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New Approaches of Ovarian Tissue Cryopreservation from Domestic Animal Species

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1. Introduction

Cryopreservation has been attempted for most of the developmental stages of both male and female reproductive cells, ranging from the immature gametes residing in ovarian or testicular tissues through the mature oocytes and spermatozoa.

However, each variant of the reproductive cells has introduced their own problems, and it has been realized that many aspects of the particular physiology of the cells will dictate how they respond to cryopreservation. Both male and female gametes have acquired highly specialized structural components (essential to fertilization and development) that may respond to the freezing process in ways different from that of basic cell structures.

1.1 Cryopreservation of spermatozoa

Semen is one of the most practical means of storing germplasm due to its abundant availability and ease of application (Holt and Pickard, 1999; FAO, 2007). Stored spermatozoa could be introduced back into existing populations either immediately or decades or centuries afterwards. In this way, cryopreservation of spermatozoa associated with artificial insemination (AI) or *in vitro* fertilization (IVF) facilitates the management of domestic animals herds, especially cow dairy herds where it is now used since 60 years. Cryopreservation better allows the use of semen from genetically superior males of threatened livestock breeds and has the potential to protect existing diversity and maintain heterozygosity while minimizing the movement of living animals and the transmission of venereal diseases (Johnston and Lacy, 1995; Andrabi and Maxwell, 2007).

Spermatozoon is a very small cell containing low amounts of cytoplasm and consequently low quantity of water. Furthermore sperm nuclear material is compacted and protected

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against physical injuries. For these reasons, cryopreservation of spermatozoa gives excellent results in term of viability and fertility, and is today widely used in human and animal assisted reproduction.

Semen from most mammalian and a few avian species has been successfully frozen in the past several years (FAO, 2007). Indeed, much better results have been obtained with sperm cryopreservation than with oocytes (and embryos) in term of viability. For these reasons, sperm cryobanking is used since the middle of the last century in domestic animal species and lately in human. Numerous storage facilities such as the French National Cryobank were also created for the preservation of valuable or endangered species. However, protocols currently used to conserve semen are still suboptimal and cannot be easily applied across species (Woods et al., 2004). First-service conception rates vary drastically between different breeding programs, but on average conception rates are fairly high in cattle, pigs, goats, and sheep. In wild species cryopreservation of gametes is currently used to preserve endangered species or breeds, and to overcome some fertility troubles. Breed reconstruction solely from semen is possible through a series of back-cross generations; however, the entire genetics of the original breed will not be recovered (Boettcher et al., 2005).

In human, gametes cryopreservation has also been developed to overcome fertility troubles (eg. genital duct problems, sexual dysfunction ...). Sperm cryopreservation that has produced live birth has been available for over 50 years (Sherman and Bunge, 1953). Anonymous donor sperm banking has been a fundamental concept of reproductive medicine for several decades, and artificial insemination and donor sperm cryobanking are widely used in reproductive medicine centers (Critser, 1998). The availability of frozen donor sperm has become a mainstay for the treatment of serious male infertility worldwide. More recently, gametes cryopreservation has been used to preserve fertility of men (or women) submitted to gonadotoxic treatments and elective sperm cryopreservation programs have been provided from cancer patients all over the world. Using vitrification of sperm obtained from testicular biopsy, epididymal fluid, or a semen sample after electroejaculation could create new hope for infertile men (Edge et al., 2006).

1.2 Cryopreservation of testicular tissue

Cryopreservation of testicular tissue has been studied since about ten years in animals (Jezek et al., 2001; Jahnukainen et al., 2007; Milazzo et al., 2008; Zeng et al., 2009; Abrishami et al., 2010; Milazzo et al., 2010; Curaba et al., 2011) and human (Bahadur and Ralph, 1999; Bahadur et al., 2000; Guerin, 2005; Revel and Mejia, 2010). It is the only available solution for pre-pubertal boys who must receive a gonadotoxic treatment (eg. cancer therapy; (Keros et al., 2005; Keros et al., 2007; Wyns et al., 2007; Wyns et al., 2008; Wyns et al., 2010).

In contrast to the situation in the ovary, it is well established that spermatogonial stem cells exist in the testis and are responsible for maintaining spermatogenesis from puberty for the lifetime of the male. Human testicular cells might be harvested, cryopreserved before a gonadotoxic treatment and re-introduced into the testis upon its completion. Possibilities include transplantation back into the inactive testes (ipsigenic germ cell transplantation), maturation *in vivo* in another host (xenogenic germ cell transplantation), or *in vitro* spermatogenesis. Mature sperm could then be used for fertilization by ICSI.

Sperm obtained after stem cell transplantation were shown to be able to fertilize mouse oocytes. Fertile offspring were obtained through artificial reproductive technologies following the establishment of complete spermatogenesis by grafting testis tissue from newborn mice, pigs, or goats into mouse host (Honaramooz et al., 2002; Schlatt et al., 2002). Different freezing protocols have been developed in several species but without a clearly identified procedure (Travers et al., 2011). Testicular tissue from prepubertal boys facing gonadotoxic treatment could be banked for several years for spermatogonial stem cell transplantation. Pregnancies have been achieved with ICSI using immature spermatids and secondary spermatocytes extracted from testicular tissue in men with spermatogenic arrest (Fishel et al., 1997; Vanderzwalmen et al., 1997; Sofikitis et al., 1998).

1.3 Cryopreservation of oocytes

Oocyte cryopreservation offers many advantages. It permits to preserve endangered species and low effective breeds, and to preserve fertility of high genetic value females. In human it permits to overcome some fertility problems. Oocyte banks would also enlarge the gene pool, facilitate several assisted reproductive procedures, salvage female genetics after unexpected death, and avoid controversy surrounding the preservation of embryos (Ledda et al., 2001; Checure and Seidel, 2007). Like semen, oocyte cryopreservation is beneficial for international exchange of germplasm, as it avoids injury and sanitary risks involved in live animal transportation (Pereira and Marques, 2008). But oocyte cryopreservation gives much lower results when compared with spermatozoa.

This is due essentially to the much important size and complexity of oocytes. For example, nuclear material is much more exposed in prophase I or metaphase II oocyte than in compacted chromatin of sperm cells. Oocytes collected from slaughterhouse derived ovaries are at the germinal vesicle (GV) stage in which the genetic material is contained within the nucleus. Since this stage has no spindle present, GVs are assumed to be less prone to chromosomal and microtubular damage during cryopreservation. However, oocytes can also be cryopreserved at the metaphase II stage of maturation. During the metaphase II stage, the cumulus cells surrounding the oocyte are expanded, microfilaments of actin are involved in cell shape and movements, and microtubules form the spindle apparatus (Massip, 2003). Moreover, cryopreservation of oocytes necessitates the success of the following steps: *in vitro* maturation, *in vitro* fertilization and embryo culture. Progress in female gametes cryopreservation has gone hand in hand with that for *in vitro* maturation and embryo culture. In livestock animals, oocytes collected by *in vivo* pickup or at slaughter can be frozen for extended periods of time for subsequent IVF to produce embryos. However, in some species such as *canidae* the collection of oocytes is difficult and *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo culture are not yet under control (Luvoni et al., 2006). For these reasons precise cooling and thawing rates and the use of a programmable cell freezer are necessary for oocyte (and also embryo) cryoconservation and very few studies have been conducted in animals.

Oocytes are extremely sensitive to chilling, and the technique is not as established as in semen or embryos, due to the fact that oocytes typically have a low permeability to cryoprotectants (Woods et al., 2004). The major differences between oocytes and embryos are the plasma membrane, presence of cortical granules, and spindle formation at

metaphase II stage of meiosis (Chen et al., 2006; Salvetti et al., 2010). To date, there has been no consistent oocyte cryopreservation method established in any species, although, there has been significant progress and offspring have been born from frozen-thawed oocytes in cattle, sheep, and horses (Otoi et al., 1996; Maclellan et al., 2002; Woods et al., 2004). During the process of cryopreservation, oocytes suffer considerable morphological and functional damage, although, the extent of cryoinjuries depends on the species and the origin (*in vivo* or *in vitro* produced). The mechanism for cryoinjuries is yet to be fully understood, and until more insight is gained, improvement of oocyte cryopreservation will be difficult. Also, it is noticeable that immature oocyte present in primordial follicles seems more resistant to cryopreservation when compared to mature oocyte. Consequently, cryopreservation of ovarian cortex may constitute an interesting alternative to isolated mature oocyte cryopreservation.

1.4 Cryopreservation of ovarian tissue

At birth, the ovaries contain the lifetime complement of primary oocytes which are arrested in the prophase stage of meiosis 1 and are surrounded by a single-layered epithelium to form the primordial follicles. Ovarian cortex presents several advantages when compared with isolated oocytes: (i) it contains the important pool of growing follicles; (ii) it does not necessitate the *in vitro* maturation /*in vitro* fertilization /embryo culture steps if it is associated with grafting; (iii) no previous ovarian stimulation is necessary. Consequently cryopreservation of ovarian cortex is an alternative to cryopreservation of isolated oocytes or embryos. It could be used as an emergency preservation and as infertility therapy method for valuable animals. Ovarian cortex cryopreservation has been developed in human in order to preserve fertility in young women submitted to gonadotoxic therapy (Stahler et al., 1976; Gook et al., 2004). In human newborns were obtained after orthotopic autograft of frozen-thawed ovarian cortices (Donnez et al., 2004).

It is obvious that, to achieve successful cryopreservation of ovarian tissue, it is essential to maintain the functional status of the whole mixture of different cell types: oocytes, granulosa cells, epithelial cells, fibroblasts... This represents a major difficulty, because the optimum kinetic of cooling is different for each cell type. Oocytes are large cells, with a low surface to volume ratio, surrounded by zona pellucida. Immediately adjacent to the oocyte are corona radiata cells that have long cytoplasmic extensions which penetrate the zona pellucida, ending in oocyte membrane. These processes and gap junctions are important in the metabolic cooperation between the oocyte and surrounding layers of granulosa cells, which form the cumulus-oocyte complex during the growth phase. Consequently, at the opposite to cryopreservation of isolated cells, a cryopreservation protocol for a tissue represents a compromise between the requirements of the different constitutive cells.

The early work on ovarian tissue cryopreservation was performed in animal studies: rabbit (Smith, 1952) and rat (Parkes and Smith, 1953; Deanesly, 1954). The earliest positive results were obtained when glycerol (15%) plus serum were used as cryoprotective agents (CPAs) for cryopreservation of rabbit granulosa cells, via a slow cooling protocol (Smith, 1952). An equilibration period was necessary to achieve CPA penetration into the tissue. For this reason small samples were recommended. A rapid rewarming by plunging the samples into a water bath at 40°C was the most effective procedure (Parkes and Smith, 1953; 1954). Normal offspring were obtained from mice with orthotopic ovarian grafts of tissue that had

been frozen and stored at -79°C (Parrott, 1960). Vitrification of ovarian tissue was also investigated. Nevertheless, Isachenko et al suggested that in human, low freezing protocols were more promising than vitrification protocols (Isachenko et al., 2009).

This technique has also been developed in rabbit (Neto et al., 2007a), mouse (Candy et al., 1997), rat (Aubard et al., 1998), ewe (Gosden et al., 1994; Demirci et al., 2001), cow (Paynter et al., 1999). We have obtained newborn rabbits after autografting of cryopreserved ovarian cortex (Neto et al., 2007b). Also, our team developed this technique in cat (Neto et al., 2006) and dog (Commin et al., 2011).

Several techniques have been applied to ovarian cortex cryopreservation: slow freezing, vitrification. Simultaneously to ovarian tissue cryopreservation, numerous researches have been conducted about ovarian tissue grafting: orthotopic, heterotopic, auto-, allo- and hetero-grafting (Pullium et al., 2008).

2. Development of optimized methods for the cryopreservation of the ovarian tissue in domestic mammalian species

The most common cryopreservation method is the slow freezing procedure, consisting of an initial slow, controlled-rate cooling to subzero temperatures followed by rapid cooling as the sample is plunged into liquid nitrogen for storage (-196°C). At such a low temperature, biological activity is effectively stopped, and the cells functional status may be preserved for centuries. However, several physical stresses damage the cells at these low temperatures. Intracellular ice formation is one the largest contributors to cell death; therefore, freezing protocols use a combination of dehydration, freezing point depression, supercooling, and intracellular vitrification in an attempt to avoid cell damage.

Currently used ovarian cortex cryopreservation protocols have been direct, or slight modifications of the methods developed for isolated oocytes and embryos. There were primarily developed by trial and error adjustments of cooling and warming rates, and choice of CPA and CPA concentrations. However, because there are a large number of protocol variables potentially affecting cell viability, an exhaustive experimental search for the optimal combination of these parameters has long been considered to be prohibitively expensive in terms of time and resources.

2.1 Chemical and physical parameters affecting equilibration and freezing processes of ovarian tissue in mammalian species

The result of a cryopreservation process is influenced by several chemophysical parameters affecting directly or not the functions and the integrity of the ovarian cells along the freezing process, from the equilibration to the thawing. Among these parameters, the method of equilibration, the freezing rate, the composition of the freezing solution and notably the nature of the permeating CPAs and the non-permeating CPAs, the concentration of each CPA, the use of serum, or the rate of thawing may be investigated to know the relative influence of each of them and the induced cell injuries.

In general, we can expect coupled flows of water and CPAs when CPAs are added, during freezing, thawing and when CPAs are removed from the cells, resulting in a series of

anisosmotic conditions. During freezing, the cells dehydrate and shrink and remain shrunken during storage, but return to their isosmotic volume upon thawing. Finally, the cells are subjected to potentially lethal swelling upon CPA dilution and removal. During the controlled slow cooling extracellular ice formation is induced (seeding) at a temperature just below the solutions' freezing point, and then the cooling continues at a given rate in the presence of a growing extracellular ice phase, which raises the extracellular solute concentration in the unfrozen fraction and results in water being removed from the cell via exosmosis.

Permeating CPAs, such as glycerol, dimethyl sulfoxide, ethylene glycol or propylene glycol are typically included in the cryoprotective medium, to protect the cells against injury from the high concentrations of electrolytes that develop as water is removed from the solution as ice. During the equilibration step the inner cell water is partly replaced by the permeating CPAs. However, the CPAs can be damaging to the cells, especially when it is used at high concentrations. The toxicity can be reduced by decreasing the time or the temperature of the equilibration step (Karlsson and Toner, 1996). But equilibration at low temperatures requires increasing the exposition time to freezing solution. Furthermore, the CPAs may have dramatic osmotic effects upon the cells during their addition and their removal. Consequently, the use of several steps of increasing concentrations of CPAs during the equilibration allows reducing the osmotic gradient. The cells exposed to such permeating CPAs undergo initial dehydration, followed by rehydration, and potential gross swelling upon removal. This osmotic shock may generate membrane damages by mechanical means and predisposition of the cell to injuries during the other steps of cryopreservation, or even cell death (Mazur and Schneider, 1986). These kinds of damages could be reduced by using cells surfactant such as serum. During the freezing step, the follicular preservation depends on the nature and the concentration of the CPAs.

Control of the cooling and warming rates is also crucial, as the freezing/thawing rates and the temperature of seeding also influence the ice properties. If cells are cooled too rapidly during the controlled slow cooling process, water does not exit the cells fast enough to maintain equilibrium and, therefore, the oocytes and other ovarian cells freeze intracellularly, resulting in death in most cases. If cooling is too slow, the long duration can cause 'solution effects' injury resulting from the high concentration of extra- and intracellular solutes, probably due to the effects of the solutes on the cellular membrane or through osmotic dehydration. During warming the small intracellular ice crystals might subsequently undergo recrystallization, forming bigger ice crystals that rupture the cell membrane, thus leading to fatal damage. Finally, the thawing and the removal of the CPA depend on the temperature and on the presence of non-permeating CPA limiting the osmotic swelling during rinsing.

2.2 Use of fractional experimental design

The influence of the multiple chemical and physical parameters cannot be exhaustively performed as it would require too much time and resources. Even if the number of factors, k , in a design is small, the 2^k runs specified for a full factorial can quickly become very large. For example, $2^5 = 32$ runs is for a two-level, full factorial design with five factors. To this design we would need to add a good number of centerpoint runs and we could thus quickly run up a very large resource requirement for runs with only a modest number of factors.

Moreover, while the approach is sequential in nature, it is potentially increasing in complexity as the knowledge and understanding of the application and domain evolves. Design of experiments techniques provides a systematic, effective and efficient approach to the investigation of a phenomenon. The main advantages of this strategy were the saving in times and resources expended compared to other approaches and the resulting mathematical models that helps us to better understand the phenomena under investigation more fully. To analyze the response of ovarian cortices from different species to the freeze/thaw process, the authors decided to use fractional (2^{n-p}) experimental designs (Mechakra et al., 1999).

Using this statistical tool, the authors used only a fraction of the runs specified by the full factorial design; which runs to make and which to leave out was one of our subjects of picked. The authors used various strategies that ensure an appropriate choice of the runs. As for an example, fractional experimental designs $2^{(5-2)}$ presented in table 1 aim to evaluate the combined effect of five different factors according to two modalities for each of them. For each experimental design, eight combinations of factors were performed. For each of them, the ratio of morphological preservation and the ratio of viability of isolated preantral follicles were recorded. While the designs were similar for each of the species that were evaluate, the parameters were chosen according to each species (Table 2 to Table 5).

Run	Variable						
	I	X ₁	X ₂	X ₃	X ₄	X ₅	X ₁ .X ₂
1	+1	-1	-1	-1	+1	-1	+1
2	+1	+1	-1	-1	+1	+1	-1
3	+1	-1	+1	-1	-1	+1	-1
4	+1	+1	+1	-1	-1	-1	+1
5	+1	-1	-1	+1	-1	+1	+1
6	+1	+1	-1	+1	-1	-1	-1
7	+1	-1	+1	+1	+1	-1	-1
8	+1	+1	+1	+1	+1	+1	+1

Table 1. Fractional experimental design $2^{(5-2)}$ used to evaluate the cryopreservation protocols in the doe rabbit, in the queen and in the cow

Variables	Level -1	Level +1
X ₁ : Permeating CPA	DMSO	PROH
X ₂ : Concentration of permeating CPA	1.5M	2M
X ₃ : Non permeating CPA	trehalose	sucrose
X ₄ : Freezing rate	0.3°C/min	2°C/min
X ₅ : Equilibration	1 step	3 steps

Table 2. Dependent variable list evaluated in the rabbit doe

These $2^{(n-2)}$ experimental designs allowed discriminating between five factors influencing the cryopreservation process (variables X_1 to X_5) and the simultaneous interactions between two of them. The linearity (structural and estimated model) of the experimental model was evaluated by an ANOVA test. One randomly chosen assay was replicated three times to estimate the experimental error (E).

So, eleven experiments were randomly performed. Multi-linear regression was performed using all the variables in order to evaluate experimental results according to this model:

$$\hat{y} = \beta_0 + \beta_1.X_1 + \beta_2.X_2 + \beta_3.X_3 + \beta_4.X_4 + \beta_5.X_5 + \beta_{1,2}.X_1.X_2 + E$$

Variables	Level -1	Level +1
X1: Permeating CPA	PROH	DMSO
X2: add of sucrose	no	yes
X3: Freezing rate	0.5°C/min	2°C/min
X4: manual seeding	no	Yes
X5: Freezing rate after -40°C	In the freezing chamber	Direct immersion in LN ₂

Table 3. Dependent variable list evaluated in the queen

Variables	Level -1	Level +1
X1: Permeating CPA	PROH	DMSO
X2: Concentration of permeating CPA	1.5M	2.5M
X3: Non permeating CPA	sucrose	trehalose
X4: Medium	Euroflush®	Medium with choline
X5: Cell surfactant	Fetal calf serum	Albumax®

Table 4. Dependent variable list evaluated in the cow

Variables	Level -1	Level +1
X1: Permeating CPA	DMSO	PROH
X2: Non permeating CPA	trehalose	sucrose
X3: Freezing rate	0.3°C/min	2°C/min
X4: Equilibration	1 step	3 steps

Table 5. Dependent variable list evaluated in the bitch

Results of experimental designs for each species were completed by at least one additional biological evaluation and one quantitative evaluation of normal and viable follicle rates after freezing, according to the best combination of factors chosen with experimental designs.

2.3 Criteria to assess the quality of frozen-thawed cortices

A survey of nearly all quality assays available to the preservation scientist reveals that they can be grouped into different categories. The following assay tier is not specific to

cryopreservation, but is presented below as a support that can guide those who work with preserved tissues. The authors decided to assess the quality of the protocols developed in different species using all or part of alternate test presented below. The morphology and the viability of the ovarian follicles were systematically assessed, in combination with the investigation of the ultrastructure of the follicles, and their capacity to resume folliculogenesis after graft.

2.3.1 Morphology and ultrastructure

Assessment of the morphology of ovarian cells required the ovarian fragments were fixed in a preliminary defined fixative agent adapted to the species, before being processed for classical light microscopy. Primordial to primary follicles – from oocytes surrounded by flattened granulosa cells until oocytes surrounded by one layer of cuboidal granulosa cells (Gougeon and Chainy, 1987) – were usually classified into four types of morphological defects: (*Type I*) follicle without any morphological defect - follicle is regular, with joined follicular cells; cytoplasm of the oocyte is homogeneous and chromatin is diffused and regular; (*Type II*) follicle with cytoplasmic defect - cytoplasm of the oocyte is vacuolated or eosinophil; (*Type III*) follicles with nuclear defect - nucleus of the oocyte is picnotic, without apparent nuclear membrane or with an irregular nuclear membrane; (*Type IV*) degenerated follicle – oocyte with combined cytoplasmic and nuclear defects or follicle with irregular shape or with disjoined follicular cells or with swelled follicular cells.

The ultrastructure of ovarian follicles was also examined for the presence of apoptotic and para-apoptotic cell death. Ultrastructurally apoptosis is characterized by margination of condensed chromatin, nuclear fragmentation, and the formation of apoptotic bodies. Para-apoptosis, nonclassical apoptosis, is a specific morphologic type of non-necrotic cell death and is characterized by cytoplasmic vacuolization, condensed chromatin (but not early margination of the chromatin), and swollen mitochondria.

2.3.2 Cytolysis live/dead assay

The cytolysis assays have both a very positive and a negative attribute to them. On the positive side, there are a variety of assays that can reveal cell membrane leakage that occurs as a final stage in most forms of cell death. Yet given that cytolysis is the last stage of preservation-induced cell death, these assays do not reveal early-stage mechanisms underlying preservation-induced cell death and thus have limited use in the future as a diagnostic means to develop improved preservation formulations and protocols. The LDH assay continues today to be useful for measuring preservation-induced cytotoxicity. The concept behind this cytolysis assay is simply that if the cell membrane is compromised, then LDH will leak into the extracellular milieu where it can be measured. The trypan blue assay is also one of the most commonly used cytolysis assays. A number of investigators has used the trypan blue exclusion assay in studying preservation efficacy. It does however share the same handicap as the LDH assay given that neither can be analyzed using fluorescence and/or bioluminescence. Currently, the best cytolysis live/dead assays are those that employ fluorescent indicator dyes. Available probes can be subdivided into two different subsets, one of which is trapped by the cell and leaks out only if a membrane rupture occurs. The other subtype, exemplified by ethidium homodimer or propidium iodide, is membrane insoluble and only stains the cell if it gains access through a compromised

plasma membrane. In this way, the authors did evaluate the viability of the frozen-thawed follicles using Calcein-AM and Ethidium homodimer I stains (Live/Dead® Viability/Cytotoxicity Kit, Molecular Probes) on enzymatically isolated follicles (Fig. 2).

2.3.3 In vivo functionality (autografting model) and in vivo growth potential (xenografting model)

One of the key components of any preservation protocol must be a functional assay that matches the type of cells or tissue being analyzed. In some cases this determination is quite easy. As for example, the sperm motility is a well-accepted functional assay for this system, whereas the capacity to resume folliculogenesis sounds reasonable for the ovarian cortex.

Fresh and cryopreserved rabbit doe ovarian tissues were autografted into young females. Fresh ovarian tissue was grafted on the ipsilateral ovarian pedicle immediately after ovary resection (2 grafts per female), contrary to cryopreserved ovarian tissue grafted on the controlateral ovarian pedicle 24 hours after freezing (one graft per female). Control females were ovariectomised, according to the same surgery resection that used before graft. From height week after graft, grafted rabbit doe were inseminated every 3 weeks in case of negative pregnant diagnosis. Eleven months later, ovarian grafts were removed at necropsy to observe follicular structures by histology.

In the bitch, the growth ability of frozen-thawed ovarian tissue has also been assessed by implantation of small pieces of ovarian tissue into adult female SCID mice. After removing mice ovaries, the canine frozen thawed ovarian tissue was placed intramuscularly in the gluteal superficial muscle. To study the setting up of graft resumption, the graft was harvested at one, eight or 16 weeks and the resumption of the ovarian activity was controlled by vaginal cytology assessment. After harvesting, the grafts were processed for histological assessment.

2.3.4 Vascularization

In the bitch, the alpha smooth muscle actin (alpha-SMA), a marker of the mature blood vessel, was used to assess the vessel density within the ovarian tissue, using a primary antibody directed against alpha-SMA. For each slice, image analysis was performed under direct light microscope to determine the tissue areas. The stained vessels were counted in several fields of the same slice and in several slices per animal to deduce a blood vessel density.

2.4 Experimental results

To illustrate the interest of the use of fractional experimental design, the authors decided to present some of their results obtained in the doe rabbit, in the queen, in the bitch and in the cow during the last 6 years. The doe rabbit was used as a model for the human and the animal applications of the ovarian tissue cryopreservation, because of its biological and breeding characteristics. The cat was considered as a model for the ovarian tissue cryopreservation studies of endangered wild felids; from all the felids, the domestic cat is the only non endangered feline species. The cow was used as model for ruminants, with a special interest for preserving high valuable individuals in combination to embryos and to

semen cryopreservation. Finally, the bitch was studied for preserving the reproductive potential of future guide dogs submitted to neutering surgery before training.

2.4.1 In the doe rabbit

The experimental variability expressed as the repetition of one single combination, showed that both the structural and the estimated models of the experimental design were valid when considering the morphological preservation ratio of the follicles. The concentration of the permeating CPA ($P = 0.67$) and the number of equilibration steps ($P = 0.19$) seemed to have no significant effect on the morphological preservation ratio of ovarian follicles. The nature of the permeating and non-permeating CPA seemed to influence the morphological preservation ratio of the follicles ($P = 0.08$ and $P = 0.07$ respectively) although the non-significant difference. DMSO tended to reduce the morphological preservation ratio, as compared with PROH. Morphological preservation ratio was increased in the presence of trehalose compared with sucrose. The freezing rate seemed to be the factor that had the greatest impact on the morphological preservation ratio of the doe rabbit follicles. At a freezing rate of $0.3^{\circ}\text{C}/\text{min}$ we observed a significant increasing of the follicular morphological preservation ratio, as compared with $2^{\circ}\text{C}/\text{min}$ ($P < 0.01$). No significant interaction was observed between the nature of the permeating CPA and its concentration.

According to the results of the experimental design, the precise evaluation of the best combination of factors influencing positively the morphological preservation ratio (3 steps equilibration protocol, 1.5M DMSO or 1.5M PROH, medium supplemented with either sucrose or trehalose) was performed. Ovarian pieces were treated according to the results obtained with experimental design. Ovarian cortices were equilibrated (3 steps) in the freezing media based on TCM 199 and 10% FCS, at room temperature. The freezing media was supplemented with 1.5 M DMSO or 1.5M PROH and 0.2 M sucrose or 0.2M trehalose. Freezing of ovarian fragments was slowly performed at $0.3^{\circ}\text{C}/\text{min}$ from the temperature of seeding ($-7^{\circ}\text{C}/\text{min}$) up to -35°C . Thawing, histology, viability tests and electron microscopy evaluation process were performed before and after cryopreservation as described previously.

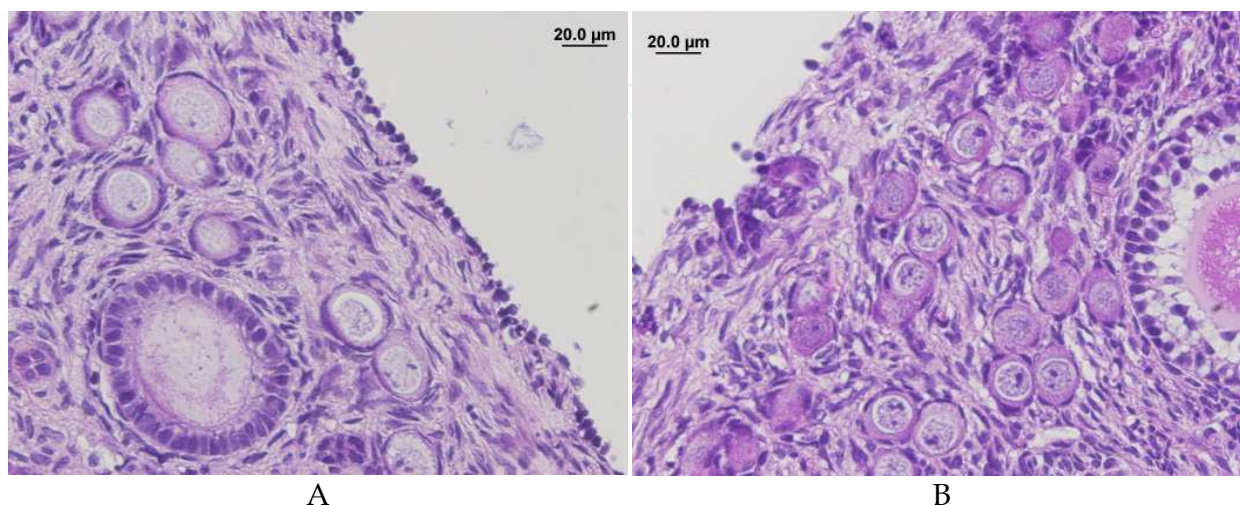


Fig. 1. Rabbit follicular morphology before (A) and after (B) cryopreservation with PROH and trehalose, with a post-seeding freezing rate at $0.3^{\circ}\text{C}/\text{min}$

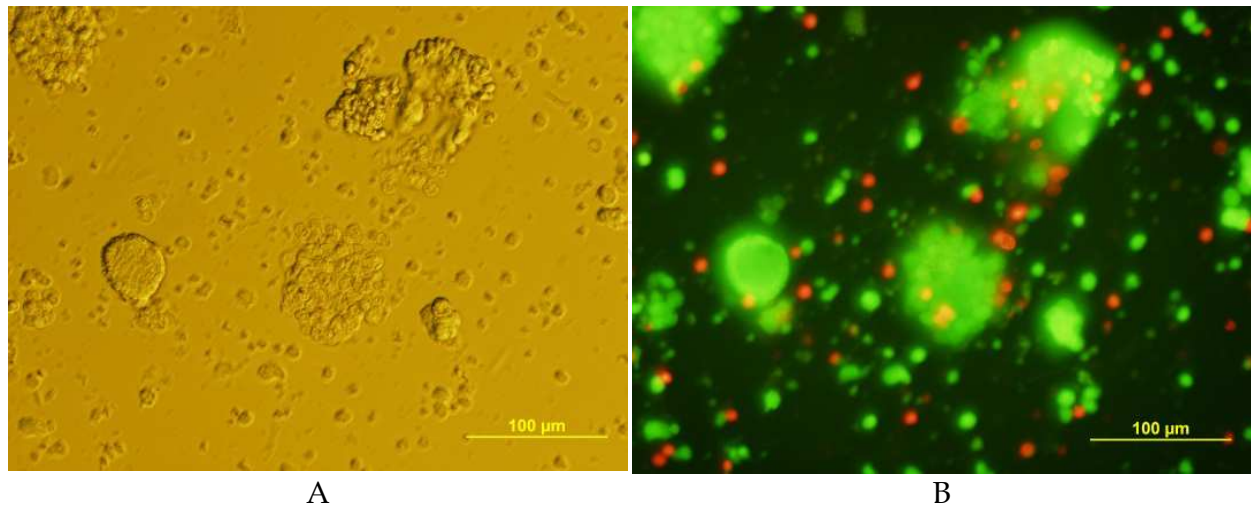


Fig. 2. View of rabbit isolated follicles under direct light for selection (A) and under fluorescent light (B) after calcein AM/ethidium homodimer I stains to evaluate viability after cryopreservation with PROH, with a post-seeding freezing rate at $0.3^{\circ}\text{C}/\text{min}$

In control fragments, we observed $72.6 \pm 2.8\%$ and $77.7 \pm 3.9\%$ of type I follicles (no significant difference) for sucrose and trehalose control groups respectively. After cryopreservation, no statistical difference of the proportions of type I follicles was found between sucrose and trehalose ($50.2 \pm 4.1\%$ vs. $51.1 \pm 1.8\%$ respectively) when using DMSO for cryopreservation. When using PROH as permeating CPA, the proportion of type I follicles was lower after cryopreservation with sucrose as compared to trehalose ($55.0 \pm 3.8\%$ vs. $65.0 \pm 3.3\%$ respectively; $P < 0.05$). When freezing with trehalose the proportion of type I follicles was higher with PROH as compared to DMSO ($65.0 \pm 3.3\%$ vs. $51.1 \pm 1.8\%$ respectively; $P < 0.01$). Nevertheless, the proportions of type I follicles were significantly reduced after cryopreservation (from $P \leq 0.01$ to $P < 0.001$), whatever the permeating and the non-permeating CPA. No significant difference was observed between the different groups of frozen ovarian cortices, when considering the morphological preservation ratio.

According to these results, the cryopreservation protocol based on a post-seeding freezing rate at $0.3^{\circ}\text{C}/\text{min}$ and using a freezing medium composed of 1.5M PROH, supplemented with 0.2M trehalose was finally evaluated by orthotopic autografting to observe the potential of the cryopreserved follicles to resume follicular growth and to be fertilized.

Before freezing, type II follicles represented the most important part of follicles with morphological defect ($19.1 \pm 2.9\%$ and $16.1 \pm 3.2\%$ in sucrose and trehalose groups respectively). After cryopreservation, follicular defect of type IV (degenerated follicles) was the most important type of morphological defect: $32.5 \pm 4.8\%$ and $24.0 \pm 1.9\%$ after freezing using DMSO, with sucrose and trehalose respectively; versus $27.2 \pm 5.6\%$ and $18.1 \pm 3.0\%$ after freezing using PROH, with sucrose and trehalose respectively. The general aspect of ovarian tissue before and after cryopreservation showed a good preservation of structural architecture (follicular structure and connective tissue). Spaces were observed in some case,

in the ovarian stroma and the albuginea. Epithelial cells were often absent as compared to the fresh ovarian tissue.

Ultrastructural analysis of the preantral follicles was performed without preliminary selection on semi-thin sections. TEM analysis showed that most follicles of control ovarian tissue had normal ultrastructure, according to mitochondria, nucleus and nuclear membrane, Golgi apparatus and endoplasmic reticulum cisternae observation. They often had vacuoles in cytoplasm and vesicles. Nevertheless, vacuoles were not characteristic of apoptosis. Cellular membranes of the oocyte and follicular cells were in close connection. In general, ovarian stroma was well organised. Fibroblasts and collagen fibres were distinguishable (Fig. 3).

After cryopreservation, oocyte ultrastructure appeared to be similar to the control especially for mitochondria, Golgi apparatus, endoplasmic reticulum, interdigital structure between oocyte and follicular cells (Fig.3). Vesicles and vacuoles were rarely observed. Chromatin of the oocyte was diffused and well preserved. Nevertheless, dark follicular cells or follicular cells without any content were most frequently observed, whereas some follicles showed partial or total disruption of their nuclear membrane whatever the evaluated cryoprotective solution. The most important damage observed after cryopreservation was the disorganisation of the ovarian stroma (Fig.3). Fibroblasts showed lack of cytoplasm or important vacuolisation. In general, these damages were less frequently observed after cryopreservation using PROH and trehalose.

2.4.2 Investigations in the queen

The experimental variability showed that neither the structural, nor the estimated models of the experimental design were valid when considering the morphological preservation ratio of the follicles or the viability preservation ratio. So, global discrimination of the chemo-physical parameters was not possible. Nevertheless, the influence of the freezing rate after seeding and after -40°C , and the influence of the addition of sucrose in the freezing medium composed of 1.5M CPA were evaluated and analyzed by classical ANOVA test.

Before freezing, ovarian tissue presented $72.2 \pm 3.6\%$ and $83.8 \pm 2.9\%$ of normal follicles (type I) for group $2^{\circ}\text{C}/\text{min}$ and $0.5^{\circ}\text{C}/\text{min}$ post-seeding freezing rate respectively. When freezing with PROH, and whatever the post-seeding freezing rate, proportions of morphologically normal follicles were not significantly reduced after freezing compared to before freezing ($69.2 \pm 9.1\%$ for $2^{\circ}\text{C}/\text{min}$ group vs. $67.4 \pm 2.9\%$ for $0.5^{\circ}\text{C}/\text{min}$ group). After freezing with DMSO, and whatever the post-seeding freezing rate, proportions of type I follicles were significantly reduced ($40.8 \pm 6.6\%$ after freezing at $2^{\circ}\text{C}/\text{min}$ and $51.6 \pm 5.1\%$ after freezing at $0.5^{\circ}\text{C}/\text{min}$; $P < 0.05$). Whatever the post-seeding freezing rate, type III defects were the most frequently observed after freezing. General observation of the ovarian tissue showed a good preservation of the ovarian stroma cells and structure after cryopreservation.

Before freezing, ovarian tissue submitted to a free fall into the freezing chamber during the third phase of the freezing process presented $72.2 \pm 3.6\%$ of type I follicles without any

difference with samples directly immersed into the liquid nitrogen ($86.8 \pm 2.5\%$). Proportion of normal follicles decreased significantly after cryopreservation except after freezing with PROH according to a free fall into the freezing chamber ($68.2 \pm 9.1\%$ of type I). After freezing using a direct immersion into liquid nitrogen after -40°C , and whatever the CPA, proportions of type I follicles were decreased compared to fresh control.

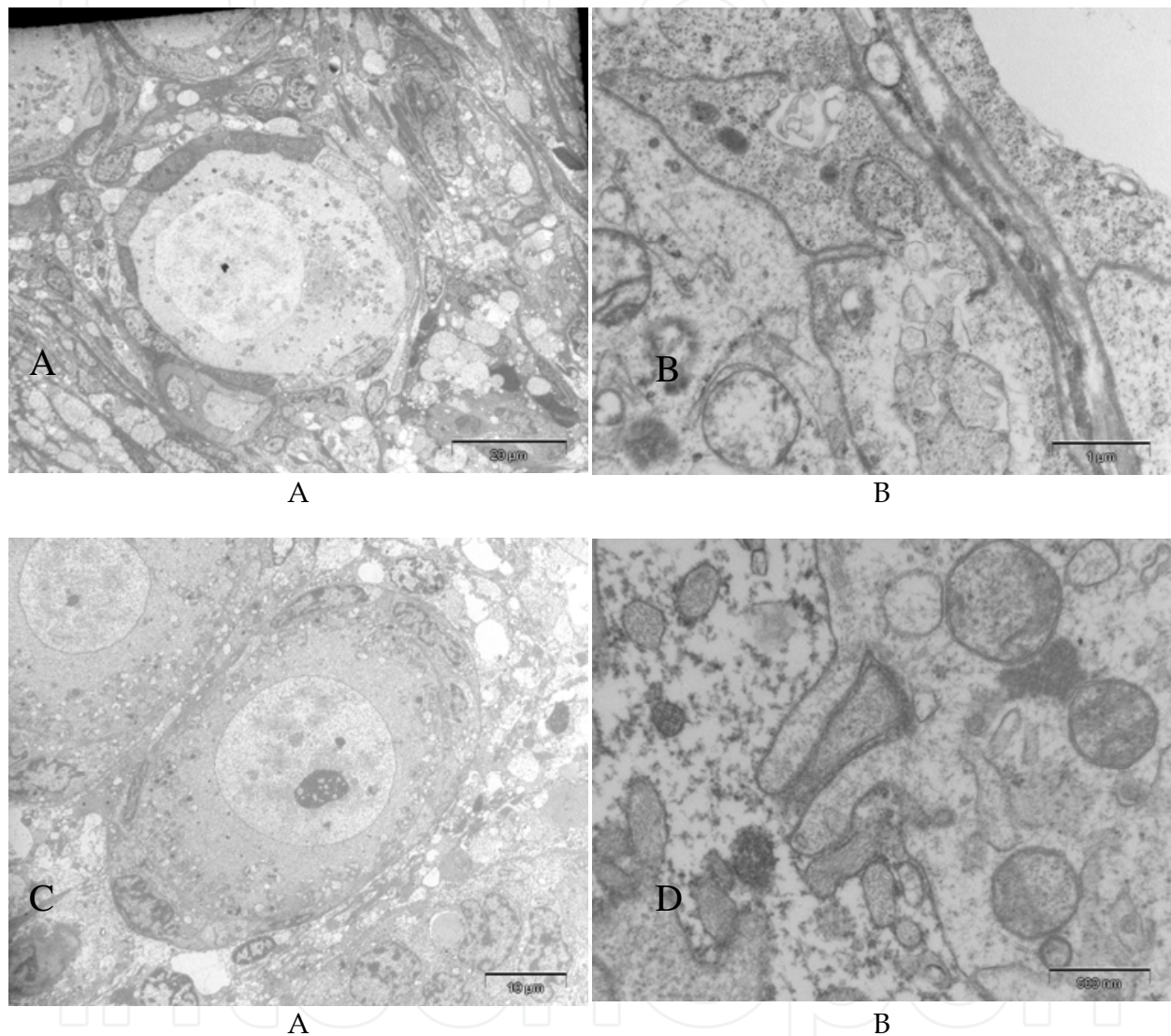


Fig. 3. Rabbit follicular ultrastructure before (A & B) and after (C & D) cryopreservation with PROH and trehalose, with a post-seeding freezing rate at $0.3^\circ\text{C}/\text{min}$

Before freezing, queen ovarian tissue showed $72.2 \pm 3.6\%$ and $74.8 \pm 6.3\%$ of type I follicles respectively for group without and with sucrose without any difference between the two control groups. After freezing, addition of sucrose allowed preserving $63.2 \pm 13.6\%$ of normal follicles versus $68.2 \pm 9.1\%$ without sucrose when associated with PROH, without any significant difference. Contrary to the results after freezing with DMSO, proportion of type I follicles was not significantly different after freezing with PROH compared to fresh

control. Morphological defect of type III was the most frequently observed after freezing with PROH. Queen ovarian stroma was well preserved.

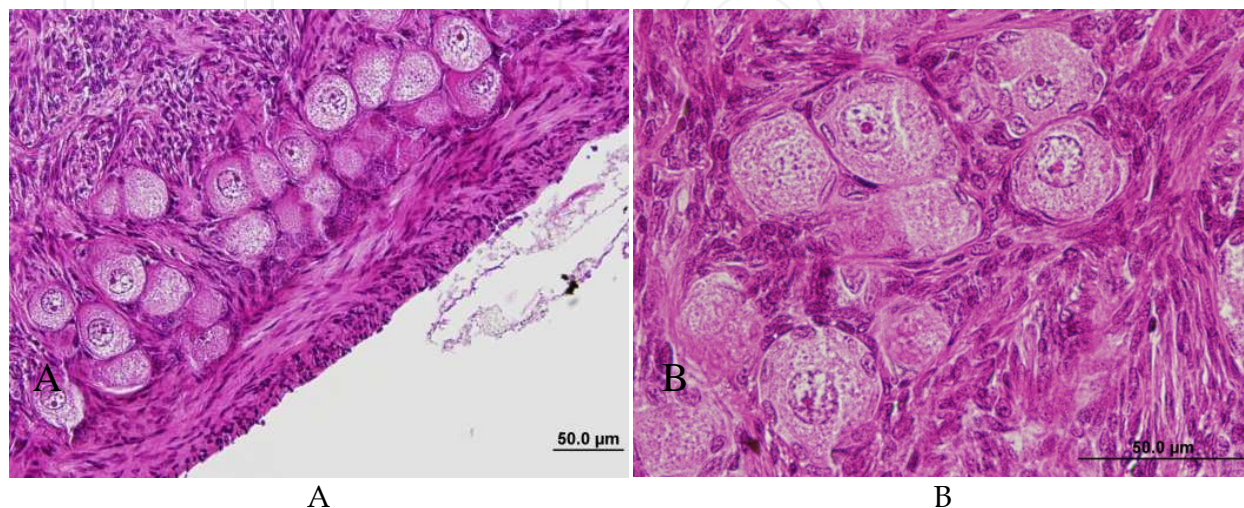


Fig. 4. Queen follicular morphology before (A) and after (B) cryopreservation with 1.5M PROH and 0.2M sucrose. Post-seeding freezing rate at 2°C/min.

In conclusion, queen ovarian tissue seems to be well preserved, without any difference compared to the fresh control when freezing with PROH (according to a free fall in the freezing chamber, without influence of neither sucrose nor post-seeding freezing rate) (Fig. 4).

2.4.3 Investigation in the bitch

In the bitch, the estimated model was validated when the viability preservation ratio was considered. The nature of the non-permeating CPA ($P = 0.37$) did not influence the post thawing viability rate of the ovarian follicles. However all the other factors investigated in this experimental design presented a significant effect on the viability rate. The permeating CPA nature ($P < 0.0001$) was the factor that influenced more the viability rate of the follicles. Thus, contrarily to the observations in the other species, the DMSO better preserved the evaluated parameter than PROH. The freezing rate had also a major effect on the viability rate ($P < 0.0001$) and a slow freezing rate (0.3°C/min) was less deleterious than the rapid freezing rate for the follicles viability. Moreover the equilibration step also significantly affected the follicles viability, with a beneficial effect of the one step equilibration compared to the 3 steps equilibration. However, no interaction was observed in this model. As a result, the fractional experimental design developed in the bitch, suggested that ovarian tissue

should be cryopreserved in a solution containing 2 M DMSO as permeating CPA supplemented with 0.2 M sucrose or trehalose in a one-step equilibration and frozen at a 0.3°C/min freezing rate (Fig. 5).

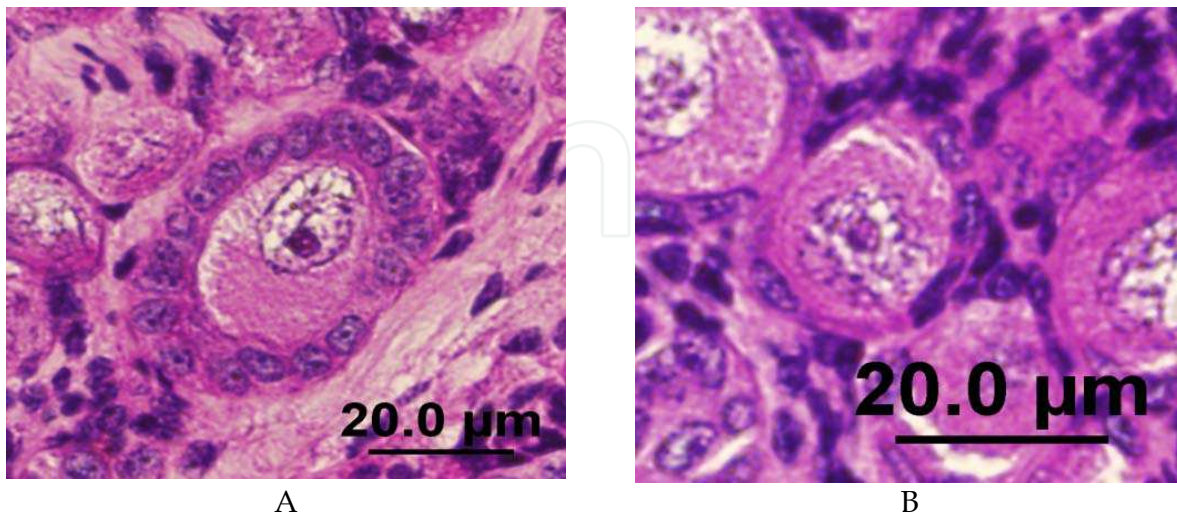


Fig. 5. Bitch follicular morphology before (A) and after (B) cryopreservation

Consequently, these parameters validated by the fractional experimental design for viability assessment were used and applied for morphological assessment of frozen-thawed bitch ovarian tissue and comparison with fresh tissue. So, the morphology of preantral follicles was compared between fresh and cryopreserved tissue. The histological analysis revealed that no significant difference was observed between fresh (89.1 ± 6.1 % type I follicles) and cryopreserved ovarian tissue (82.4 ± 4.4 % type I follicles) when type I follicles were observed. The main abnormality observed on preantral follicle after cryopreservation in the bitch was the oocyte nucleus defect (~8%). In this case, the nucleus often appeared pycnotic, with a reduced size and a densely packed chromatin. Sometimes the nuclear membrane was ruptured. However, ooplasm defect were rarely observed alone, but combined with nuclear defects. In some cases few granulosa cells were absent in the primordial or primary follicles. It is probable that ice crystal formation occurring during cooling be responsible of this partially denuded pattern, by destroying or dislodging some granulosa cell during ice expansion.

2.4.4 Investigations in the cow

As for the rabbit doe, the experimental design was valid when considering the morphological preservation ratio of the follicles, but not when considering the viability ratio of the follicles. The concentration of the permeating CPA ($P = 0.59$) and the medium ($P = 0.76$) seem to have no significant effect on the morphological preservation ratio of ovarian follicles. The nature of the permeating CPA seemed to influence the morphological preservation ratio of the follicles ($P = 0.07$) although the non-significant difference. PROH tended to improve the morphological preservation ratio, as compared with DMSO. The nature of the non-permeating CPA ($P = 0.002$) and the cells surfactant ($P = 0.04$) had significant influence. Trehalose and Albumax® improved morphological preservation ratio

compared respectively with sucrose and FCS. No significant interaction was observed between the nature of the permeating CPA and its concentration.

In order to discriminate permeating CPAs, ovarian fragments from 5 cows were frozen using 1.5M DMSO or 1.5M PROH with 4 g/L Albumax® and 0.2M trehalose according to a post-seeding freezing rate at 0.3°C/min. Before freezing, ovarian tissue showed $40.6 \pm 12.6\%$ of type I preantral follicles. Proportion of type I follicles was reduced to $20.2 \pm 3.9\%$ after cryopreservation using DMSO and to $23.8 \pm 3.4\%$ when using PROH. No statistical difference was found between DMSO and PROH. Morphological defects of type II were the most important kind of defect ($47.0 \pm 11.7\%$). Proportion of type IV follicular defects was significantly improved compared to control for the both CPAs ($47.2 \pm 7.8\%$ and $44.8 \pm 4.4\%$ for DMSO and PROH respectively). Proportion of type III follicles was constant before and after freezing. Ovarian stroma seems to be affected by cryopreservation and shows spaces and disjoined cells.

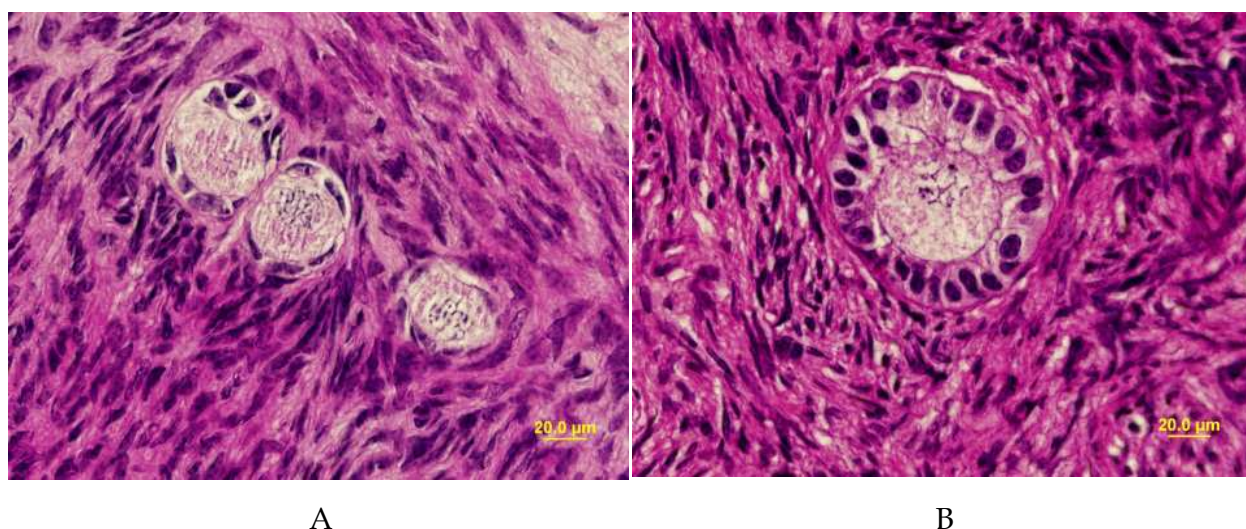


Fig. 6. Cow follicular morphology before (A) and after (B) cryopreservation using PROH and slow freezing rate

As for the other species, the influence of the post-seeding freezing rate was evaluated when freezing with 2M PROH. Proportions of morphologically normal follicles were significantly reduced after freezing compared with fresh tissue, whatever the post-seeding freezing rate $17.6 \pm 6.2\%$ after freezing at 2°C/min vs. $57.8 \pm 13.0\%$ before freezing and $17.8 \pm 6.5\%$ after freezing at 0.3°C/min vs. $60.0 \pm 4.9\%$ before freezing).

2.4.5 In vivo follicle resumption from cryopreserved ovarian tissue

In the doe rabbit, nine pups were born from cryopreserved grafted group, suggesting the efficiency of the cryopreservation protocols based on PROH and trehalose and using very slow freezing rate. At necropsy, follicular structures were observed in most of females.

In the bitch, the cryopreservation method optimized with the fractional experimental design and validated by in vitro assessment (morphology, viability, and toxicity) was then evaluated by heterotopic xenografting to determine whether the ovarian tissue integrity and

the follicular growth potential were maintained. The frozen-thawed tissues were grafted to female SCID mice as previously described. The histological assessments of the follicle population revealed a significant increase in the density and distribution of secondary follicles from eight weeks post grafting compared to the follicle population at 1 week ($P<0.05$). Consequently, the shift from primordial-primary follicles to secondary follicles occurred in a time laps of eight weeks. Moreover morphologically normal follicles were observed until 16 weeks post transplantation and intact secondary follicles with more than 3 layers of granulosa cells and a normal oocyte surrounded by a well-defined zona pellucida were present at this time. Despite a massive follicular loss touching particularly the early follicles and occurring just after grafting, the graft survived long term xenografting. Similarly, after an important loss just after grafting (one week grafts) the stromal cell number increased during the graft period, to reach a density comparable to fresh ovarian tissue at 16 weeks. Otherwise, the vascularization setting-up was assessed by immunohistochemistry as developed previously. The vessel density analysis revealed that already at one week post grafting the vessel density within the graft was comparable to the fresh control ovarian tissue. Moreover, the vessel density tended to increase at 16 weeks post grafting compared to the other groups (fresh control, one and eight weeks, $P<0.05$) even if no significant difference was registered. No antral follicles was present at the end of the graft time, however, persisting vaginal cornification was noticed on the recipients from 5-9 days post grafting, and estrus behavior was observed several times during the graft period in the recipients cages indicating an hormonal activity resulting from the graft. Taken together, these *in vivo* results confirm the good preservation of the bitch ovarian tissue by applying our cryopreservation method.

2.5 Discussion

The greater challenge of studies related to the cryopreservation of the ovarian tissue is to define a freezing protocol adapted to the different cell types such as oocytes, follicular cells, stroma cells, etc. One of the objectives of our team was to compare the effects of different freezing parameters based on the morphology and the viability of the follicles, the evaluation of the ultrastructure of the ovarian tissue, the DNA fragmentation of the oocytes or the graft of the ovarian tissue. When the mathematical model was validated, the use of experimental fractional designs allowed us to know simultaneously the individual and the relative effects of different chemo-physical freezing parameters for each species. This statistical method firstly allows a global evaluation of cryopreservation protocols and discriminates the most valuable factors. Finally, the factors which seemed to have a discriminating effect on follicular morphological preservation were evaluated in a wider population. (Neto et al., 2008).

The results of the experimental design in the doe rabbit and in the bitch show that the post-seeding freezing rate is one of the most important chemophysical factors influencing the morphology or the viability of ovarian follicles. A slow freezing rate ($0.3^{\circ}\text{C}/\text{min}$) seems to be more appropriate for the cryopreservation of the doe rabbit and bitch ovarian tissue. Nevertheless, no influence of the freezing rate was observed in the queen and in the cow. Most of the authors use a very slow freezing rate, which is derived from embryo freezing protocols. However, few studies show the importance of this freezing parameter. In contrast to our results in the bitch and in the doe rabbit, but in accordance with our result in the

queen and in the cow, Demirci *et al.* observed a high (but not significant) proportion of follicles without any morphological defect after a post-seeding freezing rate of 2°C/min in the ewe (Demirci *et al.*, 2001). Nevertheless, Gook *et al.* also observed a better follicular preservation when using a slow freezing rate (0.3°C/min) with human ovarian tissue (Gook *et al.*, 1999). Whereas Cleary *et al.* observed no difference in terms of follicular growth after grafting, between a conventional embryo freezing protocol (0.3°C/min) and a passive cooling at 1°C/min from 0°C to -84°C on the mouse ovarian tissue (Cleary *et al.*, 2001). Although these two cooling rates (0.3°C/min and 2°C/min) could be considered as slow, these results may be explained by a difference in cell dehydration during the post-seeding step. With rapid cooling rates, we can hypothesise that time required for the exosmosis of the cell water is insufficient and consequently promotes the formation of lethal intracellular ice. While at very slow cooling rates, high level of dehydration occurs with concomitant increasing in solute concentration (salting out). Investigations on the freezing rate were extended in the queen with the evaluation of the third freezing phase. When associated with PROH, slow cooling in solid phase seems to be more appropriate. Nevertheless, several authors use cryopreservation protocols with a direct immersion into liquid nitrogen after -40°C, such as Rodrigues *et al.* (2004) in the goat, Lucci *et al.* (2004) in the zebu cow, Santos *et al.* (2006) in the ewe or Lima *et al.* (2006) in the cat. Births had been obtained after graft of ovarian fragments frozen with such a protocol in the rabbit doe (Almodin *et al.*, 2004b) and in the ewe (Almodin *et al.*, 2004a), but not in the cat where *in vivo* follicular growth were observed when using a freezing protocol with controlled third phase (Bosch *et al.*, 2004).

The experimental designs revealed a crucial role of the permeating CPA in the doe rabbit, in the cow and in the bitch, added to a crucial role of the non permeating CPA in the doe rabbit and in the cow. Among the various freezing protocols described in the literature, those using DMSO or PROH as permeating CPA seems to be more efficient, whatever the species. Our results suggest that PROH improve the follicular quality after freezing in the doe rabbit and in the queen. Results of experimental design obtained in the cow suggested a better morphological preservation rate when using PROH. However, in this species, standard comparison between DMSO and PROH doesn't confirm these results. Contrary to that, bitch ovarian tissue seems to be better preserved in freezing medium composed of DMSO. These results were confirmed by the follicular growth observed after xenograft. It can be hypothesized that DMSO penetrate better within the tissue than PROH. Indeed, the bitch ovarian tissue as the goat or the ewe is rich in collagenous fibers and more fibrous than the doe rabbit ovarian tissue for example. Therefore, a good ability to penetrate within the ovarian tissue is an important characteristic for the chosen CPA. Nevertheless, both CPA have sensibly the same molecular weight (PROH: 76.10 g/mol, DMSO: 78.14 g/mol) with a lower weight for PROH. Thus a better penetration of the DMSO cannot be explained by this physical parameter. The explanation may come from the toxicity of both CPAs. Our team also investigated the toxicity of the both CPAs on bitch ovarian tissue after equilibration steps at room temperature without freezing and registered a deleterious effect of PROH compared to DMSO on preantral follicle viability in this species.

Except in canine and feline models, addition of non-permeating CPA in the freezing medium seems to improve the protective effect of CPAs. The protective effect of PROH seems to be improved when it is associated with trehalose, in the doe rabbit and in the cow. This observation was confirmed by electron microscopy evaluation of the doe rabbit ovarian

tissue subjected to cryopreservation. Ultrastructure of doe rabbit follicles after cryopreservation was well preserved, but stromal cells and fibroblasts were damaged. Such alterations have been observed in human tissues after cryopreservation (Navarro-Costa et al., 2005; Santos et al., 2010). However, fibroblasts can easily be reproduced by cell division, indicating that damage to the stroma can be repaired. Collagen fibers did not seem to be damaged by cryopreservation in this study, but they were sparse in the doe rabbit ovary. This observation may explain the fragility of doe rabbit ovarian tissue during the equilibration, freezing and thawing steps (Neto et al., 2005).

Sugars are not systematically associated with permeating CPA, but Marsella et al. (2008), showed the advantageous effect of sucrose. Trehalose has been frequently used in embryo cryopreservation, but not in ovarian tissue cryopreservation. Sucrose and trehalose share the property to stabilise cellular membranes and proteins via the formation of hydrogen bonds with polar residues of phospholipidic membrane. This property allows preserving the membrane integrity under anhydrous conditions. Moreover, it modifies the temperature at which the separation of lipid phase occurs during cooling (Crowe et al., 1984; Crowe et al., 1985; Crowe et al., 2001). As compared to other sugars, trehalose seems to have a higher capacity to preserve biomolecules, cellular membrane and cells in a drying or in a freezing state (Crowe et al., 1996; Storey et al., 1998; Sano et al., 1999; Welsh and Herbert, 1999).

Few comparable studies have been reported in the cryopreservation of different mammalian species. Despite encouraging results in the different studied species, and except in the queen, none of evaluated protocols allows preserving the same proportion of normal follicles than before freezing. Most of authors observed a reduction of normal follicles in frozen/thawed ovarian tissue compared with fresh control when using similar freezing protocols in the mouse (Candy et al., 1997), the goat (Rodrigues et al., 2004), the cow (Lucci et al., 2004), and the ewe (Demirci et al., 2002). As for the queen, no morphological difference was observed in human follicles before and after cryopreservation (Hovatta et al., 1996; Fabbri et al., 2006). Newton observed similar proportions of “viable” follicles after freezing when using DMSO, ethylene glycol or PROH and xenografting (Newton et al., 1996).

Live births in the rabbit doe and follicular growths observed in the bitch after grafting of cryopreserved ovarian tissue shows the efficacy of evaluated freezing protocols. Almodin et al. obtained live offspring after grafting of small fragments of cryopreserved rabbit ovarian tissue using 1.5 M DMSO and a very slow post seeding freezing rate (Almodin et al., 2004b). In the bitch, results about *in vivo* growth obtained after cryopreservation are relatively scarce compared to other species. Ishijima et al. (2006) tried to transplant vitrified ovarian tissue (2M DMSO, 3M PROH, 1M acetamide) into immunodeficient mice during 4 weeks and observed signs of growth in the early follicles (primordial-primary follicles). However, they noticed an important follicular loss occurring just after grafting which is in accordance with our results obtained with slow-frozen tissue. The time necessary for setting up of the neovascularization within the grafted tissue seems to be more deleterious for the cells than the cryopreservation technique itself. Except our results obtained on the bitch ovarian tissue cryopreservation no other results using slow freezing of female germ cells was obtained in this species. Furthermore, live birth has not yet been obtained after ovarian tissue cryopreservation, but the difficulties to master *in vitro* maturation and fertilization steps in canines do not contribute to the development of this technique.

For the first time a complete evaluation process of important factors influencing the morphology and the viability of preantral follicles has been performed after equilibration process and freezing in different species. These results suggest that cryopreservation of ovarian tissue is a promising and suitable technique that could be used as complementary tool to embryo cryopreservation, to preserve the animals' genetic resources by the female pathway. Doe rabbit could also be used as a biomedical model to investigate the long term consequences of cryopreservation on ovarian follicles and the birth of future progenies.

3. Perspectives

In definitive, the use of factorial fractional experimental design approach allowed us to develop suitable cryopreservation protocol in different species, while reducing the number of experiments and increasing the number of parameters evaluated. However it can be noticed in our model species, but also in the literature, that results can be radically different according to the species. Moreover, among the numerous articles published on ovarian tissue cryopreservation heterogeneous results can be observed in the same species after applying roughly or widely different slow-freezing protocols. One of the candidates to explain such disparity in the obtained results is the amount of ice crystals formed during slow freezing. Indeed, the strategy of slow cooling is to decrease cell temperature enough slowly to allow removal of most of the freezable intracellular water before reaching the ice nucleation temperature. The main objective of this method is to avoid intracellular ice crystal formation which is known to be lethal. However ice crystallization still occurs extracellularly with the risk of tissue shrinkage or disorganization of the tissue components.

As ice formation and melting are exothermic and endothermic phenomena respectively, they can be objectified and studied by thermodynamical measures. Among the various physical methods of analysis, Differential Scanning Calorimetry (DSC) is an interesting tool. In fact, DSC gives the opportunity to measure important parameters of a cryopreservation solution under dynamic conditions. A cryopreservation solution can thus be characterized by its thermal properties such as temperatures of phase transitions and quantity of ice crystallized and melted. Two types of DSC are commonly used: power-compensation DSC and heat-flux DSC. Our team chose the first one in order to study cryopreservation solutions with a more fundamental approach than with biological methods.

The power compensation DSC is based on the "zero balance principle" as explained as follow. The sample and a reference are placed in two microfurnaces which are continuously cooled by liquid nitrogen. The temperature of each microfurnace can be, on the one hand, precisely measured by a temperature sensor and, on the other hand, precisely adjusted by a heating resistor. Each microfurnace contains one sensor and one resistor. The principle of the power compensation DSC is to maintain the two microfurnaces under the same temperature regardless of phase transitions or reactions occurring in the sample. Thus, when a phase transition occurs in the sample, the heat released or absorbed by the sample has to be compensated by the heating resistor which is below the sample. Consequently, the calorimeter measures a difference between the heating powers provided by the two resistors. This difference reveals the phase transition. When this phase transition is crystallization, this difference allows also us to measure the quantity of ice formed.

Since recent years, several strategies are developed to avoid deleterious effects of the ice crystal formation during cooling, and thermodynamical approaches are more and more associated to these strategies.

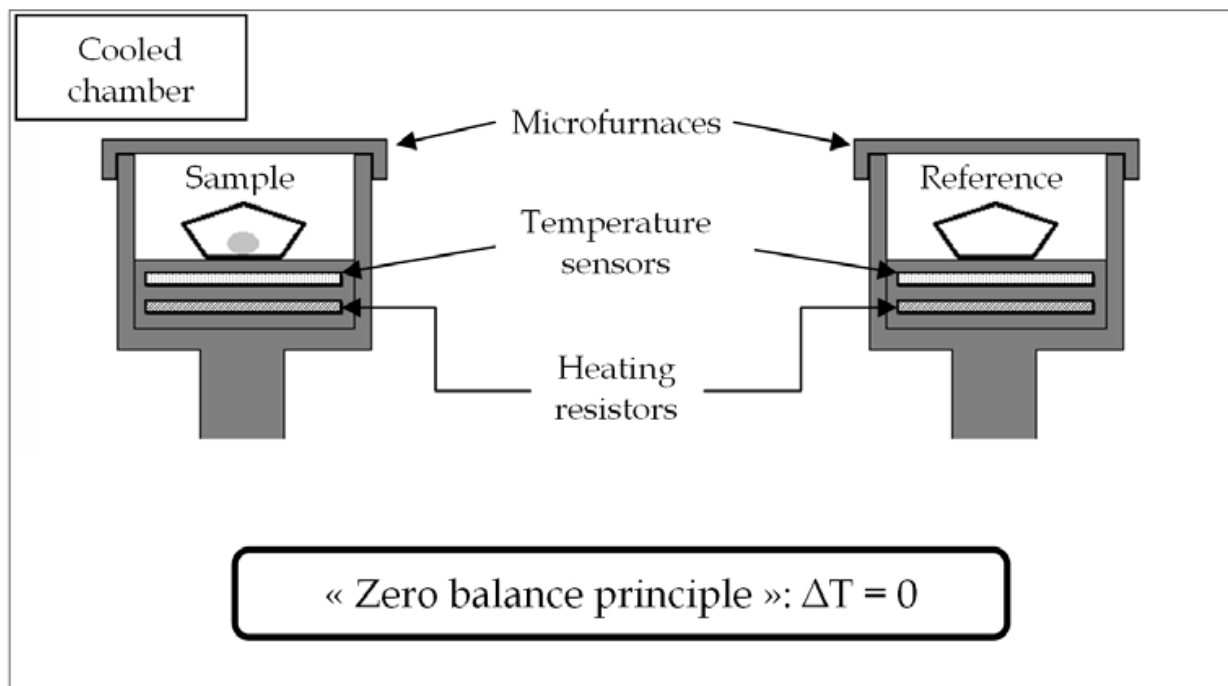


Fig. 7. Scheme of power-compensation DSC

The specimen and reference temperatures are controlled independently using separate (identical) ovens. The temperature difference between the sample and reference is maintained to zero by varying the power input to the two furnaces. This energy is then a measure of the enthalpy or heat capacity changes in the test specimen (relative to the reference).

3.1 Prevent the ice formation

The physical definition of vitrification is the glass-like solidification of solutions at low temperatures, without the formation of intracellular ice crystals. The vitrification technique is the solidification of a liquid without ice crystal formation. This phase is obtained by increasing the solute concentration of the vitrification media (increased viscosity that makes water solidify without expansion) or by using very fast cooling rate to avoid molecular rearrangement into ice crystals but into an amorphous glass. (Vajta, 2000). Consequently, the risk of injuries due to intra- or extracellular ice crystallization is avoided, which constitutes the main advantage of this technique.

Despite the fact that slow freezing is the most widely used cryopreservation technique, vitrification is a viable and promising alternative that is increasingly becoming more attractive to the commercial sector. Vitrification has been used for oocyte and tissue cryopreservation since the 1980s (Rall and Fahy, 1985). Since the first use of this approach in ovarian tissue cryopreservation, it has been well optimized, particularly by decreasing the concentration of CPAs used to reduce their toxicity, but also by increasing the cooling rate applied (Chen et al., 2006; Keros et al., 2009). These improvements of vitrification in ovarian

tissue but also in embryo and oocyte vitrification place the vitrification as a gold standard method for germ cells cryopreservation (Saragusty and Arav, 2011). It has been suggested that with time, conventional slow freezing will be replaced entirely by vitrification techniques (Vajta and Kuwayama, 2006).

As mentioned above, DSC can be a precious aid for the study of vitrification. In fact, DSC allows the detection of phase transitions, both crystallization or glass transition, and can thus determine three thermal properties of vitrification solutions: the vitreous transition temperature, the critical cooling rate and the critical warming rate (Baudot et al., 2007). These three thermal properties are specific to each vitrification solution and could be used for a better utilization of the solutions. The critical cooling rate and the critical warming rate are the cooling and warming speeds above which the crystallization cannot occur between the vitreous transition temperature and the crystallization temperature. The calculation of these critical cooling and warming rates is based on a semi-empiric model developed by Boutron in 1986 according to the “classic” theory of crystallization (Boutron, 1986). This model reproduces well analytically the experimental results, but some approximations are introduced.

3.2 Limit the ice formation

As described in the introductory part, extracellular crystallization occurs during slow freezing. The penetrating CPAs like DMSO, PROH or ethylene glycol, are mainly used to reduce the volume of freezable ice within the cells, but their respective toxicity for the cells limit the potential concentration usable. However, since the last decades, non-penetrating CPAs like disaccharides have been widely used in cryoprotective solution to improve cell preservation and reduce the penetrating CPA amount necessary. To our knowledge, the first use of sucrose as cryoprotectant of ovarian tissue was in 1996 with human ovaries (Hovatta et al., 1996). Sugars have been proved to play a role on ice crystal formation and stability (Kuleshova et al., 1999). Previous studies on mouse embryos revealed that sugars increase the homogeny ice transition temperature in freezing solutions. By this way, dehydration of the cells occurs when they are still permeable to water without reaching ice nucleation temperature. Moreover, the trehalose was reported to reduce the size of ice crystals and shorten their elongation during freezing (Sei et al., 2002).

On the basis of the contribution of DSC in other fields of cryopreservation [vitrification of ovary (Baudot et al., 2007), slow-freezing or vitrification of plants (Volk and Walters, 2006; Skyba et al., 2011), slow-freezing of aquatic crustaceans (Issartel et al., 2006)], our team chose a thermodynamic approach to study slow-freezing solutions for ovarian tissue. In fact, DSC allows the measure of the maximal quantity of ice formed in a solution (Q_{\max}). Q_{\max} corresponds to the definition of Boutron : “The heat of solidification are represented by the numbers q of grams of ice whose solidification at 0°C would liberate the same amount of heat as that from 100g of solution on crossing the corresponding peaks. They are close to the real quantities of ice crystallized in % (w/w) of the solution when it is ice which crystallizes. One obtains the heat in calories per 100g of solution by multiplying q by 79.78” (Boutron, 1984). Our team has thus quantified the quantity of ice formed in solutions used for the cryopreservation of ovarian tissue of different species (table 6).

Species	Q_{\max} in percentage (w/w) of solution
Bitch	36.81
Doe rabbit	45.79
Cow	37.64

Table 6. Maximal quantity of ice crystallized (Q_{\max}) in solutions used for the cryopreservation of ovarian tissue of bitch, doe rabbit and cow.

Then, our team decided to explore two research areas. On the one hand, DSC can compare the quantity of ice formed in two different cryopreservation solutions. Thus, it seems possible to select the most suitable cryopreservation solutions for slow freezing methods. The first results obtained in doe rabbit seem to confirm this hypothesis. In fact between two cryopreservation solutions tested, those for which the quantity of ice formed was the lowest, was also the one with the best biological results. On the other hand, for a given solution, DSC allows the measure of the quantity of ice formed for different freezing kinetics. Consequently, it seems also possible to select the most suitable kinetics according to the cryopreservation solutions.

3.3 Promote the formation of a non-vulnerable extracellular ice

Another approach to optimize cryopreservation process should be to control the ice crystal growth and shape in order to promote the less deleterious crystallization. It is already assumed that intracellular ice formation is lethal. Nonetheless, the recent observations of rabbit ovarian tissue by cryoscanning electronic microscopy and cryofracture reveal that depending on the cooling rate, the extracellular ice shape is modified. Moreover, according to these results the seeding temperature influences the shape and regularity of the ice crystals resulting in large uniform crystals when seeding was induced close to the solution melting point (Gosden et al., 2010).

Otherwise, a better understanding of intracellular ice formation can also be advantageous to improve cryopreservation processes. Indeed, Han *et al* (2009) investigated the size of intracellular ice crystals in mouse oocytes by cryomicroscopy. They conclude that increasing the concentration of macromolecules in the cells by increasing the extracellular non permeating solute concentration significantly reduced the required permeating CPA concentration for intracellular vitrification. Moreover they also observed that intracellular ice melting point was always lower than extracellular ice. Taken together, this information can be helpful to optimize the cryopreservation protocols.

Regarding DSC, a recent study showed that it is possible to differentiate the crystallization of intra and extracellular ice depending on freezing kinetics (Seki et al., 2009). Consequently, in addition to the measure of the quantity of crystallized ice, DSC can provide a better control of ice formation.

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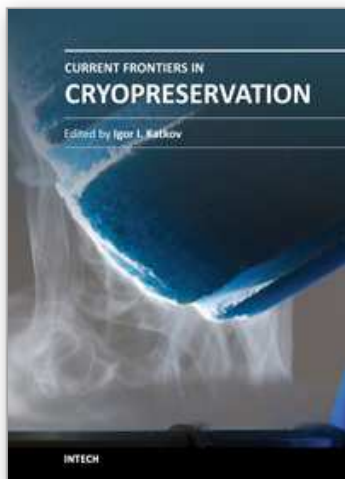
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Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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