in a Petri dish in HEPES-buffered Medium 199. Spermatozoa were obtained from vas deferens and cauda epididymis by slicing with scissors at room temperature, and flushing sperm suspension through a 30 µm filter (Sysmex Partec GmbH, Görlitz, Germany). Subjective motility and concentration by Bürker chamber were determined.

After 24 hours of IVM, oocytes were removed from the IVM dishes, washed twice and transferred into 50 µL microdrops of fresh IVF medium [305] (Medium 199 supplemented with 3 mg/mL BSA, 0.1 mg/mL cysteine, 1.4 mg/mL HEPES, 0.25 mg/mL sodium pyruvate, 0.6 mg/mL sodium lactate, 0.15 mg/mL L-glutamine, 0.055 mg/mL gentamicin and 2.2 IU/mL heparin) covered by mineral oil in Petri dishes. Diluted spermatozoa, previously centrifuged (500 g, 5 min) and resuspended in IVF medium, were added to the microdrops containing the oocytes to reach 100 µL volume with a final concentration of 1×10^6 motile spermatozoa/mL. Each IVF drop contained 7-11 oocytes from the same experimental group. Oocytes and spermatozoa were co-incubated in a controlled atmosphere (39ºC and 5% CO_2 in air) for 18 hours.

3.9.3. *In vitro* embryo culture

After IVF, all oocytes were gently washed in *in vitro* culture (IVC) medium (Ham's F-10 supplemented with 5% FBS, 0.11 mg/mL sodium pyruvate, 0.075 mg/mL L-glutamine, 0.6 mg/mL gentamicin) to remove spermatozoa and residual cumulus cells with the help of a stripper micropipette (The Stripper, BioTipp, Waterford, Ireland) equipped with 155 µm

and 125 µm tips (RI-Tip, Gynemed, Lensahn, Germany). Presumptive embryos were moved to 20 µL microdrops of IVC medium covered by mineral oil in Petri dishes, where they were cultured singly for up to 8 days in a controlled atmosphere (39ºC, 5% CO_2 and 5% O_2). The medium was not changed during embryo culture. During IVC, assessment of embryo development was performed every 24 hours under an inverted microscope at 200× magnification (Axiovert 100, Zeiss).

3.9.4. Assessment of maturation, fertilization and embryonic developmental rates

Two days after IVF, uncleaved oocytes were deprived of remaining cumulus cells and bound spermatozoa by mechanical displacement with a stripper micropipette equipped with a 125 µm tip, placed on a slide, air-dried, and fixed in 80% ethanol overnight at 4°C. Chromatin configuration was then evaluated by PI staining (Thermo Fisher Scientific; 1 mg/mL, 1:100 in PBS). Growing embryos were cultured until day 8 or until they showed signs of degeneration and then fixed and stained with PI to ascertain their developmental stage based on the number of blastomere nuclei. Maturation stage (MII) in unfertilized oocytes was identified as described before (i.e. presence of a tightly packed group of chromosomes in the form of the first polar body, and of another well-spread group which allows for the identification of individual chromosomes [224]).

Nuclear evaluation was performed under a fluorescence microscope (Axiovert 200M, Zeiss) at 200× magnification. The total number of *in vitro* matured oocytes was calculated as the sum of unfertilized MII oocytes, fertilized oocytes (i.e. uncleaved but showing pronuclei) and cleaved embryos; accordingly, the number of fertilized oocytes was calculated as the sum of fertilized oocytes and cleaved embryos. For the assessment of embryonic development, cleaved embryos (2-4 cells), 5-8 cells, 9-16 cells, morulae, and blastocysts stages were recorded.

3.10. Statistical analysis

In Paper II, to have a normal and homoscedastic distribution, data of E2 and P4 concentrations (Experiment I) were recalculated with logarithmic transformation by the relation $y' = Ln (y + 1)$, where y' is the new transformed value and y is the original concentration value; transformed E2 and P4 data were analyzed by a two-way ANOVA followed by Tukey's test. Maturation rates of VOs (Experiment II) were analyzed by chi-square test.

In Paper III, in Experiments I and II, TUNEL data were analyzed by Fisher's exact test, whereas caspases activation (i.e. fluorescence intensity) was analyzed by Kruskal-Wallis non-parametric one-way ANOVA followed by Dwass-Steel-Critchlow-Fligner pairwise comparisons (data were not normally distributed by Shapiro-Wilk test - $p<0.001$ for Experiment I, $p=0.005$ for Experiment II). In Experiment III, maturation, fertilization and embryo development rates were analyzed by Fisher's exact test.

For all the experiments, the level of significance was set at $p<0.05$.

4. Results

4.1. Post-warming viability of domestic cat VOs (Paper I)

After warming and staining with FDA/PI (Figure 8), 395 out of 435 (90.8%) VOs survived. Survival rates were consistent among replicates, as shown in Table 2. Therefore, the vitrification protocol was considered efficient and was used throughout the project, although some morphological changes could be noticed in warmed oocytes (Figure 9), most commonly changes in ooplasm shape and granulation, partial (or, rarely, total) loss of cumulus cells and (rarely) zona pellucida fractures.

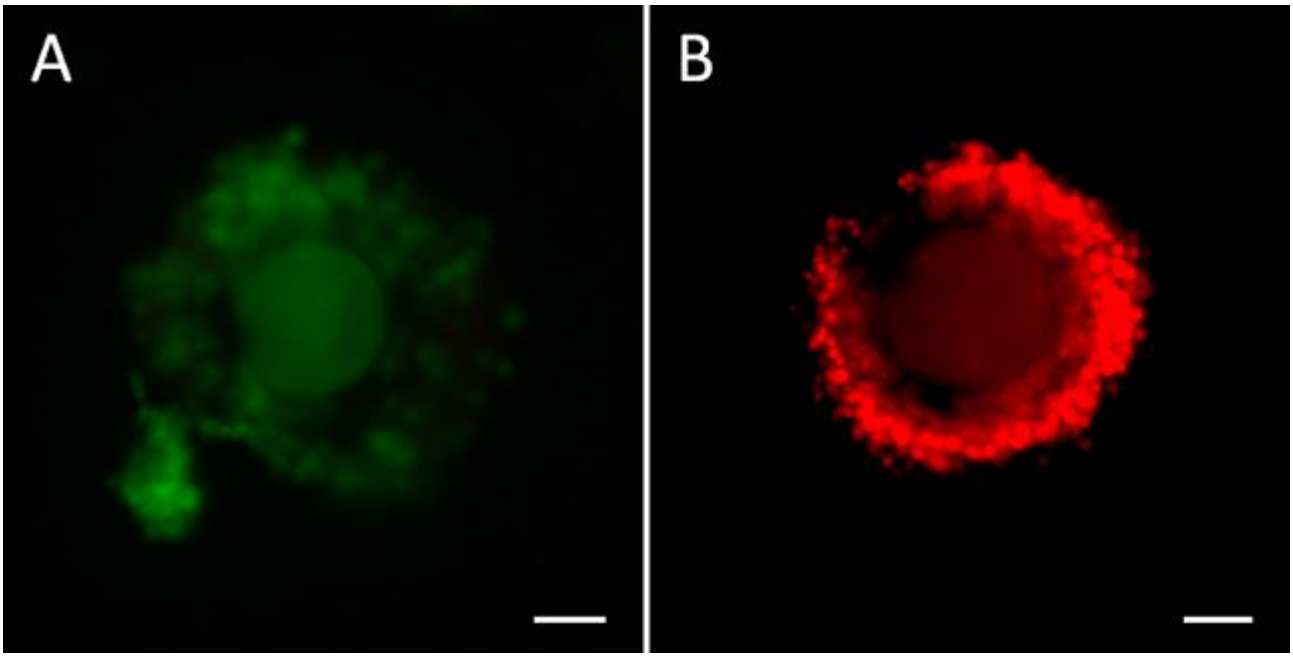

Figure 8*. Fluorescent micrographs of domestic cat vitrified cumulus-oocyte complexes stained with fluorescein diacetate/propidium iodide (FDA/PI) after warming (from Colombo & Luvoni, 2020* [292]*). (A) Viable vitrified-warmed cumulus-oocyte complex, showing green fluorescence; (B) Dead vitrified-warmed cumulus-oocyte complex, showing red fluorescence. Scale bar: 50 µm.*

***Table 2**. Post-warming viability of vitrified domestic cat immature vitrified oocytes, divided by replicate (from Colombo & Luvoni, 2020* [292]*).*

Experiment	Group	Warmed oocytes n.	Viable oocytes n.	Viability %	Viability mean % ± SD
1†	1	13	12	92.3	91.5 ± 3.7
	2	47	44	93.6	
	3	52	45	86.5	
	4	26	24	92.3	
	5	41	40	97.6	
	6	21	19	90.5	
	7	50	44	88	
2‡	1	17	17	100	91.5 ± 9.2
	2	17	17	100	
	3	9	8	88.9	
	4	21	21	100	
	5	30	23	76.7	
	6	26	23	88.5	
	7	17	13	76.47	
	8	10	10	100.00	
	9	15	14	93.33	
	10	23	21	91.30	

Data were extrapolated from a previous work (†Colombo et al. 2019 [112]*) and Paper II (‡Colombo et al. 2020* [293]*).*

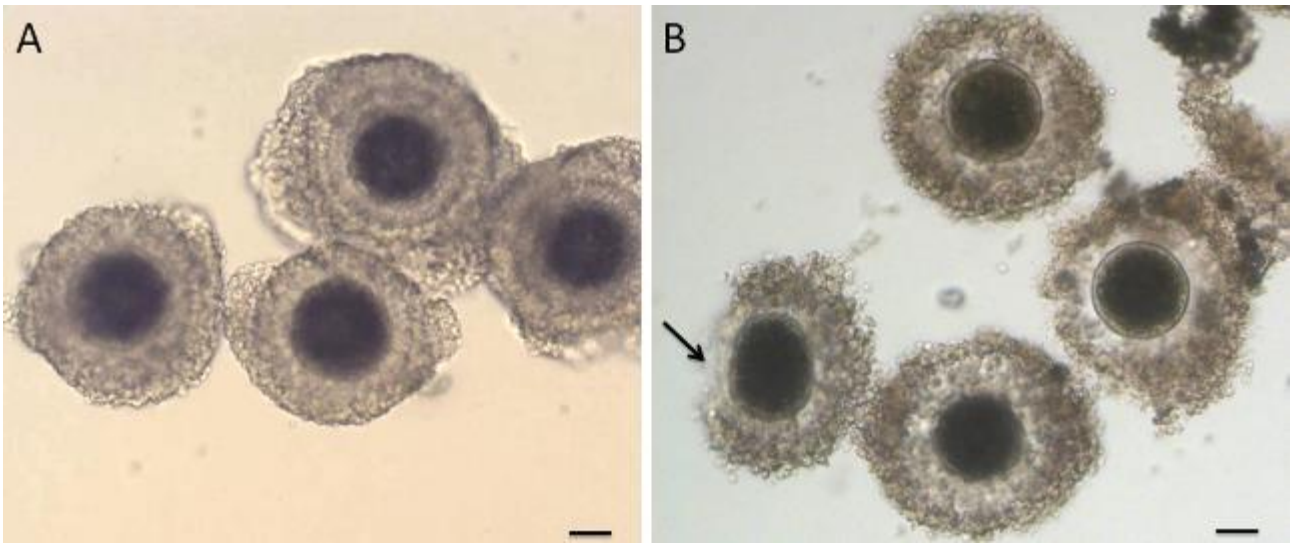

***Figure 9**. Light micrographs of (A) fresh and (B) vitrified-warmed domestic cat immature cumulus-oocyte complexes (from Colombo & Luvoni, 2020* [292]*). Vitrified oocytes show some vitrification-induced morphological damages after warming (changes in shape and loss of cumulus cells, black arrow). Scale bar: 50 µm.*

4.2. Nursing ability of granulosa cells for the IVM of cat VOs (Paper II)

4.2.1. Steroidogenic activity of cat granulosa cells in follicle-like structures and monolayers (Paper II, Experiment I)

To assess the functionality of cat GCs cultured in 3D FLS and in 2D MONO, and so their suitability to be employed as an enriched culture environment for VOs, GCs secretion of E2 and P4 was determined (Table 3). For both the hormones, culture systems differed significantly (P4, $p<0.001$; E2, $p=0.02$) on day 6 of GCs culture, but not on day 2. Progesterone levels, but not E2 ($p=0.825$), were significantly higher on day 6 than on day 2 ($p=0.018$) in both culture conditions, although GCs in FLS had a lower steroid secretion. Comparisons between the P4/E2 ratio were not significant on any day of culture, but this ratio had a tendency to increase more along culture in MONO than in FLS

(MONO: Day 2, 0.006 ± 0.004 and Day 6, 0.034 ± 0.047; FLS: Day 2, 0.015 ± 0.013 and Day 6, 0.025 ± 0.020; p=0.411).

***Table 3**. Hormonal secretion of cat granulosa cells cultured for different time spans (2 or 6 days) in different culture systems (from Colombo et al., 2020* [293]*).*

Culture system	Day of culture	E2 (pg/mL)	P4 (ng/mL)
3D follicle-like structure	Day 2	151.3 ± 121.8[a]	1.4 ± 0.7[a]
	Day 6	172.6 ± 144.8[a]	2.4 ± 1.3[b]
2D monolayer	Day 2	20283.8 ±18223.1[a]	107 ± 153[a]
	Day 6	26708.8 ± 17921[a]	610.4 ± 607.3[b]

Different superscripts ([a,b]) indicate significant differences within columns of the same culture system (p<0.05). Data are reported as mean ± SD. E2 = estradiol; P4 = progesterone.

4.2.2. Meiosis resumption of cat VOs *in vitro* matured in granulosa cells-enriched culture systems (Paper II, Experiment II)

After IVM in 3D FLS (Figure 10), 2D MONO or standard 2D microdrops of medium (control group), VOs nuclear configurations were as reported in Table 4. Vitrified oocytes resumed meiosis at higher rates (45.5% and 48.5%, respectively) in FLS-2d and MONO-2d compared to FLS-6d and MONO-6d (p=0.03). Concerning full maturation, in all the experimental conditions, except in FLS-2d, around 12% of VOs achieved MII stage. Lower VOs degeneration rates were recorded when GCs-2d (in both FLS and MONO) were used as culture environment compared to GCs-6d (p<0.001) or microdrops of medium (p=0.003).

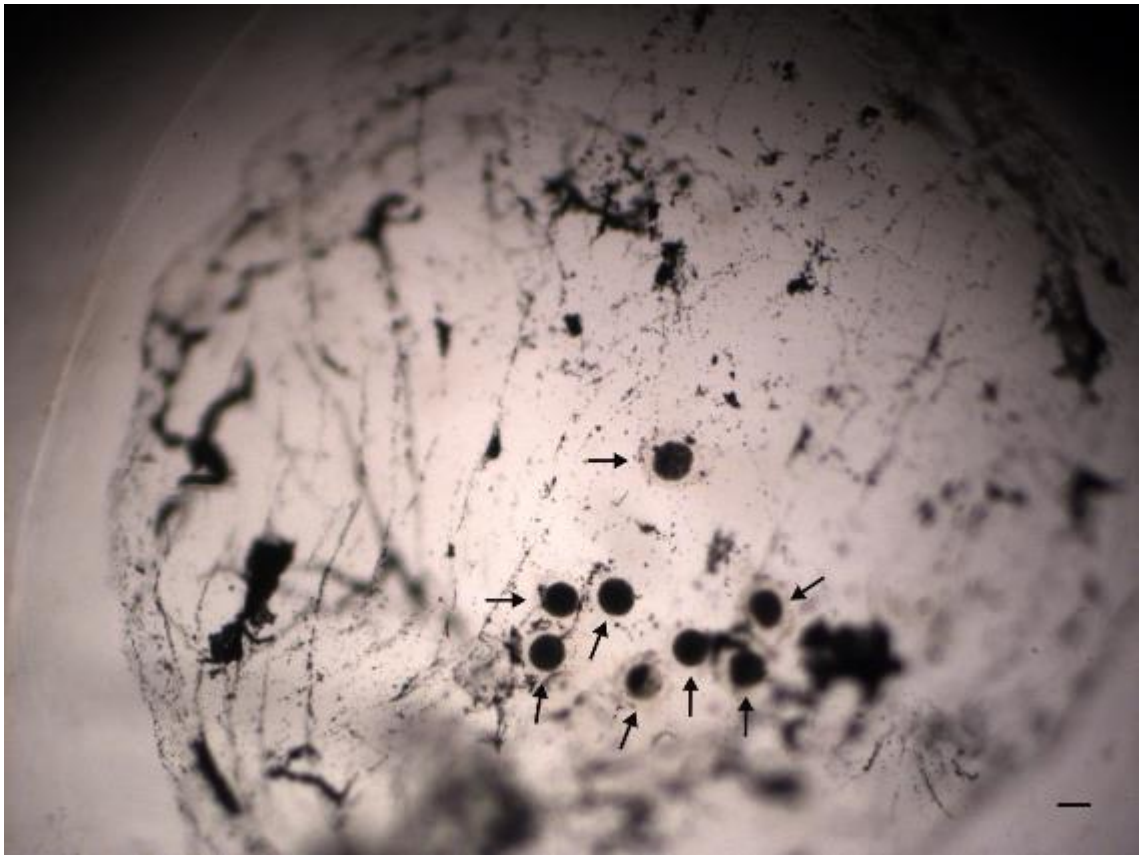

Figure 10*. Three-dimensional follicle-like structure (barium alginate microcapsule enriched with domestic cat granulosa cells) during the in vitro maturation of conspecific vitrified oocytes (black arrows; from Colombo et al., 2020* [293]*). Scale bar: 100 μm.*

***Table 4**. Nuclear configurations of vitrified-warmed domestic cat oocytes after 24 hours in vitro maturation in 3D or 2D systems enriched with granulosa cells cultured for 2 or 6 days (from Colombo et al., 2020* [293]*).*

Culture system	Day of GCs culture	Immaturity (GV)	Meiosis resumption (GVBD-AI)	Full maturation (TI-MII)	Degeneration
		n. of oocytes (%)	n. of oocytes (%)	n. of oocytes (%)	n. of oocytes (%)
3D follicle-like structure	Day 2	8/33 (24.2)[a]	15/33 (45.5)[a]	0/33 (0)[a]	10/33 (30.3)[a]
	Day 6	0/34 (0)[b]	5/34 (14.7)[b]	4/34 (11.8)[b]	25/34 (73.5)[b]
2D monolayer	Day 2	7/33 (21.2)[a]	16/33 (48.5)[a]	3/33 (9.1)[a,b]	7/33 (21.2)[a]
	Day 6	0/33 (0)[b]	6/33 (18.2)[b]	4/33 (12.1)[b]	23/33 (69.7)[b]
Control group	/	4/34 (11.8)[a]	7/34 (20.5)[b]	4/34 (11.8)[b]	19/34 (55.9)[b]

Different superscripts ([a,b]) within the same column indicate significant differences ($p<0.05$). GV = germinal vesicle; GVBD = germinal vesicle break-down; MI = metaphase I; TI = telophase I; MII = metaphase II.

Control group: vitrified oocytes cultured in traditional microdrops of medium without granulosa cells.

4.3. Detection and inhibition of apoptosis for the *in vitro* embryo development of cat VOs (Paper III)

4.3.1. Caspase activity and DNA integrity of cat VOs (Paper III, Experiment I)

The activation of apoptotic pathways in fresh (FOs, negative controls) and VOs incubated for 2 or 24 hours is reported in Table 5, together with data of positive controls (hydrogen peroxide-treated oocytes, HPOs).Representative pictures are shown in Figure 11. DNA fragmentation (TUNEL-positive oocytes) was the lowest in FOs and 2hVOs ($p=0.106$). The number of oocytes with fragmented DNA increased ($p=0.023$) in VOs incubated longer (24hVOs), even if it was lower than in HPOs ($p=0.004$). Caspase activity was higher in VOs and HPOs than in FOs ($p=0.001$), and there were no differences between VOs incubated for different time spans after warming (2hVOs vs 24hVOs, $p=0.989$) or between VOs and HPOs ($p=0.766$).

4.3.2. Caspase activity and DNA integrity of cat VOs treated with the apoptosis inhibitor Z-VAD-FMK (Paper III, Experiment II)

Based on the results of Experiment I, in which 24hVOs had a higher DNA damage, and since the standard length of post-warming incubation in VOs is 24 hours (i.e. IVM length for cat oocytes), this time was chosen for incubation to assess the effect of an apoptosis inhibitor. Results of the use of Z-VAD-FMK during vitrification-warming or during vitrification-warming and incubation on DNA fragmentation and caspase activity are reported in Table 6. Representative pictures are shown in Figure 12. Compared to the control [VOs(-/-)], Z-VAD-FMK-treated VOs had lower percentages of TUNEL-positive oocytes ($p=0.010$), especially those both vitrified-warmed and incubated in the presence of the inhibitor [VOs(+/+)], in which more oocytes had intact DNA than in the other groups ($p=0.005$).

Caspase activity was the lowest in VOs(+/+) ($p<0.001$), while it did not differ between VOs(+/-) and VOs(-/-) ($p=0.588$).

***Table 5**. Activation of apoptotic pathways in vitrified and fresh domestic cat oocytes, assessed as DNA fragmentation (TUNEL-positive oocytes) and caspase activity (from Colombo et al., 2020* [294]*).*

Oocytes	n.	DNA fragmentation n. (%)	Caspase activity Fluorescence intensity mean value ± SD
2hVOs	31	9 (29) [a]	321.7 ± 212.3 [a]
24hVOs	32	19 (59.4) [b]	414.6 ± 326.8 [a]
FOs	31	3 (9.7) [a]	199.6 ± 178.3 [b]
HPOs	33	30 (90.9) [c]	420.1 ± 346.1 [a]

Different superscripts (a,b,c) within the same column indicate significant differences among groups ($p<0.05$).

2hVOs = vitrified oocytes incubated in Medium 199 for 2 hours after warming; 24hVOs = vitrified oocytes incubated in Medium 199 for 24 hours after warming; FOs = fresh oocytes (negative control); HPOs = oocytes treated with hydrogen peroxide to induce apoptosis (positive control).

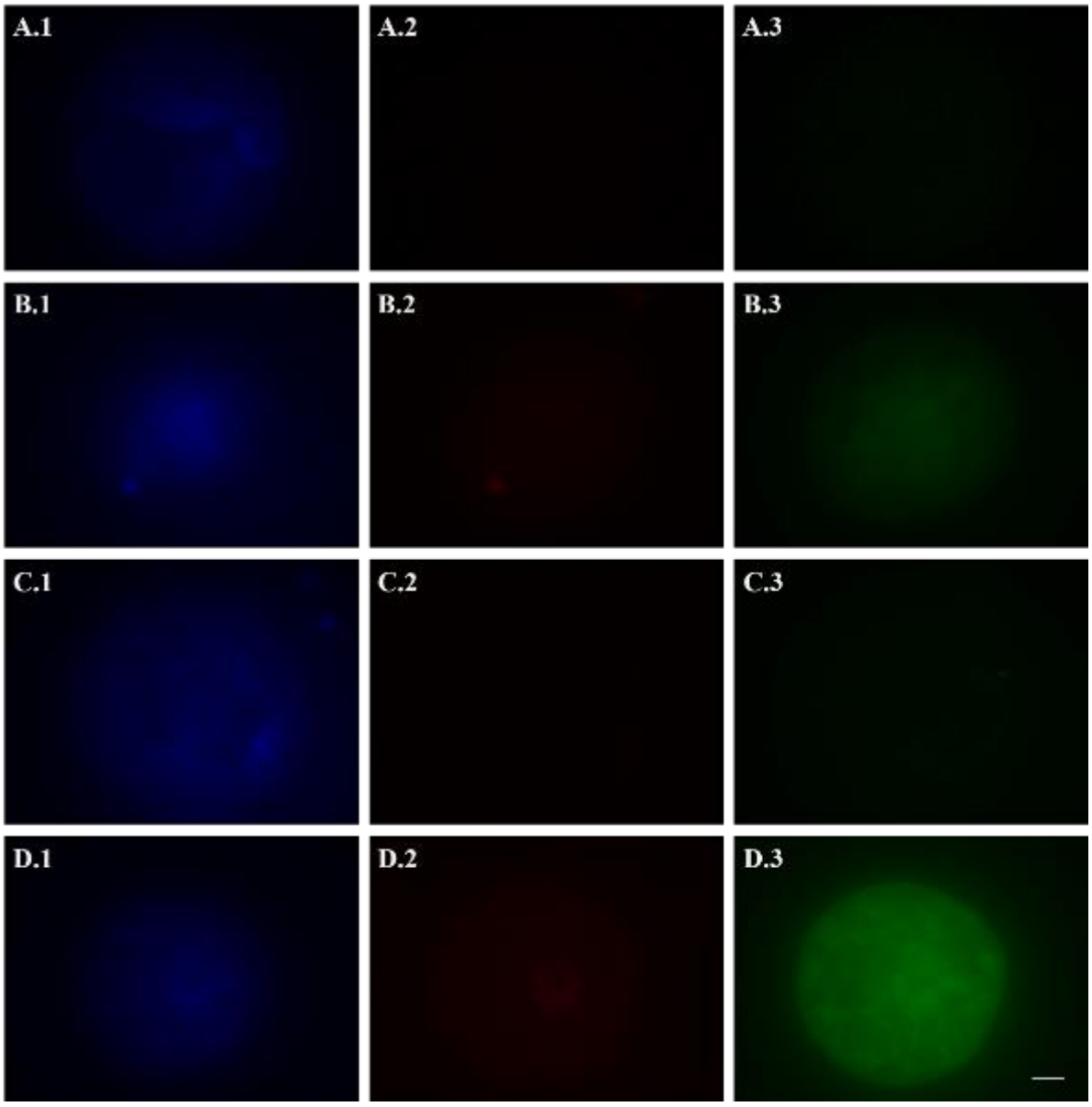

Figure 11*. Representative fluorescence micrographs of (A, B) vitrified, (C) fresh (negative control) and (D) hydrogen peroxide-treated (positive control) domestic cat oocytes stained with (1) Hoechst 33342, (2) TUNEL assay and (3) a caspase activity assay, to assess the activation of apoptotic pathways (from Colombo et al., 2020* [294]*). Vitrified oocytes were stained two (A) or twenty-four (B) hours after warming. Bright blue fluorescence (A.1, B.1, C.1, D.1) indicates the nuclear material. Bright red fluorescence (B.2, D.2) in the nuclear area indicates DNA fragmentation by TUNEL assay. Green fluorescence in the ooplasm (A.3, B.3, C.3, D.3) indicates, according to its intensity, the extent of caspase activity. Images were captured in black and white and pseudo-colored after acquisition with the Imaging Software ZEN 2.5 blue edition. Black and white balance of Hoechst (1) and TUNEL (2) images was adjusted after coloring to make nuclear stainings more visible in print. Caspase images, which were used for quantification, were not modified. Scale bar: 20 µm.*

***Table 6**. Activation of apoptotic pathways in domestic cat vitrified oocytes (VOs), vitrified-warmed and/or incubated after warming with the addition of the pan-caspase inhibitor Z-VAD-FMK, assessed as DNA fragmentation (TUNEL-positive oocytes) and caspase activity (from Colombo et al., 2020* [294]*).*

Oocytes	n.	DNA fragmentation n. (%)	Caspase activity Fluorescence intensity mean value ± SD
VOs(+/+)	34	3 (8.8) [a]	243.7 ± 106.9 [a]
VOs(+/-)	37	14 (37.8) [b]	653.9 ± 591.6 [b]
VOs(-/-)	36	25 (69.4) [c]	434.5 ± 248.3 [b]

Different superscripts (a,b,c) within the same column indicate significant differences among groups ($p<0.05$). VOs(+/+): oocytes vitrified-warmed and incubated for 24 hours with the addition of Z-VAD-FMK; VOs(+/-): oocytes vitrified-warmed with the addition of Z-VAD-FMK and incubated for 24 hours in plain Medium 199; VOs(-/-): oocytes vitrified-warmed and incubated for 24 hours in Medium 199-based media without the addition of Z-VAD-FMK (control).

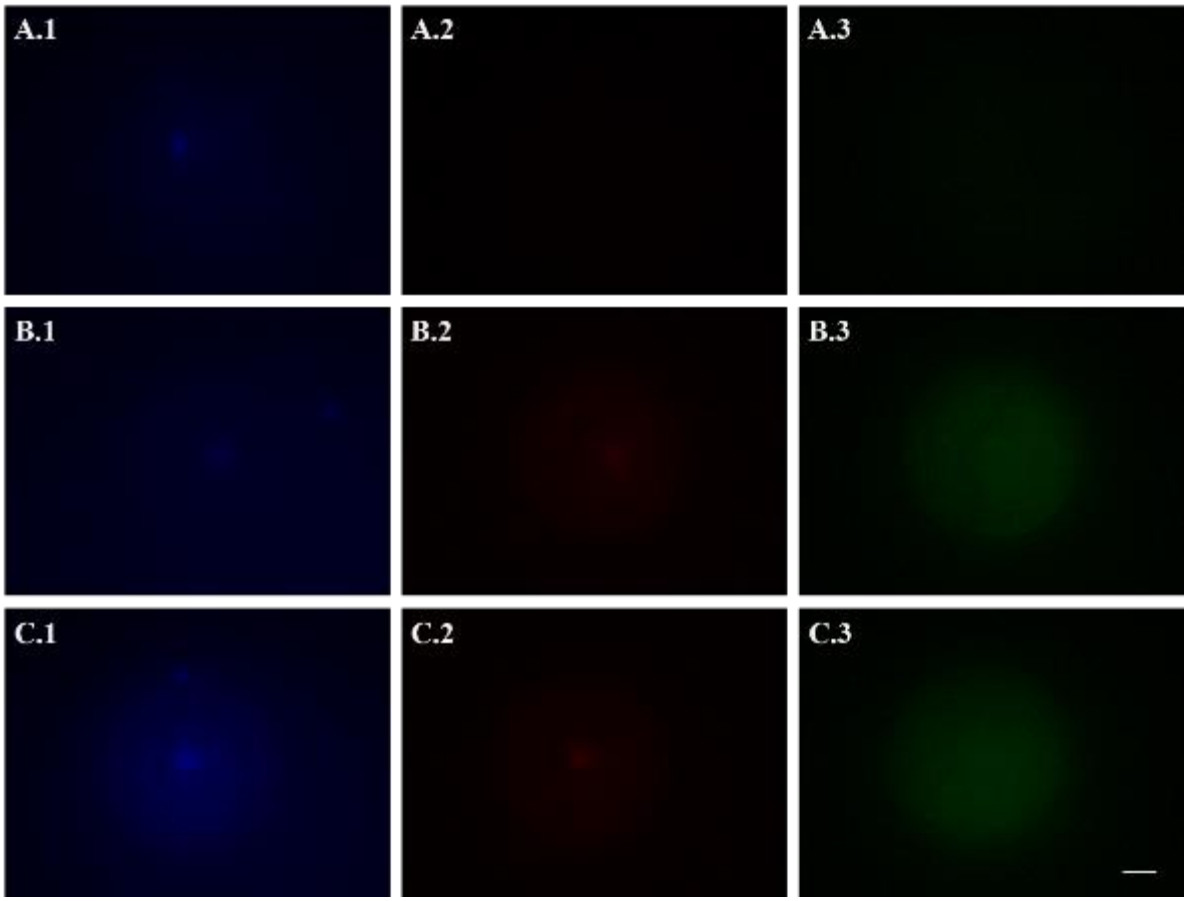

***Figure 12**. Representative fluorescence micrographs of domestic cat vitrified oocytes stained 24 hours after warming with (1) Hoechst 33342, (2) TUNEL assay, and (3) a caspase activity assay, to assess the activation of apoptotic pathways. (A) Oocytes vitrified-warmed and incubated for 24 hours in the presence of the pan-caspase inhibitor Z-VAD-FMK; (B) Oocytes vitrified-warmed in the presence of Z-VAD-FMK and incubated for 24 hours without it; (C) Control oocytes, vitrified-warmed and incubated for 24 hours without Z-VAD-FMK (from Colombo et al., 2020* [294]*). Bright blue fluorescence (A.1, B.1, C.1) indicates the nuclear material. Bright red fluorescence (B.2, C.2) in the nuclear area indicates DNA fragmentation by TUNEL assay. Green fluorescence in the ooplasm (A.3, B.3, C.3) indicates, according to its intensity, the extent of caspase activity. Images were captured in black and white and pseudo-colored after acquisition with the Imaging Software ZEN 2.5 blue edition. Black and white balance of Hoechst (1) and TUNEL (2) images was adjusted after coloring to make nuclear stainings more visible in print. Caspase images, which were used for quantification, were not modified. Scale bar: 20 µm.*

4.3.3. Developmental competence of cat VOs treated with the apoptosis inhibitor Z-VAD-FMK (Paper III, Experiment III)

Based on the results of Experiment II, VOs vitrified-warmed and incubated (i.e. *in vitro* matured) for 24 hours with Z-VAD-FMK [VOs(+/+)] were chosen for the *in vitro* embryo production to assess their developmental ability compared to control VOs(-/-) and FOs. Maturation, fertilization and embryo development rates are summarized in Tables 7 and 8. As expected, maturation rates of control VOs(-/-) were lower than those of FOs ($p=0.004$), while VOs(+/+) had similar rates to both VOs(-/-) ($p=0.384$) and FOs ($p=0.057$). In addition, fertilization did not differ among the three groups ($p=0.082$), as well as cleavage/MII ratios ($p=0.294$). At later stages, embryo development rates did not differ between VOs(-/-) and VOs(+/+) ($p=0.226$ for 5-8 cells stage), but they were always higher in FOs ($p=0.001$). This was especially true for late embryonic developmental stages, which are really challenging to reach for VOs, as confirmed by the inability of both VOs(-/-) and VOs(+/+) to form blastocysts.

***Table 7**. Maturation, fertilization and degeneration rates of vitrified and fresh domestic cat oocytes following in vitro embryo production (from Colombo et al., 2020* [294]*).*

Oocytes	n.	Maturation† n. (%)	Fertilization‡ n. (%)	Deg/N.A. n. (%)
VOs(+/+)	128	68 (53.1) [a,b]	57 (44.5) [a]	45 (35.2) [a]
VOs(-/-)	129	61 (47.3) [a]	53 (41.1) [a]	50 (38.8) [a]
FOs	130	85 (65.4) [b]	68 (52.3) [a]	18 (13.9) [b]

Different superscripts (a,b) within the same column indicate significant differences among groups (p<0.05). Percentages calculated on the total number of oocytes in each group. VOs(+/+): oocytes vitrified-warmed and in vitro matured with the addition of the pan-caspase inhibitor Z-VAD-FMK; VOs(-/-): oocytes vitrified-warmed and matured without the inhibitor Z-VAD-FMK (control); FOs: fresh control oocytes; Deg: degenerated; N.A.: not assessable. † Sum of cleaved embryos, uncleaved fertilized oocytes (i.e. with pronuclei) and unfertilized metaphase II oocytes. ‡ Sum of cleaved embryos and uncleaved fertilized oocytes.

***Table 8**. Embryonic developmental rates of vitrified and fresh domestic cat oocytes following in vitro embryo production (from Colombo et al., 2020* [294]*).*

Oocytes	Matured n.	2-4 cells n. (%)	5-8 cells n. (%)	9-16 cells n. (%)	Morulae n. (%)	Blastocysts n. (%)
VOs(+/+)	68	44 (64.7) [a]	20 (29.4) [a]	6 (8.8) [a]	1 (1.5) [a]	0 (0) [a]
VOs(-/-)	61	41 (67.2) [a]	12 (19.7) [a]	4 (6.6) [a]	1 (1.6) [a]	0 (0) [a]
FOs	85	62 (72.9) [a]	53 (62.4) [b]	42 (49.4) [b]	30 (35.3) [b]	13 (15.3) [b]

Different superscripts (a,b) within the same column indicate significant differences among groups ($p<0.05$).

Percentages calculated on the total number of matured oocytes in each group.

VOs(+/+): oocytes vitrified-warmed and in vitro matured with the addition of the pan-caspase inhibitor Z-VAD-FMK;

VOs(-/-): oocytes vitrified-warmed and matured without the inhibitor Z-VAD-FMK (control); FOs: fresh control oocytes.

5. General discussion

This thesis was aimed at assessing whether the *in vitro* developmental competence of domestic cat VOs could be improved through physical and/or chemical enrichments to the culture microenvironment. Indeed, cryopreservation induces changes in the oocyte *milieu* and several cellular damages, and VOs often degenerate after warming or mature and develop into embryos at very low rates [16]. For this purpose, this project firstly described a vitrification protocol useful to maintain high rates of post-warming viability (**Paper I**), and then explored the effects of a GCs 3D co-culture (**Paper II**) and of an apoptosis inhibitor (**Paper III**) on VOs *in vitro* outcomes.

In **Paper I**, the described vitrification protocol gave high (>90%) post-warming viability rates and mostly maintained oocyte morphological integrity. The protocol is based on the use of laboratory-made media and of a device for minimum volume vitrification (i.e. Cryotop) and should be performed by trained operators, who are probably the most critical factor for the success of the method.

The commercial vitrification device used for these experiments is easy to handle and safe to store [111], and it allows the vitrification volume to be reduced to less than 0.1 µL, providing the chance to reach better cooling and warming rates (-23,000°C/minute and 42,000°C/minute respectively, according to the manufacturer) compared to other devices. For example, Cryoloop (whose structure is similar to a microbiology inoculation loop, made by a ring supporting a film of solution on which the oocytes are loaded) allows good reduction of vitrification volume, but it is fragile and prone to accidental warming. Straws (0.25 mL) or OPSs, instead, cannot reduce the vitrification volume enough, and their loading and unloading is challenging and might lead to oocyte loss [306]. Other supports exist, but they also bring some drawbacks with them, and this is why minimum

volume vitrification with Cryotop is probably the most common choice for oocyte preservation in human ARTs, also thanks to its good outcomes in terms of live birth rate [111]. In addition, the comparison with a completely different oocyte cryopreservation technique, i.e. slow freezing, could also be made, but this also has some disadvantages compared to vitrification. While slow-freezing requires programmable freezers, vitrification is field-feasible, and whilst the former takes around one hour and half to be carried out [20], the vitrification protocol hereby described can be completed in approximately 17 minutes.

On the other hand, vitrification has intrinsic limitations due to the cell exposure to toxic cryoprotectants and to sub-zero temperatures [122], which cause cryoinjuries even when timings and temperatures are controlled. Among cellular damages, some can be evident upon microscopical observation (e.g., cumulus cell loss, alteration of cellular shape and size), while others (e.g. meiotic spindle and DNA alterations, oxidative stress, cytoskeleton damages and zona pellucida hardening) are not [13,119]. Oocytes vitrified following the present protocol are sometimes affected by some morphological alterations at warming, but they are mostly viable. There is no correlation between morphology and viability, which also depend on intracellular injuries [19,20].

Moreover, due to cryoprotectant toxicity [122], compliance with protocol temperatures and timings is crucial, and this is the reason why staff training is required. The publication of this protocol not only showed the efficiency of the method, but it might contribute to reach a higher intra- and inter-laboratory standardization. If performed flawlessly, consistent and repeatable post-warming viability rates will be obtained. Therefore, such Cryotop vitrification protocol was judged suitable to guarantee good VOs survival in the following experiments.

In **Paper II**, 3D follicle-like structures, consisting of functional GCs encapsulated in barium alginate microcapsules, were created, and they proved to be a suitable microenvironment for the IVM of VOs, although without significant improvements in VOs meiosis resumption compared to GCs 2D monolayers or standard 2D microdrops of maturation medium.

Because of the beneficial effects they could give, i.e. physical presence of companion cells and chemical intercellular signalling, co-cultures have been applied during IVM or IVF of immature or matured VOs in pigs and buffaloes [286,287,307], resulting in an enhancement of developmental competence in pigs. However, a 3D co-culture with GCs has never been tested for VOs, even if it could combine the positive influence of companion cells with the structural benefits of 3D scaffolds [200]. Follicular cells have already been cultured in 3D systems in human [239], bovine and swine [238] species, and they were able to support the full maturation of co-cultured oocytes (humans [236] and pigs [237]).

The hormonal determination performed in **Experiment I** revealed that GCs adapted to grow in both 2D monolayers and 3D follicle-like structures, even if secreting E2 and P4 at different extents (i.e. higher concentrations in monolayers). We hypothesized that this might be due to the differences in proliferation and metabolism in cells cultured in 2D or 3D conditions [308], for instance due to the faster cell cycles and to the upregulation of gene expression that occur in monolayers. Instead, GCs in FLS might need more time to proliferate and secrete hormones, and longer cultures could help understanding whether 3D-cultured cells would produce hormone to the same extent, as well as a transcriptomic study could highlight the specific mechanisms and peculiarities of hormone secretion in 2D- and 3D-cultured cells.

Despite this difference, GCs maintained the same secretion pattern in both the culture systems, with a stable E2 secretion and an increase in P4 secretion over time. This was expected, since GCs are known to luteinize spontaneously *in vitro* [309]. In a previous report, it was observed that the luteinization index (i.e. P4/E2 ratio) was lower in 3D FLS than in 2D MONO for bovine and swine GCs [238]. In the present study, the P4/E2 ratio increased more (even if not significantly) in MONO than in FLS, potentially indicating that cat GCs might also behave differently in different culture conditions and that the 3D culture might have hindered their luteinization. The avoidance of cell flattening on 2D surfaces and the maintenance of an *in vivo*-like spatial and physiological organization might better preserve the aromatase activity of 3D-cultured cells, reducing luteinization [310].

The functional GCs were then used as enriched culture condition for the IVM of VOs in **Experiment II**. Follicle-like structures and GCs MONO sustained the maturation of cat VOs, but no differences were observed neither between the systems nor with the GCs-free control (i.e. microdrops of medium). Similar meiosis resumption rates were observed in FLS and MONO, indicating that the differences in steroid secretion between culture systems did not influence meiosis resumption to the GVBD-AI stages, likely because hormones are not the only players in oocyte meiotic progression. Culture length of GCs (2 or 6 days) influenced IVM outcomes. Meiosis resumption was benefitted by a shorter culture (FLS-2d and MONO-2d), while full maturation was enhanced when GCs cultured longer (FLS-6d and MONO-6d) were employed. Degeneration rates, instead, were lower in FLS-2d and MONO-2d. These differences might be due to the different production of signalling molecules, which could dynamically change during GCs culture and could differently influence VOs outcomes.

However, these results were similar to those observed in our previous work on enriched culture conditions for VOs. When a 3D alginate system was used for the IVM, no differences were noticed compared to control 2D microdrops in terms of maturation and embryo development [112]. When this system was enriched with fresh COCs, results were also similar, and the 3D co-culture only seemed to be beneficial as it was the only condition leading to blastocyst development [112].

Taking together the results of our studies on enriched culture conditions for VOs ([112] and **Paper II**), the next step was trying to understand the underlying alterations of VOs to better target them. Therefore, the project developed towards an investigation on the mechanism of vitrification-induced cell death and on its possible inhibition.

In **Paper III**, DNA fragmentation and caspase activity were assessed in VOs, revealing that immature cat oocytes suffer vitrification-induced apoptosis. The subsequent use of the pan-caspase inhibitor Z-VAD-FMK led to a decrease in the same apoptosis markers and brought VOs maturation rates close to those of fresh oocytes, even if it did not influence embryo development.

Apart from the use of enriched culture conditions to improve VOs competence after warming, a strategy to enhance VOs outcomes could be the modification of the vitrification procedure itself. Some studies in the domestic cat model applied this idea, but they obtained contrasting results [21,23,24,113]. Degeneration rates remained significant, and the mechanisms which lead to cell death and poor development are still scarcely investigated [135]. Therefore, we assessed whether vitrification might induce the activation of apoptotic pathways in cat VOs and tested the effects of the apoptosis inhibitor Z-VAD-FMK on two apoptotic markers and on VOs developmental competence,

considering that similar molecules were useful for vitrified embryos [274,275] and mature oocytes [278] in other species.

The main players of apoptosis are caspases, which are proteolytic enzymes usually activated after cleavage by other proteases in a molecular cascade [262]. Based on their function, they can be classified as initiators (e.g. caspases 8 and 9) or effectors (e.g. caspases 3, 6 and 7). Activated caspases 3 and 7 were identified with the staining used in **Experiments I and II**, and they can affect other subcellular structures, including DNA [263,264], whose fragmentation was hereby detected by TUNEL assay.

In **Experiment I**, caspase activity was higher in both 2hVOs and in 24hVOs than in fresh oocytes, while DNA fragmentation increased only in 24hVOs. This might indicate that caspase activity started soon after warming, but a delay occurred before some enzymes reached the nucleus and fragmented DNA to the point it was detectable by TUNEL assay. Previous studies also described high VOs viability right after warming, which then decreased during *in vitro* culture [112,295]. A recent report on cat immature VOs detected an overexpression of apoptosis and DNA repair proteins after 24 hours IVM [134], and therefore agrees with our findings.

Following the detection of apoptotic activation in cat VOs, we assessed the effects of a molecule (i.e. Z-VAD-FMK) which is known to act on some components of the apoptotic cascade (i.e. caspases) and partially inhibit apoptosis. Z-VAD-FMK binds to the catalytic sites of caspases and hinders their activity.

In **Experiment II**, it was noticed that the addition of Z-VAD-FMK only during the vitrification-warming procedure was not enough to avoid DNA damage, while its further addition during 24 hours incubation reduced caspase activity and DNA damage, bringing them to values which were numerically similar to those observed for FOs in the previous experiment. We hypothesized that this difference between VOs(+/+) and VOs(+/-) could

be owed to the length of exposure to the inhibitor. While in VOs(+/+) the presence of Z-VAD-FMK lasted more than 24 hours, VOs(+/-) were only exposed to the inhibitor for vitrification-warming phases, which lasted only a little more than 25 minutes considering that molecular interactions are negligible during liquid nitrogen storage, and this was probably not enough to benefit VOs. Consequently, since the longest incubation with Z-VAD-FMK demonstrated the strongest apoptosis inhibitory power, it was chosen for the following experiment to give to VOs the best chances of development.

In **Experiment III**, VOs cleaved at encouraging rates (>30%) compared to those obtained in other studies [16], suggesting that the combination of our Cryotop vitrification protocol with our IVM-IVF-IVC protocol was a good choice for the IVEP from cryopreserved cat oocytes. Nevertheless, there were no differences between VOs(+/+) and VOs(-/-), which also developed to late embryo stages to similar, and equally poor, rates (about 1.5% morulae, 0% blastocysts). Z-VAD-FMK, though, was beneficial for maturation rates, which were similar in VOs(+/+) and FOs. This probably indicated that the effect of the inhibitor lasted as long as it was present, and no long-lasting effects were developed to prevent later degeneration of VOs (i.e. after IVF). In cats, another molecule targeting a different component of apoptotic pathways was previously tested, and it improved the fertilization competence of VOs [135], unlike Z-VAD-FMK. The blockage of ROCK, a kinase activated during apoptosis [311] which is also involved in cytoskeleton organization and therefore in oocyte viability and competence [312] was performed in [135], while in this study Z-VAD-FMK was used to broadly inhibit caspases, which are mostly involved in cell death and inflammation, and this could explain the differences in IVEP results.

Looking at **Experiments II and III** together, it was easy to notice that in VOs(+/+) there was not any relation between the decrease in DNA fragmentation and caspase activity and the developmental competence. It is hard to distinguish healthy and dying

cells, and no threshold exist. Neither the fluorescent staining hereby used, nor other methods (e.g. immunohistochemistry, RT-PCR) can supply an absolute quantification of caspase activity, so the evaluation of DNA integrity might better predict the developmental potential of VOs. Yet, although there were differences between VOs(+/+) and VOs(-/-) for what concerns both caspase activity and DNA fragmentation, their embryo development was comparable. Much more than DNA integrity is needed for embryo development, and probably here lies the reason for impaired VOs development. This observation, though, could also lay the foundations for other investigations on VOs developmental competence.

Overall, these observations suggest that, although Cryotop vitrification was efficient in guaranteeing high post-warming viability rates and gave reproducible and consistent results, improvements in the post-warming development of cat immature VOs are still strongly needed. Even if mature oocytes better develop into embryos [115], immature oocyte vitrification is a crucial germplasm preservation technique. Besides often being the most commonly available source of gametes, immature oocytes can be preserved even in the absence of a specialized laboratory and dedicated equipment, and they should be the focus of more investigations.

Future perspectives of our studies on immature oocyte vitrification may include further developments of physico-chemical enrichments on both the culture microenvironment and the vitrification procedure itself. As previously discussed, in an attempt to find a better IVM co-culture system, different GCs culture lengths could be tested, and cell molecular features could be analyzed to fully comprehend their metabolic and steroidogenic functionality, understand how their secretions change during culture and how they could influence oocyte meiosis resumption. Besides alginate, other 3D

scaffolds, made of different biomaterials such as agarose, hyaluronan and collagen, which already proved their suitability for the *in vitro* culture of reproductive cells [313] could be exploited, as well as microfluidic systems for the creation of dynamic conditions.

On the chemical side, instead, considering the positive effect of Z-VAD-FMK on maturation outcomes, other inhibitors against different targets could be tested to assess whether they have a better influence. Their use both during IVM and IVC should be considered to have long-lasting effects and to improve embryo development to late stages, which remains the most critical challenge for VOs. However, this choice should be made carefully since it could alter the delicate balance of embryo growth. Indeed, growing embryos need apoptosis to control the selection of healthy blastomeres [314] and since the effects of inhibitors on embryos is unknown, their survival after transfer to recipient animals should also be investigated.

In a wider view, the developmental competence of immature VOs could be improved also in other ways. For instance, in-depth analysis of molecular alterations induced by vitrification by transcriptomic or metabolomic approaches, or further studies of subcellular structures by electron microscopy might help identify sensitive spots and give new ideas to contrast oocyte cryoinjuries. In addition, more attention should be given to the development of VOs to late embryo stages, which was not well investigated, so far. After fertilization, cellular divisions begin, in a complex and well-coordinated fashion [315], but we ignore whether vitrification-induced damages affecting the oocytes might be inherited by the embryos. A recent study seemed to support this hypothesis, since it showed that mice embryos derived either from fresh or cryopreserved mature oocytes had different transcriptomic profiles [316]. DNA, membranes, and cytoskeleton are also affected by vitrification, and their functional integrity in the blastomeres of nascent embryos should be assessed. This could help to understand how to design better strategies

to enhance the developmental competence of cat VOs. Finally, automated (microfluidic) systems for vitrification might allow a thorough control of cryoprotectant addition/removal and temperature decrease/increase, reducing osmotic stress and volume changes [317]. Hopefully, soon these systems will reduce cellular damages to a minimum and give optimal chances of development to cryopreserved oocytes.

6. Conclusions

Vitrified immature oocytes are an accessible and valuable source of genetic material to preserve the fertility potential of humans, valuable domestic animals, and wild, endangered species. Nonetheless, cold-induced damages, such as the loss of supporting cumulus cells, lead to the impairment of physiological, biochemical, and molecular abilities of such cryopreserved oocytes to resume meiosis and properly develop into embryos *in vitro*.

In this project, a vitrification protocol based on the minimum volume approach with the Cryotop device was employed. Physico-chemical enrichments to the vitrification protocol or to the post-warming culture environment were proposed to improve VOs developmental competence.

Minimum volume vitrification with the Cryotop protocol gave good, consistent and repeatable results in terms of VOs post-warming viability, which allowed its use in following studies (**Paper I**).

Cat granulosa cells could successfully be cultured in 3D follicle-like structures, as well as in 2D monolayers, and maintained their steroidogenic ability. These systems, used as enriched culture *milieu* for the IVM of VOs, allowed meiosis resumption, even if without differences with the standard IVM conditions (**Paper II**).

A possible reason for VOs degeneration was determined. Vitrification induced an increase in DNA fragmentation and caspase activity, which could be reverted with the addition of the pan-caspase inhibitor Z-VAD-FMK during vitrification-warming and the following 24 hours of culture. Z-VAD-FMK brought the maturation capacity of treated VOs close to that of fresh control oocytes, but it did not influence embryo development (**Paper III**).

Further studies to assess more in depth the molecular mechanisms involved in the onset of cryoinjuries and in VOs post-warming development will support the design of new strategies for cryopreservation and culture of immature cat oocytes. The application of customized enriched culture systems or chemical inhibitors should be considered, as well as innovative systems such as automated vitrification.

Hopefully, these studies will contribute to enhance the efficiency of cat oocytes vitrification and foster the creation of feline gamete biobanks for biodiversity preservation.

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Paper I

Minimum volume vitrification of immature feline oocytes

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Paper II

Granulosa cells in three-dimensional culture: A follicle-like structure for domestic cat vitrified oocytes

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Paper III

Inhibition of apoptotic pathways improves DNA integrity but not developmental competence of domestic cat immature vitrified oocytes

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12. Appendix

Links to co-authored publications:

- Nuclear competence and genetic expression of growth differentiation factor-9 (GDF-9) of canine oocytes in 3D culture
  https://doi.org/10.1111/rda.13336
- The never-ending search of an ideal culture system for domestic cat oocytes and embryos
  https://doi.org/10.1111/rda.13331
- Developmental competence of domestic cat vitrified oocytes in 3D enriched culture conditions
  https://doi.org/10.3390/ani9060329
- Morphological indices for canine spermatozoa based on the World Health Organization laboratory manual for human semen
  https://doi.org/10.1111/rda.13440
- Reproductive technologies in companion animals *(book chapter)*
  https://doi.org/10.1016/b978-0-12-817107-3.00009-6
- Cold case: Small animal gametes cryobanking
  https://doi.org/10.1016/j.theriogenology.2020.02.047
- Ovary cold storage and shipment affect oocyte yield and cleavage rate of cat immature vitrified oocytes
  https://doi.org/10.1016/j.cryobiol.2020.11.003
- Maturation and fertilization of African lion (*Panthera leo*) oocytes after vitrification
  https://doi.org/10.1016/j.cryobiol.2020.11.011