Reproduction in Domestic Animals

Reprod Dom Anim **47** (Suppl. 6), 266–268 (2012); doi: 10.1111/rda.12040
ISSN 0936–6768

Cryosurvival of *Ex Situ* and *In Situ* Feline Oocytes

GC Luvoni

Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale, e la Sicurezza Alimentare, Università degli Studi di Milano, Milano, Italy

Contents
Cryosurvival of feline oocytes preserved as isolated cells (*ex situ*) or enclosed in ovarian follicles (*in situ*) has been demonstrated, and significant advances have recently been achieved. However, an ideal protocol for oocyte cryopreservation has not been established to date because of extreme sensitivity of the structural complex to chilling injury. Several factors, such as stage of maturation, membrane permeability and plasticity of the cytoskeleton, affect cryosurvival of the oocyte. Also, intercellular communications between cumulus cells and oocyte are compromised after freezing or vitrification of *ex situ* or *in situ* cumulus–oocyte complexes, which has a detrimental effect on oocyte maturational competence. Despite these issues, embryo development, pregnancies and live kittens have been obtained after *in vitro* fertilization (by ICSI) and transfer of embryos derived from cryopreserved oocytes. It is a general belief that the efficiency of cryopreservation would increase through a better understanding of oocyte responses to cryoprotectants, cooling rates and all the physical events occurring during the exposure of feline oocytes to low temperatures. Cryobanking of feline oocytes would significantly contribute to the preservation of rare genotypes and to the maintenance of a valuable source of genetic material for research applications.

Introduction

Significant advances in feline oocytes cryopreservation have recently been achieved.

Even though the oocyte is considered more susceptible to cooling damage than embryos and spermatozoa, recent results demonstrate that banking of cryopreserved female gametes is an attainable goal.

In felids, as well as in other mammals, oocyte cryopreservation would significantly contribute to the improvement in assisted reproductive technologies aimed to the preservation of biodiversity. Besides the advantages in animal conservation, the use of animal models, such as the domestic cat, provides the opportunity for further investigations of the principles of oocyte cryobiology, which can help to improve current technologies applied to both humans and animals. A review of the literature regarding survival of feline oocytes cryopreserved as isolated cells (*ex situ*) or enclosed in the ovarian follicles (*in situ*) is provided.

Ex situ oocytes

The first report on cryopreservation of *ex situ* feline oocytes was published by Luvoni et al. (1997). Cryosurvival of germinal vesicle (GV) stage oocytes was demonstrated as resumption of meiosis and maturation to the metaphase II (MII) stage after thawing and culture. The results indicated that 1.5 M dimethyl sulphoxide (DMSO) or ethylene glycol (EG) were both suitable cryoprotectants for slow freezing of immature cat oocytes.

Conversely, rapid cooling of feline GV oocytes did not allow resumption of meiosis *in vitro* after warming, despite maintenance of oocyte morphology. The occurrence of subcellular freezing damage, which was not revealed by morphological evaluation, negatively affected nuclear maturation.

Ex situ oocytes may be cryopreserved at the MII stage rather than at the GV stage. However, the need for preventive hormonal treatment of the female to cause oocyte maturation or *in vitro* culture makes MII stage oocytes less readily accessible than immature (GV stage) oocytes that are now preferred for cryobanking, although their cryosurvival is not as efficient as expected.

The absence of the fragile meiotic spindle and the presence of a nuclear membrane surrounding decondensed chromatin might favour higher cryosurvival of immature oocytes as compared to mature oocytes; however, it has been shown that development *in vitro* after fertilization of thawed oocytes was enhanced when MII oocytes were slow frozen with 1.5 M EG (Luvoni and Pellizzari 2000).

Following these demonstrations, there have been further reports on cryosurvival of feline oocytes at different maturational stages and, in addition, vitrification has been applied to feline oocytes. Different carrier tools, including straws, OPS (Open Pulled Straws) and Cryoloop, have been tested for vitrification of *ex situ* oocytes, and embryo development after *in vitro* fertilization of mature (Murakami et al. 2004; Merlo et al. 2008) and immature oocytes (Comizzoli et al. 2009; Cocchia et al. 2010) has been achieved.

Recently, the first pregnancy was established in a recipient receiving embryos derived from vitrified immature oocytes (Tharasanit et al. 2011), but foetal resorption occurred during mid-gestation. The commercial introduction of innovative cryodevices, such as the Cryotop, prompted further investigations and, very recently, the first kittens, were born after vitrification of mature oocytes, ICSI and transfer of derived embryos into recipients (Pope et al. 2012).

Cryosurvival of *ex situ* feline oocytes has been widely documented, but the sensitivity of oocytes at different maturational stages to cryopreservation deserves further investigation.

It is important to point out that immature oocytes are selected for freezing based on their morphology, but a 'normal' appearance that does not guarantee maturational competence. Meanwhile, MII oocytes can be considered as cells with higher intrinsic developmental ability.

Furthermore, viability and competence of immature oocytes largely depends on preserving structural and functional integrity of the cumulus–oocyte complex.

Several layers of cumulus cells surround the immature oocyte, and the functional coupling between somatic and germinal compartments, other than between cumulus cells, is essential to the transfer of nutrients and messenger molecules during oocyte growth and maturation.

In feline immature oocytes, hyperosmotic conditions (Comizzoli et al. 2008) and freezing/vitrification (Luciano et al. 2009) caused a decrease in cumulus–oocyte gap junction-mediated communication, which was detrimental to oocyte competence. Different size, structure and permeability of the oocyte and cumulus cells make it difficult to determine a cryopreservation procedure that is suitable for both cellular types. Additionally, the oocyte itself is particularly vulnerable to cold injury. The exposure to low temperatures can cause an irregular distribution of cytoskeletal elements, as demonstrated in feline GV oocytes following slow freezing or vitrification (Luciano et al. 2009). In mature oocytes, cryodamage of cytoskeleton results in disassembly of the meiotic spindle apparatus that is associated with dispersion of metaphase chromosomes.

Microtubules and microfilaments of the cytoskeleton play an important role during the maturation and fertilization processes. Tubulin is involved in the formation of the meiotic spindle driving the alignment of chromosomes on the metaphase plate, while actin microfilaments are necessary for cortical granule migration and polar body expulsion, both crucial mechanisms for appropriate fertilization.

Cytoskeleton disorganization is only one portion of the subcellular damage not revealed by morphological evaluation of thawed/warmed oocytes. Alterations of DNA, cellular membranes and intracellular organelles might also occur during cryopreservation.

Attempts to stabilize the cytoskeleton system and the nucleus of GV oocytes prior to cryopreservation have been made using cytochalasin B to increase cytoskeleton flexibility, and resveratrol, an enhancer of histone deacetylase activity to promote DNA compaction and decrease GV volume. It has been reported that cytochalasin B mitigates the deleterious effect of extreme hyperosmotic conditions on cumulus–oocyte communications (Comizzoli et al. 2008), while the GV chromatin compaction induced by resveratrol is beneficial to survival of feline oocyte during vitrification (Comizzoli et al. 2009).

However, an ideal protocol for cryopreservation of *ex situ* oocytes has not yet been established. The cryotolerance of the gamete is affected by several factors (stage of maturation, membrane permeability, plasticity of the cytoskeleton) that impair its functional integrity after thawing/warming.

In situ oocytes

Interest in banking of mammalian ovarian tissue has increased progressively over the last several years.

The preservation of functional interactions between cells, follicular integrity and enclosed (*in situ*) oocytes is the main goal of the cryopreservation of ovarian cortex. However, cryopreservation of tissue is generally more demanding than that of oocytes because of the presence of several cellular types with differing cryotolerance. Ovarian tissue is rich in primordial and primary follicles, and transplantation of fragments of thawed ovarian cortex can result in follicular and oocyte development. Follicular development to antral stages, although at low rates, was obtained following xenografting of cortex fragments from frozen/thawed domestic cat ovaries into SCID mice (Bosch et al. 2004). These results provided the initial demonstration that cat ovarian cortex could survive cryopreservation. Freezing of cat ovarian tissue (Lima et al. 2006) or small preantral follicles (Jewgenow et al. 1998) resulted in the preservation of follicular morphology and in the survival of enclosed (*in situ*) oocytes, respectively.

Ovarian cortex contains a population of antral follicles and healthy immature oocytes that can be retrieved from cryopreserved tissue and matured/fertilized *in vitro*.

We firstly demonstrated that feline immature oocytes retrieved from vitrified ovarian tissue (*in situ* oocytes) maintain the capability of resuming meiosis after warming (Luvoni et al. 2012). Evaluation of cytoskeletal and chromatin organization after vitrification indicated that damage to cytoskeleton occurred mainly to actin rather than to the tubulin network, but GV morphology was not altered. Also, integrity of follicles, irrespective of developmental stage, was maintained after vitrification. Sufficient permeation of the whole tissue fragments by cryoprotectants is crucial for their cryopreservation. In our experiment, very small ovarian fragments (1.5–2 mm^3) were vitrified with a combination of cryoprotectants (DAP 213 solution: 2 M DMSO, 1 M acetamide and 3 M propylene glycol). The rationale for the use of more than one cryoprotective agent in vitrification protocols is that a combination of different cryoprotectants increases the viscosity of the solution, a physical condition needed for the development of a glass-like state, and, thereby, reduces their toxic effect on the cells.

Recent findings on comparative cryosurvival of *ex situ* and *in situ* feline oocytes demonstrated that viability can be preserved using different procedures of vitrification (DAP 213 and Cryotop). Their ability to resume meiosis after warming indicated that oocytes vitrified *ex situ* or *in situ* have comparable cryotolerance; however, the incidence of oocytes with complete layers of viable cumulus cells was remarkably decreased after warming and after culture in both vitrification procedures as compared to fresh oocytes (Alves et al. 2012).

These findings further confirm that cumulus cells are highly susceptible to cryopreservation and their integrity is poorly preserved. Hence, an enriched culture system optimized to support maturation of oocytes deprived of cumulus cells by cryopreservation would be advisable.

Conclusions

Cryopreservation of feline oocytes started in 1997, and the birth of kittens from vitrified oocytes has been obtained this year (Pope et al. 2012). This success will give further support to continue research aimed at developing procedures for cryopreservation of *ex situ* and *in situ* oocytes that will provide consistently high rates of survival.

Protocols applied to feline oocytes were generated empirically from those previously developed for other species. Criticism has been expressed about the empirical approach of cryopreservation, but it is unquestionable that it has allowed the achievement of significant progress.

A further impetus to the research would be the combination of empirical and theoretical knowledge. A better understanding of oocyte response to cryoprotectants, cooling rates and all the physical events occurring during exposure to low temperatures could greatly contribute to improving the efficiency of cryopreservation. Preservation of rare genotypes and maintenance of valuable sources of genetic material for research applications will hopefully be the culmination of all the efforts.

Conflicts of interest

None of the authors have any conflicts of interest to declare.

References

Alves AE, Kozel AC, Luvoni GC, 2012: Vitrification with Cryotop and DAP213 of *ex situ* and *in situ* feline cumulus-oocyte complexes. Reprod Domest Anim doi: 10.1111/j.1439-0531.2012.02006.x.

Bosch P, Hernandez-Fonseca HJ, Miller DM, Wininger JD, Massey JB, Lamb SV, Brackett BG, 2004: Development of antral follicles in cryopreserved cat ovarian tissue transplanted to immunodeficient mice. Theriogenology **61**, 581–594.

Cocchia N, Ciani F, Russo M, El Rass R, Rosapane I, Avallone L, Tortora G, Lorizio R, 2010: Immature cat oocyte vitrification in open pulled straws (OPSs) using a cryoprotectant mixture. Cryobiology **60**, 229–234.

Comizzoli P, Wildt DE, Pukazhenthi BS, 2008: Impact of anisosmotic conditions on structural and functional integrity of cumulus–oocyte complexes at the germinal vesicle stage in the domestic cat. Mol Reprod Dev **75**, 345–354.

Comizzoli P, Wildt DE, Pukazhenthi BS, 2009: In vitro compaction of germinal vesicle chromatin is beneficial to survival of vitrified cat oocytes. Reprod Dom Anim **44** (Suppl 2), 269–274.

Jewgenow K, Penfold KL, Meyer HHD, Wildt DE, 1998: Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. J Reprod Fertil **112**, 39–47.

Lima AK, Silva AR, Santos RR, Sales DM, Evangelista AF, Figueiredo JR, Silva LD, 2006: Cryopreservation of preantral ovarian follicles in situ from domestic cats (*Felis catus*) using different cryoprotective agents. Theriogenology **66**, 1664–1666.

Luciano AM, Chigioni S, Lodde V, Franciosi F, Luvoni GC, Modina SC, 2009: Effect of different cryopreservation protocols on cytoskeleton and gap junction mediated communication integrity in feline germinal vesicle stage oocytes. Cryobiology **59**, 90–95.

Luvoni GC, Pellizzari P, 2000: Embryo development in vitro of cat oocytes cryopreserved at different maturation stages. Theriogenology **53**, 1529–1540.

Luvoni GC, Pellizzari P, Battocchio M, 1997: Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. J Reprod Fertil Suppl **51**, 93–98.

Luvoni GC, Tessaro I, Apparício M, Ruggeri E, Luciano AM, Modina SC, 2012: Effect of vitrification of feline ovarian cortex on follicular and oocyte quality and competence. Reprod Dom Anim **47**, 385–391.

Merlo B, Iacono E, Regazzini M, Zambelli D, 2008: Cat blastocysts produced in vitro from oocytes vitrified using the cryoloop technique and cryopreserved electroejaculated semen. Theriogenology **70**, 126–130.

Murakami MK, Otoi T, Karja NWK, Wongsrikeao P, Agung B, Suzuki T, 2004: Blastocysts derived from in vitro-fertilized cat oocytes after vitrification and dilution with sucrose. Cryobiology **48**, 341–348.

Pope CE, Gòmez MC, Kagawa N, Kuwayama M, Leibo SP, Dresser BL, 2012: In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. Theriogenology **77**, 531–538.

Tharasanit T, Manee-In S, Buarpung S, Chatdarong K, Lohachit C, Techakumphu M, 2011: Successful pregnancy following transfer of feline embryos derived from vitrified immature cat oocytes using "stepwise" cryoprotectant exposure technique. Theriogenology **76**, 1442–1449.

Submitted: 31 May 2012; Accepted: 10 Jul 2012

Author's address (for correspondence): Gaia Cecilia Luvoni, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy. E-mail cecilia.luvoni@unimi.it