Cryopreservation of caprine ovarian tissue: recovery of gonadal function after auto-transplantation

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Cryopreservering van ovariumweefsel van de geit:
herstel van de geslachtsklierfunctie na autotransplantatie
(met een samenvatting in het Nederlands)

Criopreservação de tecido ovariano caprino:
recuperação da função gonadal após auto-transplante
(com resumo em português)

Proefschrift

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“L’essentiel est invisible pour les yeux”

Antoine de Saint-Exupéry

In: Le Petit Prince, Chapitre XXI

To:

All the goats that made this study possible; the anonymous ones and those that showed me the fascinating communication among species.

My best friends: my family and *mijn wederhelft* (Geert-Jan); all my love.

Contents

Chapter 1

General Introduction

Introduction

Cryopreservation of cells and tissues has become of high importance in reproductive biotechnology for the preservation of gametes and embryos. The use of frozen sperm for artificial insemination in domestic animals has resulted, however, in indiscriminate introduction of exotic genetic material in farms and caused dilution or loss of native breeds, and therefore the loss of genetic diversity. Thus, cryopreservation is not only of special importance for the implementation of gene banking and breeding programs of rare and endangered species but also for rare breeds of domestic animals. Also for human medicine, cryopreservation has become more relevant, especially since the advances in biotechnology have enabled diagnosis and treatment of cancer at an early stage, leading to a more favourable prognosis for the patient. Because cancer treatment often consists of chemotherapy or radiotherapy with sterility of the patients as a consequence, ovarian tissue removal and cryopreservation for subsequent auto-transplantation after cure might result in the recovery of fertility of the patient involved.

Although preservation of male gametes is a well-known procedure, the long-term storage of female gametes remains a challenge. Trying to establish a cryopreservation protocol for female germinal cells, various studies have been undertaken with laboratory animals such as mice and rats. Although the importance of these experimental models to understand how cells are affected during and after preservation is evident and encouraging results have been obtained, procedures developed for mice are not necessarily suitable for man or livestock animals. In contrast, among domestic animals, goats in particular can be used as excellent experimental models to develop cryopreservation protocols for man, because of the corresponding architecture of the ovaries and the comparable duration of oocyte development. Domestic goats are also excellent models for wild ruminants (e.g. Alpine Ibex) and endangered breeds of small ruminants (Moxotó, Canindé, Repartida, Marota, Azul, Montain Goat and Erzebirge). Transplantation of cryopreserved ovarian tissue will be performed to evaluate the feasibility of this preservation procedure.

Preceded by a brief introduction on the mammalian ovary, general aspects of cryopreservation are presented below, followed by a description of methods to evaluate freezing and vitrification protocols, and the ability of early-staged follicles to develop after cryopreservation.

Mammalian ovary

The ovary is a female reproductive organ, responsible for the production of gametes and steroid hormones that regulate various aspects of gametogenesis and the development of the female's reproductive tract and external secondary sex characters (Senger, 2003). Ovaries from ruminants, mice and man have an ovoid shape and, in contrast to the spongy structure in mice, human and ruminant ovaries are marked by the presence of a tough connective tissue. These ovaries are endowed by thousands or millions of oocytes (depending on the age and species) enclosed in ovarian follicles at different developmental stages, from primordial to pre-ovulatory (see Fig. 1). According to the absence or presence of a cavity filled with follicular fluid, the antrum, ovarian follicles are classified as: (i) preantral or early-staged follicles or (ii) antral follicles, respectively. Early-staged follicles represent more than 90% of the ovarian follicle population (Saumande, 1981) and are generally divided in primordial, primary and secondary follicles. Primordial follicles are composed of an immature oocyte surrounded by a single layer of flattened pre-granulosa cells (Gougeon, 1996; Van den Hurk, 1997). Primary follicles are constituted of an immature oocyte surrounded by one single layer of cuboidal granulosa cells. Following granulosa cells proliferation (at least two layers) and development of theca cells, secondary follicles are formed (for a review, see Van den Hurk and Zhao, 2005). In contrast to primordial follicles, primary and secondary follicles are considered growing and developing follicles. Antral follicles are the tertiary (dominant or subordinate) and pre-ovulatory follicles. These follicles are characterized by a theca layer, a granulosa, consisting of several cell layers, and an oocyte surrounded by corona radiata and cumulus cells that connect the oocyte to the granulosa. In the granulosa an antrum has been formed, in which the oocyte and its surrounding cells form a peninsula. Tertiary follicles have an immature oocyte, while the pre-ovulatory follicle contains a mature oocyte (Figueiredo, 1995). Complete folliculogenesis, i.e. the development of a primordial into a pre-ovulatory follicle, takes about 18-24 days in mice (Eppig, 1977), 10 weeks in human (Gougeon, 1985), 12 weeks in cattle (Britt, 1991) and around 17 weeks in sheep (Bordes et al., 2005). Despite the enormous follicular population present in the ovary, only around 0.1% of the primordial follicles reach ovulation (Nuttinck et al., 1993), while the rest of the quiescent follicles become atretic during follicular growth and oocyte maturation (Carroll et al., 1990; Otala et al., 2002).

Female reproductive capacity can be lost by e.g. senescence, disease, or cancer treatment (radio- or chemotherapy). Isolation of early-staged ovarian follicles from livestock animals and man followed by their in vitro culture until the oocyte can be fertilised, may be a promising procedure to restore fertility but is currently not yet applicable. In addition, rescue and in vitro culture of follicles requires time, and often results in significant decrease in follicular viability. The development of an efficient cryopreservation protocol to store ovarian tissue for an undefined period may enable the use of gametes in future efficient in vitro culture systems or in vivo by transplantation.

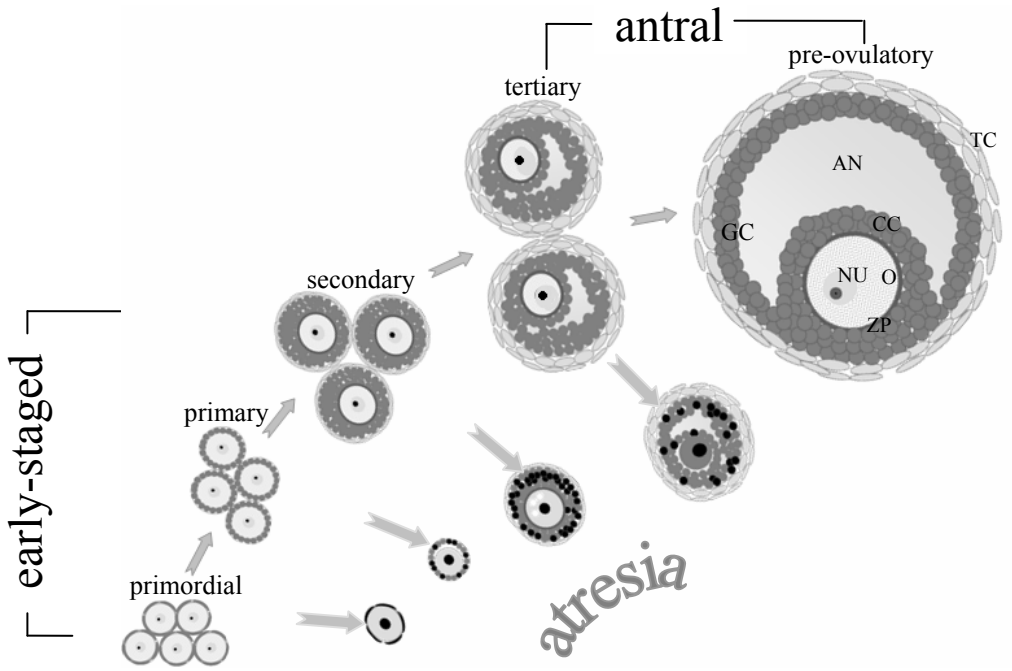


Fig. 1 Scheme presenting the development of mammalian ovarian follicles. O: oocyte; GC: granulosa cells; CC: cumulus cells; TC: theca cells; ZP: zona pellucida; AN: antrum; NU: nucleus

Brief history of cryopreservation

Almost 60 years ago, a fortunate mistake in the labelling of a bottle established a new field of science named cryopreservation. In 1949, Polge and co-workers accidentally froze chicken semen in a mixture of glycerol, albumen and water at -70^{o}C, instead of using their intended solution (levulose), obtaining spermatozoa that survived. This discovery has facilitated artificial reproductive

techniques (ART) in farm animals and man. Furthermore, it has stimulated a cascade of studies on cell tolerance to low temperatures and identified the importance of the use of "freezing protective" substances (cryoprotective agents). Almost 25 years later, the survival of mouse embryos after freezing-thawing was demonstrated (Whittingham et al., 1972), and was followed by experimental freezing studies on embryos from farm animal species such as cow, sheep, pig (Polge et al., 1974) and goat (Bilton and Moore, 1976). Several years later, a frozen-thawed human embryo resulted in the first healthy newborn (Westmore, 1984), drawing the attention of the research community to a new challenge: the preservation of oocytes. It is hypothesised that it is easier to successfully cryopreserve embryos than mature oocytes from large mammals, because DNA from embryonic cells is present at the interphase, while DNA of a mature unfertilized oocyte is compacted and aligned on a metaphase plate (Stachecki and Cohen, 2004). However, mature mouse oocytes have been successfully frozen-thawed and, after fertilisation, resulted in live offspring (Whittingham, 1977). Protocols for oocyte freezing in large mammals and man are in development, but are less efficient, because in contrast to mouse oocytes, human oocytes and those from other large mammals have a high volume:surface area ratio. As mentioned before, 90% of the oocytes present in the ovary are immature, enclosed in early-staged follicles and, because the oocyte is quiescent or in an initial growing phase, the volume:surface area ratio is much lower than that of mature oocytes. These characteristics have been the main reason to initiate cryopreservation studies on early-staged follicles and the first successful results after freezing-thawing of isolated primordial follicles followed by transplantation were achieved with the mouse as an experimental model in the '90s (Carroll and Gosden, 1993). These techniques were further optimised resulting in the live birth after in vitro culture, maturation and fertilisation of frozen-thawed mouse secondary follicles (Smitz and Cortvrindt, 1998). Simultaneously, freezing was used to preserve early-staged follicles from domestic cats (Jewgenow et al., 1998), although with low follicular survival rates (19%). Encouraging results were obtained when human early-staged follicles were frozen-thawed enclosed in ovarian tissue (Hovatta et al., 1997) with about 97% of the follicles appearing normal at histological analysis. However, for the determination of viability, only histological analysis of follicles is not sufficient (Van den Hurk et al., 1998; Santos et al., 2006). Follicular "survival" rates after freeze-thawing and subsequent in vitro culture was relatively low (67 - 40%), and complete follicular development was not obtained. As previously mentioned, the duration of folliculogenesis in mice is much shorter (18 to 24 days) than in larger

mammalian species, like man or live stock animals (2 to 4 months). In addition, follicles from large mammals need to increase in diameter around 500-fold (30 μm to 15 mm) before ovulation (Van den Hurk et al., 1999), instead of approximately 5-fold in mice (35 to 200 μm) (Eppig, 1977; Smitz and Cortvrindt, 2002). Survival and activation of in vitro cultured fresh (non-frozen) primordial follicles have been described for e.g. sheep, but in vitro development of primordial follicles from large mammals beyond the primary stage has not been achieved (Santos et al., 2007). Aubard and co-workers (1996) have speculated upon the possibility to auto-graft cryopreserved ovarian tissue, which may potentially enable the recovery of pre-ovulatory follicles from cryopreserved early-staged follicles. However, an efficient culture system (in vitro or in vivo) by itself will not guarantee successful follicular development after cryopreservation. The choice of the cryoprotective agent(s), the exposure time to these agent(s), and the applied cryopreservation protocol(s) are important determinants.

Cryoprotective agents

Cryoprotective agents protect cells against dehydration, cooling and freezing damage during cryopreservation. In general, these agents can act (i) by permeating the cells (intra-cellular cryoprotectants) and replacing water molecules within cells, (ii) by reducing the freezing point, or protecting cell membranes (extra-cellular cryoprotectants) (iii) by binding to phospholipid head groups, (iv) by increasing the viscosity of the cryopreservation medium, or (v) by decreasing the concentration of electrolytes during freezing, thereby diminishing the extent of osmotic shrinkage. However, cryoprotective agents can be toxic or may facilitate the entrance of toxic agents into cells. Glycerol is an intra-cellular cryoprotectant (low molecular mass compounds), which has antifreeze properties and is able to counteract dehydration. Its metabolic product, however, is formaldehyde, which may induce metabolic acidosis. A similar effect can be observed after conversion of ethylene glycol into oxalate, or propylene glycol into lactic acid.

Despite its antioxidant effect, dimethyl sulfoxide increases the permeability of cell membranes, which may permit entrance of toxic agents into the cell (Table 1). High molecular mass compounds, or extra-cellular cryoprotectants, may increase solution viscosity (e.g. polyvinyl-alcohol) or bind to phospholipid head groups (sugars, such as sucrose and trehalose), and thereby protect cell membranes against chilling and freezing injury. In addition, serum from foetuses or adults is

commonly added to "improve" cryoprotectant solutions (Table 2). These supplements, however, may contain infectious agents or prions, which are not destroyed by the freezing procedure and therefore can transfer diseases.

Cryopreservation has been performed in an empirical way, resulting in a limited improvement in ovarian follicle survival. Although cryopreservation studies with early-staged follicles have been performed since the '90s (Carroll and Gosden, 1993), most of the subsequent studies focused on the use of a basic medium, supplemented with a single intra-cellular cryoprotectant and serum (Amorim et al., 2003; Lucci et al., 2004; Courbiere et al., 2005). In addition, sucrose has been used for the preservation of immature oocytes obtained from antral follicles (Fabri et al., 1998) and mature oocytes (Tucker et al., 1996) for around ten years, but only few years ago sucrose has been used in combination with intra-cellular cryoprotectants to preserve early-staged follicles (Salehnia, 2002). Indeed, to define an efficient cryopreservation protocol, it is first necessary to identify toxicity of cryoprotectants at different concentrations and exposure times, and to evaluate the effects of such substances alone or in combination. Furthermore, a good cryoprotectant must not only efficiently protect cells during cryopreservation, but should subsequently be removed without impairing follicular viability and developmental capacity.

Cryopreservation procedures

Cryopreservation can be performed by a conventional method or by vitrification. Conventional freezing, also called slow cooling, is characterized by the exposure of the cells or tissues to low concentrations of cryoprotective agents (~1,5 mol/l) (Paynter, 2000) for a period ranging from 20 (Rodrigues et al., 2004) to 60 minutes (Candy et al., 1997), followed by a slow and gradual temperature reduction. In summary, the sample is slowly cooled at a speed of 2ºC/min until a temperature of -4 to -9ºC (generally -7ºC) is reached, and held at this temperature for a short period (10 to 15 minutes) to allow thermal stabilisation and to perform seeding (ice-nucleation induction). Seeding prevents super-cooling by an osmotic cell dehydration induction, and is indicated to be performed at a temperature as close to the nominal freezing point as possible (Jondet et al., 1984). Subsequently, the freezing process is re-started slowly at a speed of 0.3ºC/min, until samples are sufficiently dehydrated (-30ºC), thus avoiding lethal intracellular ice formation. Next, frozen material is stored in containers filled with liquid nitrogen (-196°C).

Table 1. Intra-cellular cryoprotective agents commonly used for oocyte and ovarian cryopreservation.

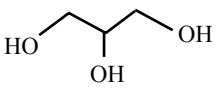
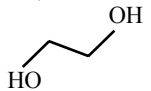
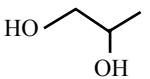
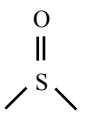
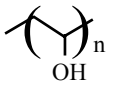
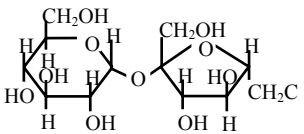
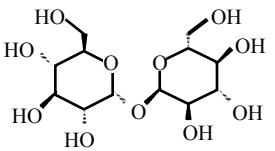
compound	source	formula	molar mass	use	metabolic product	toxicity
alcohols and derivatives						
glycerol (1,2,3-trihydroxypropane; glycerin; propane-1,2,3 -triol; glycyl alcohol)	animal and vegetable fat and oil	$C_3H_8O_3$ 	92.09 g/mol	antifreeze surfactant basis counteract dehydration prevent cell lyses	formaldehyde glucose (only in hepatocytes)	metabolic acidosis
ethylene glycol (monoethylene glycol; 1,2-ethanediol)	via reaction of ethylene oxide with water	$C_2H_6O_2$ 	62.07 g/mol	coolant antifreeze	glyoxylic acid → oxalate glyoxylic acid → glutamate	toxic if converted to oxalate
propylene glycol (1,2-propanediol; 1,2-methylethylene glycol; dihdroxypropane; trimethyl glycol; methyl glycol)	via hydration of propylene oxide	$C_3H_8O_2$ 	76.09 g/mol	solvent emulsifier humectant	lactic acid	cell acidosis
sulfoxides						
dimethyl sulfoxide (methylsulfinylmethane; DMSO)	wood pulp	C_2H_6OS 	78.13 g/mol	solvent antioxidant ↑permeability of cell membrane ↓respiratory chain activity ↓ethanol oxidation ↑cytokine/chemokine production	dimethyl sulfide	allows toxic agents to enter into the cell due to increase in cell membrane permeability

Table 2. High molar mass compounds commonly used as cryoprotective agents for oocyte and ovarian cryopreservation.

compound	source	formula	molar mass	use	metabolic product	toxicity
alcohols and derivatives						
polyvinyl alcohol (polyvinol; ethenol homopolymer; PVA)	synthetic resin obtained from hydrolysis of polyvinyl acetate	[-C_2H_4O-]n 	not applicable	preparation of other resins resistant to solvents or oxygen anticoagulant viscosity increasing agent	not applicable	non toxic; no success when used alone as cryoprotectant
proteins						
blood serum; albumin	bovine foetuses	not applicable	not applicable	nutrient source; there is no evidence to proof its role during freezing and thawing	not applicable	virus present in serum is not inactivated by the cryopreservation procedure
saccharides						
sucrose (α-D-glucopyranosyl-1-2-β-D-fructofuranose; saccharose)	produced by plants	$C_{12}H_{22}O_{11}$ 	342.30	nutrient protects cell membranes against chilling and freezing binding to the phospholipid head groups and forming a gel phase as cells dehydrate	glucose + fructose	non toxic; no success when used alone as cryoprotectant
trehalose (α-D-glucopyranosyl-1-1-α-D-glucopyranoside)	fungi, plants and invertebrate animals	$C_{12}H_{22}O_{11}.2H_2O$ 	378.33	similar to sucrose but with higher stability and less solubility	glucose	non toxic; no success when used alone as cryoprotectant

When required, samples are better quickly than slowly thawed (El-Naggar et al., 2006) and cryoprotectant can be removed in one (Leibo, 1984), three (Rodrigues et al., 2004) or even six steps (Shelton, 1992). Although one wash-out step seems to be practical for thawed embryos, more successful results with ovarian tissue were obtained after three washing steps (Lima et al., 2006; Sadeu et al., 2006; Santos et al., 2006). The advantage of conventional freezing is the feasibility to control every step, including temperature changes, and a limitation of the toxic effects of cryoprotectants by using them at low concentrations. However, disadvantages such as the use of expensive equipment and time to perform the procedure (about 110 minutes; see Fig. 2) brought researchers to return to old protocols and to try to improve a simple procedure called vitrification (Luyet, 1937).

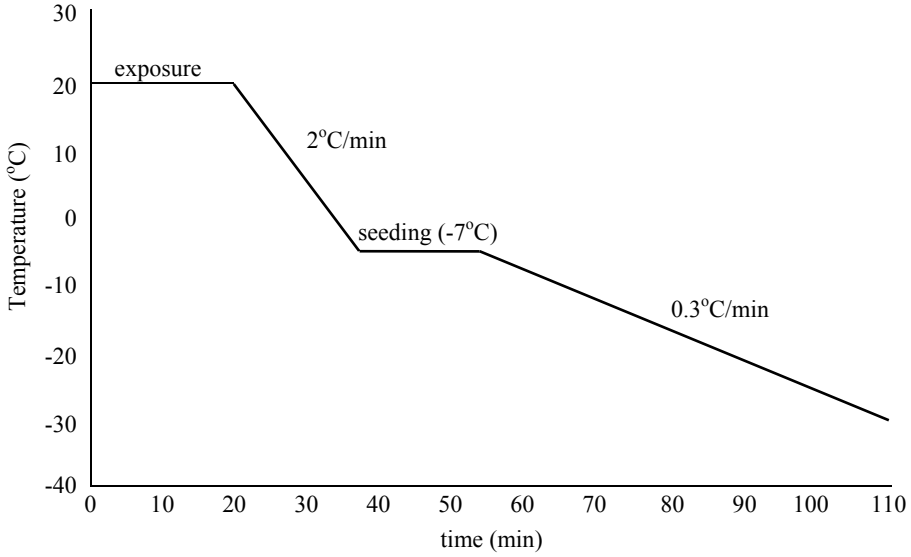


Fig. 2 Temperature (°C) versus time (min) diagram, showing an example of a complete conventional freezing procedure (Santos et al., 2006).

Vitrification was firstly idealised by Luyet in 1937 and was presented as an alternative for conventional freezing 50 years later (Rall and Fahy, 1985). In contrast to slow cooling, vitrification samples are exposed to high concentrations of cryoprotectants (~ 4 to 6 mol/l) for a shorter period (25 seconds to 5 minutes) until a glass-like amorphous status is formed, after which samples are immediately

stored in liquid nitrogen. Although there is a risk of cell damage by the high concentration of cryoprotectants, intracellular-ice formation is avoided and exposure to cryoprotectant shortened when compared to slow cooling. Samples can be warmed up and cryoprotectants removed similar to the conventional procedure. Other advantages of the vitrification method are the low costs, its practicality, since acquisition of a programmable freezer is not necessary, and the complete process can be performed in several minutes. On the other hand, during vitrification, it is not possible to control the temperature.

There are several vitrification methods including a technique with conventional straws (Park et al., 1998), Open Pulled Straws (OPS) (Vajta et al., 1998), Electron Microscope (EM) grids (Park et al., 1999), and Solid-Surface (Dynnies et al., 2000) (Fig. 3). Vitrification using straws is relatively similar to freezing but the results have not been encouraging.

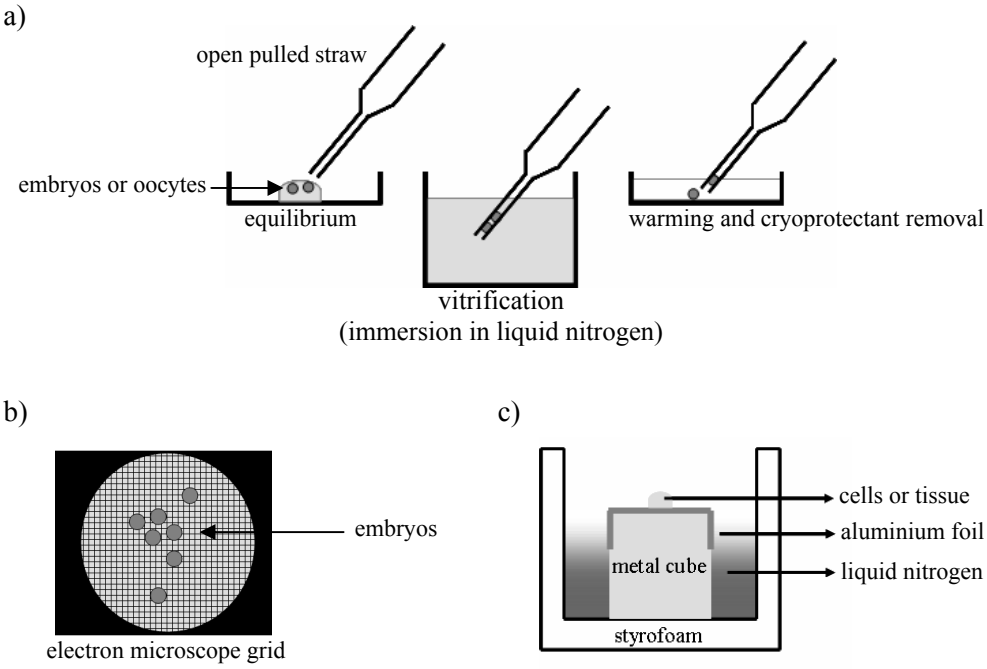


Fig. 3 Vitrification techniques currently used: Open Puled Straws (a), Electron Microscope grids (b) and Solide-Surface Vitrification (c). Adaptated from Vajta et al. (1998), Park et al. (1999) and Dynnies et al. (2000).

The OPS method was developed by Vajta and co-workers (1998), and consists of narrowing the inner diameter of 250µl straws by heat-softening and manually pulling the straws. This method allows the loading of embryos and oocytes by a capillary effect and subsequent extrusion by sedimentation (Fig. 3a). Vitrification in EM grids (Parks et al., 1999) is basically performed by using 400 µm mesh copper EM grids, which are used as a physical support to maximise the cooling rates during the vitrification process (Fig. 3b). Because both above mentioned methods are not applicable for successful ovarian tissue vitrification, an alternative consists in the Solid-Surface vitrification method (Dynnies et al., 2000). For this procedure, a metal cube covered with aluminium foil is placed in a styrofoam box and partially submerged with liquid nitrogen. Biological material is put onto the cold upper surface and instantaneously vitrified (Fig. 3c). Although this technique was developed for embryos and oocytes, it might also be applicable for ovarian tissue.

Techniques to evaluate cryopreservation protocols

For the application of cryopreservation of ovarian tissue, it is necessary to determine the best cryoprotectant, its concentration, exposure time and way of removal. Histology can be used to rapidly evaluate ovarian quality. However, morphology alone is not sufficient to assess the freezing-thawing process (Schotanus et al., 1997; Van den Hurk et al., 1998; Martinez-Madrid et al., 2004). Morphological evaluation will facilitate only the identification of primary signs of microscopically visible atresia (nuclear pyknosis, cytoplasmic damage, detachment of granulosa cells from the oocyte and irregularity of the basement membrane) (Jorio et al., 1991; Hulshof et al., 1995; Demirci et al., 2002). Cell morphology as assessed by light microscopy is not always correlated with functional integrity of organelles, but can be evaluated by transmission electron microscopy to detect e.g. the integrity of mitochondria and endoplasmic reticulum, and cell vacuolisation (Santos et al., 2006). It is also important to determine the cell's plasma membrane integrity by e.g. trypan blue staining (Santos et al., 2007) or ethidium staining (Schotanus et al., 1997; Van den Hurk et al., 1998; Fig. 4), and enzymatic activity by calcein fluorescence (Schotanus et al., 1997; Van den Hurk et al., 1998). Follicular damage caused by cryopreservation can not always immediately be observed, necessitating a few hours of in vitro culture before viability analysis. The restoration of a normal cell metabolism, which can be detected by enzymatic activity and membrane integrity, will guarantee follicular viability. Therefore,

short-term (24 hours) in vitro culture of ovarian tissue or follicles is considered to be the proper method to determine follicular quality after freezing-thawing (Santos et al., 2007). Evidently, long-term in vitro culture of frozen-thawed ovarian tissue, during which primordial follicles can grow and differentiate to the pre-ovulatory stage, would be the optimal method to control follicular viability. Complete in vitro culture of early-staged follicles is, however, still not successful for large mammals (see next paragraph), because of insufficient knowledge of suitable culture media that are necessary for all developmental steps in folliculogenesis (Van den Hurk et al., 2000). Currently, the best method to evaluate a cryopreservation procedure is transplantation of ovarian tissue by providing an adequate microenvironment for follicular survival and development.

In vitro and in vivo culture of cryopreserved ovarian tissue

In vitro culture

In vitro culture of human fetal ovarian tissue was pioneered by Wolff and Zondek in 1925. However, only during the last 20 years attempts have been made to activate primordial follicles in vitro and to enable their growth up to maturation stages (for reviews see Van den Hurk et al., 2000; Fortune, 2003; Van den Hurk and Zhao, 2005). In vitro culture systems have been studied using different mammalian species, such as goats (Silva et al., 2004), cattle (Wandji et al., 1996), non-human primates (Fortune et al., 1998) and man (Hreinsson et al., 2002). However, primordial follicles from these mammals did not grow beyond early secondary stages, not even when tissues were in vitro cultured for 20 days (Wandji et al., 1997; Fortune et al., 1998). When mouse ovarian tissue is cultured in vitro, primordial follicles can be activated and develop up to antral stages, providing a source of fertilisable oocytes. Indeed offspring has been obtained using these techniques although in the studies of O'Brien and co-workers (2003) only one mouse was born, which was obese and showed multiple malformations. The more successful in vitro culture results with mouse follicles, opposed to those from other mammalian species, are likely be due to the short time necessary to complete follicular growth in mice (~21 days). Furthermore, in contrast to oocytes from large mammalian species, mouse oocytes of secondary follicles do not grow anymore in antral stages. In large mammals, in vitro culture of primordial follicles should take at least 10 weeks, during which the oocyte must increase about 4-fold, and follicles about 500-fold in size to complete folliculogenesis. It is possible that various

sequential media are necessary for every follicular stage to obtain a mature, healthy, and fertilisable oocyte from large mammals (Abir et al., 2006). Remarkably, an antral follicle with a diameter of 1mm was obtained by Isachenko (2006) after in vitro culture of developing follicles enclosed in human ovarian tissue for 2 weeks. After 6 weeks of in vitro culture, all follicles, even primordial follicles, had degenerated in this latter study. Possibly, the observed antral follicle was the result of an advanced secondary follicle that had not been removed from the tissue to be cultured. Recently, we tried to culture caprine primordial follicles in vitro for 25 days but, although these follicles became activated and grew to secondary stages, they did not reach the antral stage (Santos, Van den Hurk and Roelen; data not published). Although some improvements have been achieved in the in vitro culture procedures of ovarian tissue, the main goals for large mammals, e.g. complete follicular development and consequent recovery of ovarian endocrine function in vitro, are yet far from practice. Currently, transplantation of cryopreserved ovarian tissue seems to be the most attractive method to evaluate folliculogenesis after cryopreservation (Fig. 4). In most studies with domestic animals and man, ovarian slices have been xeno-transplanted to SCID mice. However, allo- or auto-transplantation avoids the effects of species variability.

In vivo culture - ovarian tissue transplantation

The first human ovarian transplantation was performed more than 100 years ago, when Robert Morris transplanted ovarian tissue into the uteri of women with infertility signals (Morris, 1895). His studies were repeated one year later in rabbits, showing that the presence of ovarian tissue in the body prevented uterine atrophy (Knauer, 1896), which was the first indication for an ovarian endocrine function. Despite these exciting studies, interest in reproductive organ transplantation was only renewed at the end of the 20th century, maybe because of the advances in cancer treatments and cryopreservation studies (Carroll and Gosden, 1993).

Transplantation has been performed (i) by re-implanting ovarian tissue into the donor animal itself (auto-transplantation), (ii) into a recipient from other species than the donor (xeno-transplantation, for which SCID-mice are commonly used) or (iii) into a recipient from the same species as the donor (allo-transplantation). However, successful xeno-transplantation to SCID mice may still be hampered by immune responses mediated by macrophages (Wolvekamp et al.,

2001), which reduces the amount of recovered ovarian tissue (Fassbender et al., 2007). To perform allo-transplantation immunological suppression is required, demanding extra care for the animal subjected to the grafting. Currently, auto-transplantation is the best tool for the evaluation of cryopreservation procedures for oocytes of large mammals, when compared to xeno- and allo-transplantation.

The first allo-transplantation of frozen-thawed primordial mouse follicles was performed in 1993 by Carroll and Gosden. After that, Cox and co-workers successfully allo-transplanted frozen-thawed fetal mouse ovarian tissue to adult recipients (Cox et al., 1996). Several years later, frozen-thawed sheep ovarian tissue was auto-transplanted and cyclical ovarian function was recovered, but with a drastic reduction (up to 28%) in the total number of primordial follicles and formation of follicular cysts (Baird et al., 1999). Subsequently, human ovarian fragments were frozen-thawed and xeno-grafted to adult mice, after which viability of primordial follicles and their initial growth was observed (Oktay, 2000). Antral follicles were obtained from human fetal ovarian slices that were collected after 4 months of xeno-transplantation into the kidney capsules of SCID mice (Abir et al., 2003). More recently, offspring was obtained after auto-transplantation of cryopreserved ovarian tissue of a woman (Donnez et al., 2004) and ewes (Bordes et al., 2005), and of fresh ovarian tissue from a non-human primate (Lee et al., 2004). However, these results have not given final proof that transplantation of cryopreserved ovarian tissue can restore the reproductive function. It is unknown whether the ovum that was fertilised after transplantation of human ovarian tissue came from the re-implanted tissue or from ovarian tissue that had not been removed. Furthermore, to restore and maintain the patient's fertility, administration of hormones was required, while one of the objectives of ovarian auto-transplantation is the recovery of the female's endocrine function (Donnez et al., 2004). Non-human primate ovaries have been successfully transplanted, but these had not been cryopreserved (Lee et al., 2004). In transplantation experiments performed in ewes, whereby half of the cryopreserved ovarian cortex was transplanted to the hilus, three from six animal's studies became pregnant, of which one delivered a malformed lamb (Bordes et al, 2005).

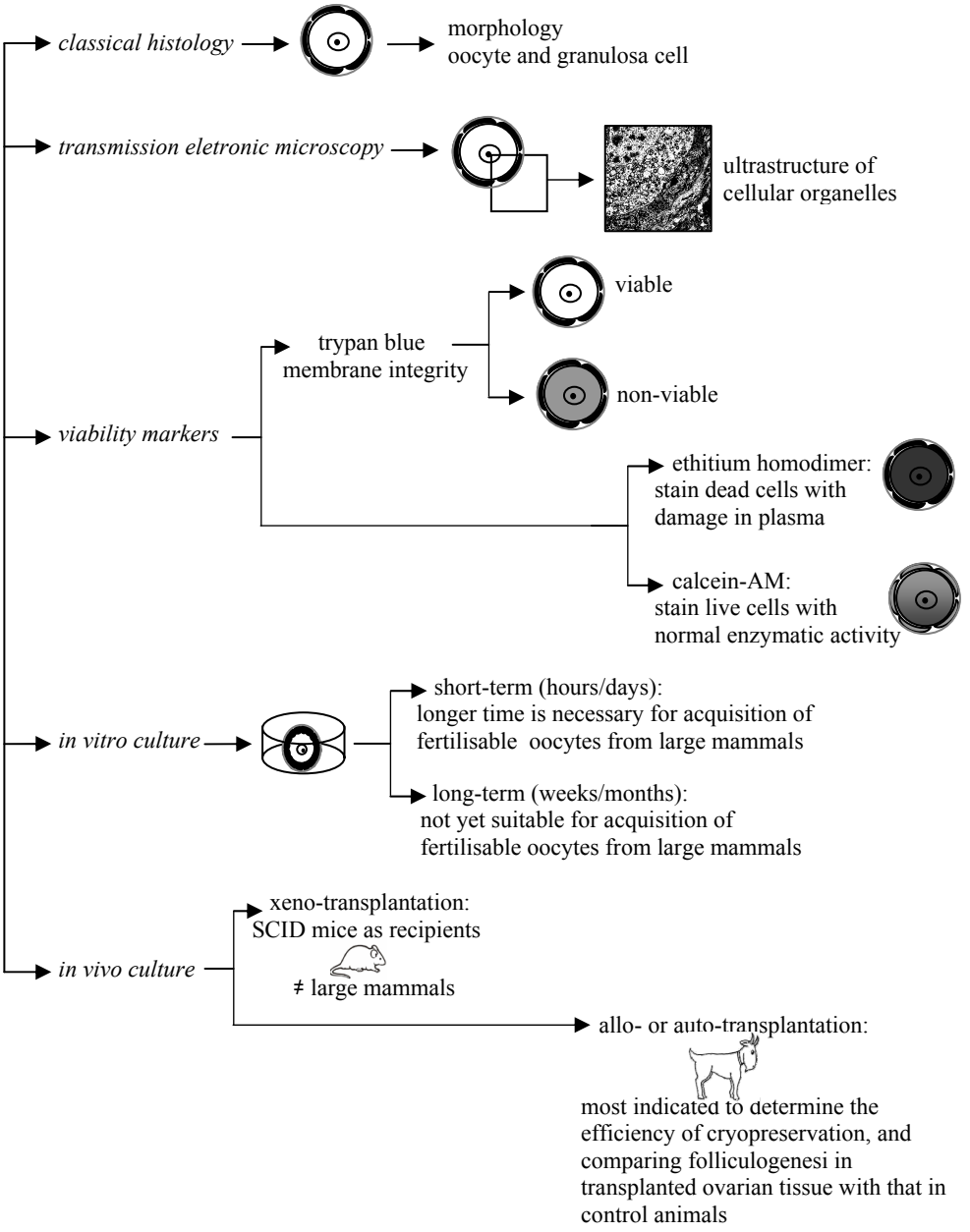


Fig. 4 Schematic presentation, showing the most common methods to evaluate the quality of follicles after cryopreservation

Scope of the thesis

The objectives of this study are to develop an efficient cryopreservation protocol to better preserve mammalian early-staged follicles, capable of normal growth and to develop to the pre-ovulatory follicle stage. In addition, it is aimed to characterise and clarify the damage caused by cryoprotectant exposure, as well as to confront some myths about cryopreservation techniques (like the toxicity of cryoprotectants and to what respect freezing can be deleterious). Finally, the suitability of goats as a model for studies on ovarian cryopreservation will be investigated. In **Chapter 2** a conventional freezing (slow-cooling/rapid-thawing) procedure, that should preserve adequate morphology and viability of caprine cryopreserved early-staged follicles after short-term in vitro culture, is evaluated. Follicular integrity is studied by using classical histology and viability by staining follicles with calcein-AM and ethidium homodimer as markers for living and dead cells, respectively. By using the same evaluation parameters, in **Chapter 3** the effects of two different vitrification procedures (conventional and solid-surface) on the integrity and viability of caprine early-staged follicles are analysed. In **Chapter 4,** the osmotic effect of ionic and non-ionic solutions on the follicular viability is investigated, to better understand the damage caused by cryoprotectant exposure. Furthermore, the effects of the cryopreservation and vitrification procedures are compared. Because it is not yet possible to obtain complete in vitro development of large mammalian primordial follicles to pre-ovulatory structures, auto-transplantation of fresh and frozen-thawed ovarian fragments is performed to study neo-vascularisation, follicular development, and recovery of ovarian cyclicity **(Chapter 5)**. The results of these studies and their possible implications for future research and practical application are summarised and discussed in **Chapter 6**.

References

Abir R, Orvieto R, Raanani H, Feldberg D, Nitke S, Fisch B (2003) Parameters affecting successful transplantation of frozen-thawed human fetal ovaries into immunodeficient mice. Fertil Steril 80:421-428

Abir R, Nitke S, Ben-Haroush A, Fisch B (2006) In vitro maturation of human primordial ovarian follicles: clinical significance, progress in mammals, and methods for growth evaluation. Histol Histopathol 21:887-898

Amorim CA, Rodrigues APR, Rondina D, Gonçalves PBD, Figueiredo JR, Giorgetti A (2003) Cryopreservation of ovine primordial follicles using dimethyl sulfoxide. Fertil Steril 79: 682–686

Aubard Y, Newton H, Oktay K, Piver P, Gosden R (1996) Follicle freezing and autografting. A new method of medically assisted procreation? Press Med 25:921-923

Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG (1999) Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196^{o}C. Endocrinology 140:462-471

Bilton RJ, Moore NW (1976) In vitro culture, storage and transfer of goat embryos. Aust J Biol Sci 19:125-129

Bordes A, Lornage J, Demirci B, Franck M, Courbiere B, Guerin JF, Salle B (2005) Normal gestations and live births after orthotopic autograft of vitrified-warmed hemi-ovaries into ewes. Hum Reprod 20:2745-2748

Britt JH (1991) Impacts of early postpartum metabolism on follicular development and fertility. Bov Practition 24:39-43

Candy CJ, Wood MJ, Whittingham DG (1997) Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. J Reprod Fertil 110:11-19

Carroll J, Gosden RG (1993) Transplantation of frozen-thawed mouse primordial follicles. Hum Reprod 8:1163-1167

Carroll J, Whittingham DG, Wood MJ, Telfer E, Gosden RG (1990) Extra-ovarian production of mature viable mouse oocytes from frozen primary follicles. J Reprod Fert 90:321-327

Courbiere B, Massardier J, Salle B, Mazoyer C, Guerin JF, Lornage J (2005) Follicular viability and histological assessment after cryopreservation of whole sheep ovaries with vascular pedicle by vitrification. Fertil Steril 84:1065–1071

Cox SL, Shaw J, Jenkin G (1996) Transplantation of cryopreserved fetal ovarian tissue to adult recipients in mice. J Reprod Fertil 107:315-322

Demirci B, Salle B, Frappart L, Franck M, Guerin JF, Lornage J (2002) Morphological alterations and DNA fragmentation in oocytes from primordial and primary follicles after freezing–thawing of ovarian cortex in sheep. Fertil Steril 77:595–600

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, Van Langendonckt A (2004) Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 364:1405-1410

Dynnies A, Dai Y, Jiang S, Yang X (2000) High developmental rates of vitrified oocytes following parthenogenetic activation, in vitro fertilisation, and somatic cell nuclear transfer. Biol Reprod 63:513-518

El-Naggar MM, Al-Mashat FM, Elayat AA, Sibiany AR, Ardawi MS, Badawoud MH (2006) Effect of thawing rate and post-thaw culture on the cryopreserved fetal rat islets: functional and morphological correlation. Life Sci 78:1925-1932

Eppig JJ (1977) Mouse oocyte development in vitro with various culture systems. Dev Biol 15:371-388

Fabbri R, Porcu E, Marsella T, Primavera MR, Seracchioli R, Ciotti PM, Magrini O, Venturoli S, Flamigni C (1998) Oocyte cryopreservation. Hum Reprod 13:98-108

Fassbender M, Hildebrandt TB, Paris MC, Colenbrander B, Jewgenow K (2007) High-resolution ultrasonography of xenografted domestic cat ovarian cortex. J Reprod Dev (epub ahead of print)

Figueiredo JR (1995) Isolement, caractérisation et culture de follicules préantaux chez les bovins. Liège-Belgique: Université de Liège, PhD thesis. 113 p

Fortune JE, Kito S, Wandji SA, Srsen V (1998) Activation of bovine and baboon primordial follicles in vitro. Theriogenology 49:441-449

Fortune JE (2003) The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim Reprod Sci 78:135-163

Gougeon A (1985) Origin and growth of the preovulatory follicle(s) in spontaneous and stimulated cycles. In: Testart J and Friedman R (ed) Human in vivo fertilisation, Insem Symposium, 24, Amsterdam, Elsevier

Gougeon A (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17:121-155

Hovatta O, Silye R, Abir R, Krausz T, Winston RML (1997) Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. Hum Reprod 12:1032-1036

Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh J, Hovatta O (2002) Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J Clin Endocrinol Metab 87:316-321

Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, Van Den Donk HA, Van den Hurk R (1995) Effects of fetal bovine serum, FSH and 17β-estradiol on the culture of bovine preantral follicles. Theriogenology 44:217–226

Isachenko V (2006) Effective method for in-vitro culture of cryopreserved human ovarian tissue. RBM Online 13:228-234

Jewgenow K, Penfold LM, Meyer HH, Wildt DE (1998) Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. J Reprod Fertil 112:39-47

Jondet M, Dominique S, Scholler R (1984) Effects of freezing and thawing on mammalian oocyte. Cryobiology 21:192-199

Jorio A, Mariana JC, Lahlou-Kassi A (1991) Development of the population of ovarian follicles during the prepubertal period in D'man and Timahdite sheep. Anim Reprod Sci 26:239–250

Knauer E (1896) Einige Versuche über Ovarientransplantation bei Kaninchen. Zentralbl Gynaekol 20:524–528

Lee DM, Yeoman RR, Battaglia DE, Stouffer RL, Zelinski-Wooten MB, Fanton JW, Wolf DP (2004) Live birth after ovarian tissue transplant. Nature 428:137-138

Leibo SP (1984) One-step method for direct non-surgical transfer of frozen-thawed bovine embryos. Theriogenology 21:767–790

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

Lucci CM, Kacinskis MA, Lopes LHR, Rumpf R, Báo SN (2004) Effect of different cryoprotectants on the structural preservation of follicles in frozen zebu bovine (Bos indicus) ovarian tissue. Theriogenology 61:1101-1114

Luyet BJ (1937) The vitrification of organic colloids and of protoplasm. Biodynam 1:1–14

Martinez-Madrid B, Dolmans MM, Van langendonckt A, Defrere S, Donnez J (2004) Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. Fertil Steril 82:1390-1394

Morris RT (1895) The Ovarian Graft. NY Med J 62:436

Nutinck F, Mermillod PM, Dessy F (1993) Characterization of in vitro growth of bovine preantral ovarian follicles: a preliminary study. Theriogenology 39:811-821

O'Brien MJ, Pendola JK, Eppig JJ (2003) A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol Reprod 68:1682-1686

Oktay K (2000) Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. Fertil Steril 73:599-603

Otala M, Erkkila K, Tuuri T, Sjoberg J, Soumalainen L, Suikkari AM, Pentikainen V, Dunkel L (2002) Cell death and its suppression in human ovarian tissue culture. Hum Reprod 8:228-236

Park SP, Kim EY, Kim DI (1998) Systems for production of calves from Hanwoo (Korean Native Cattle) IVM/IVF/IVC blastocyst I. Hanwoo IVM/IVF/IVC blastocyst cryopreserved by vitrification. Kor J Anim Reprod 22:349-357

Park SP, Kim EY, Kim DI, Park NH, Won YS, Yoon SH, Chung KS, Lim JH (1999) Simple, efficient and successful vitrification of bovine blastocysts using electron microscope grids. Hum Reprod 14:2838-2843

Paynter SJ (2000) Current status of the cryopreservation of human unfertilized oocytes. Hum Reprod Up 6:449-456

Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164:666

Polge C, Wilmut I, Rowson LEA (1974) The low temperature preservation of cow, sheep and pig embryos. Cryobiology 11:560

Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at -196 degrees by vitrification. Nature 313:573-575

Rodrigues AP, Amorim CA, Costa SH, Matos MH, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004) Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology 61:1009-1024

Sadeu JC, Cortvrindt R, Ron-El R, Kasterstein E, Smitz J (2006) Morphological and ultrastructural evaluation of cultured frozen–thawed human fetal ovarian tissue. Fertil Steril 85:1130-1141

Salehnia M (2002) Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. Exp Anim 51:509–512

Santos RR, Rodrigues APR, Costa SHF, Silva JRV, Matos MHT, Lucci CM, Bao SN, Van den Hurk R, Figueiredo JR (2006) Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. Anim Reprod Sci 91:249-263

Santos RR, Van den Hurk R, Rodrigues APR, Costa SHF, Martins FS, Matos MHT, Celestino JJH, Figueiredo JR (2007) Effect of cryopreservation on viability, activation and growth of in situ and isolated ovine early-staged follicles. Anim Reprod Sci 99:53-64

Saumande J (1981) Ovogenèse et folliculogenèse. Rec Méd Vét 157:29-38

Schotanus K, Hage WJ, Vanderstichele H, Van den Hurk R (1997) Effects of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. Theriogenology 48:471-483

Senger PL (2003) Pathways to pregnancy and parturition (2nd revised edition). Cadmus Professional Communications, pp. 386. Science press Division, Ephrata, PA, USA

Shelton JN (1992) Factors affecting viability of fresh and frozen-thawed sheep demi-embryos. Theriogenology 37:713-721

Silva JRV, Van den Hurk R, Matos MHT, Santos RR, Pessoa C, Moraes MO, Figueiredo JR (2004) Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. Theriogenology 61:1691-1704

Smitz J, Cortvrindt R (1998) Follicle culture after ovarian cryostorage. Maturitas 12:171-179

Smitz J, Cortvrindt R (2002) The earliest stages of folliculogenesis in vitro. Reproduction 123:185-202

Stachecki JJ, Cohen J (2004) An overview of oocyte cryopreservation. RBM Online 9:152-163

Tucker M, Wright G, Morton P, Shanguo L, Massey J, Kort H (1996) Preliminary experience with human oocyte cryopreservation using 1,2-propanediol and sucrose. Hum Reprod 11:1513-1515

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H (1998) Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 51:53-58

Van den Hurk R, Bevers MM, Beckers JF (1997) In-vivo and in-vitro development of preantral follicles. Theriogenology 47:73-82

Van den Hurk R, Spek ER, Hage WJ, Fair T, Ralph JH, Schotanus K (1998) Ultrastructure and viability of isolated bovine preantral follicles. Hum Reprod Up 4:833-841

Van den Hurk R, Bevers MM, Dieleman SJ (1999) Folliculogenesis and oocyte development in mammals (livestock animals). In: Comparative endocrinology and reproduction, Joy KP, Krishna A, Haldar C (ed) pp.296-312. Narosa Publishing House, New Delhi, India

Van den Hurk R, Abir R, Telfer EE, Bevers MM (2000) Primate and bovine immature oocytes as sources of ferilizable oocytes. Hum Reprod Up 6:457-474

Van den Hurk R, Zhao J (2005) Formation of mammalian oocytes and their growth differentiation and maturation within ovarian follicles. Theriogenology 63:1717-1751

Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE (1996) Initiation in vitro of growth of bovine primordial follicles. Biol Reprod 55:942-948

Wandji SA, Srsen V, Nathanielsz PW, Eppig JJ, Fortune JE (1997) Initiation of growth of baboon primordial follicles in vitro. Hum Reprod 12:1993-2001

Westmore A (1984) First freeze-thaw baby is born. New Scientist 102:3

Whittingham DG, Leibo SP, Mazur P (1972) Survival of mouse embryos frozen to -196 and -269^{o}C. Science 178:411-414

Whittingham DG (1977) Fertilisation in vitro and development to term of unfertilized mouse oocytes previously stored at -196 degrees. C J Reprod Fertil 49:89–94

Wolff EK, Zondek BK (1925) Die Kultur menschlichen Ovarial- und Amniongewebes. Virchows Arch 254:1-16

Wolvekamp MCJ, Cleary ML, Cox SL, Shaw JM, Jenkin G, Trounson AO (2001) Follicular development in cryopreserved Common Wombat ovarian tissue xenografted to Nude rats. Anim Reprod Sci 65:135-147

Chapter 2

Preservation of caprine preantral follicle viability after cryopreservation in sucrose and ethylene glycol

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Abstract

Caprine preantral follicles within ovarian fragments were cryopreserved in the absence or presence of 0.5 M sucrose with or without 1.0 M dimethyl sulfoxide (DMSO) and/or 1.0 M ethylene glycol (EG). After being thawed, they were washed in minimum essential medium (MEM) with or without 0.3 M sucrose. Histological analysis of follicle integrity immediately after cryopreservation showed consistent beneficial effects of media including sucrose in the three cryoprotectant solutions analyzed when tissue was thawed without or with sucrose (53.9±14.8 – 82.4±3.2% vs. 27.6±1.6 – 38.6±6.5%, $P<0.05$). However, in further studies, the addition of sucrose to the thaw solutions proved detrimental or of no benefit. An analysis of the cryopreserved material with calcein-AM and ethidium homodimer (markers for living and dead cells, respectively) gave comparable results to those obtained by histology. Follicles cryopreserved in EG, EG plus sucrose, or sucrose alone were cultured in vitro for 24 h following thawing. During this culture period, viability fell most rapidly in material cryopreserved in sucrose alone and was no longer correlated with either the viability or the integrity estimates made immediately after thawing. By contrast, the viability of follicles cryopreserved in EG with sucrose and then cultured for 24 h was not significantly different from the cultured non frozen controls. These results indicate that cryopreservation in 1.0 M EG plus 0.5 M sucrose combined with thawing without sucrose is effective for caprine ovarian tissue.

Keywords
preantral follicles♦cryopreservation♦viability markers♦in vitro culture♦caprine

Introduction

Many studies have been carried out to improve the cryopreservation of female reproductive cells and tissues from various species, e.g. human (Gook et al., 2005), porcine (Liu et al., 2003), bovine (Lucci et al., 2004), and murine (Newton and Illingworth, 2001) ovarian tissue. Permeating (ethylene glycol, EG; dimethyl sulfoxide, DMSO) and non-permeating (sucrose) solutes have been shown effectively to reduce cryoinjury during the cryopreservation process. For example, a low concentration of sucrose (0.1 - 0.5 M) has been used to increase the viability of early-staged (preantral) follicles in mouse (Salehnia, 2002) and human (Hovatta et al., 1996), and can be used in combination with plasma membrane-permeating cryoprotectants such as DMSO (Hovatta et al., 1996) or EG (Salehnia, 2002). With regard to the cryopreservation of preantral follicles from small ruminants, most studies have focused on sheep (Amorim et al., 2003a,b, 2004; Capacchietti et al., 2004; Cecconi et al., 2004; Santos et al., 2006), whereas our knowledge in goats is more restricted (Rodrigues et al., 2004a,b, 2005). Goats are present on all continents and are commercially highly attractive livestock animals, since they can be used for many purposes, such as milk, meat, and skin production. Additionally, two endangered breeds in Brazil (Moxotó and Canindé) may benefit from assisted reproduction. Therefore, studies of caprine reproduction and preservation have become important.

The ultimate aim of ovarian cryopreservation is to store developmentally competent follicles. In studies of caprine preantral follicles, follicular survival after freezing-thawing has been determined by using histological and ultrastructural parameters (Rodrigues et al., 2004a,b) and plasma-membrane-integrity tests (Rodrigues et al., 2005). The use of morphology alone is insufficient to assess the freezing-thawing process (Schotanus et al., 1997; Van den Hurk et al., 1998; Martinez-Madrid et al., 2004) as morphological analysis is not always correlated to the viability or developmental competence of the follicles. Cryopreservation often induces cell disruption, such as the loss of cell membrane integrity, the loss of mitochondrial activity (Santos et al., 2006), and cytoskeletal damage (Smith et al., 2004). Histological studies can be used to identify signs of follicular atresia, such as nuclear pyknosis, cytoplasmic damage, detachment of granulosa cells from the oocyte, and changes in the basement membrane (Jorio et al., 1991; Hulshof et al., 1995; Demirci et al., 2002). However, cells that are morphologically intact might be non-viable (Van den Hurk et al., 1998). Fluorescent probes such as calcein-AM

and ethidium homodimer-1 have been used to assess the viability of cells. Calcein-AM is cleaved by esterase enzymes in living cells and the cleavage product, a fluorescent compound, is left in the cell (de Clerck et al., 1994), whereas ethidium homodimer-1 is used to assess plasma membrane integrity, since it binds only to the DNA of permeable cells (Poole et al., 1993). These viability markers have been previously used to evaluate the quality of isolated fresh bovine (Schotanus et al., 1997; Van den Hurk et al., 1998), frozen murine (Cortvrindt and Smitz, 2001), and human (Martinez-Madrid et al., 2004) preantral follicles.

The aim of this study has been to develop a freeze-thaw procedure that better preserves the morphology and viability of caprine cryopreserved preantral follicles, both after thawing and short-term in vitro culture. Studies have been performed with follicles cryopreserved by using 0.5 M sucrose, 1.0 M DMSO, and 1.0 M EG alone or in combination. The viability of the preantral follicles has been investigated by using calcein AM and ethidium homodimer as markers for living and dead cells, respectively.

Materials and Methods

Source and preparation of ovarian tissue

This study comprised three experiments. For each experiment, female reproductive organs (n=10) from adult mixed-breed goats (*Capra hircus*) were obtained at a local slaughterhouse in The Netherlands. The material was transported, in thermos flasks at 30°C, to the laboratory within 1 h of slaughter. At the laboratory, ovaries were denuded of adhering tissue and subsequently washed once in 70% alcohol and twice in phosphate-buffered saline (PBS). The cortex from each pair of ovaries was then removed and cut into small fragments (1 mm^3) that were placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco, Paisley, UK) at room temperature (25°C). Pieces of 1 mm^3 in size were used for all experiments. The selected ovarian fragments were randomly distributed over the experiments. All fragments contained sufficient numbers (more than 30) of preantral follicles, and no obvious differences in the numbers of preantral follicles were observed.

Experimental design
Experiment I: histology of cryopreserved preantral follicles

Fifteen 1 mm^3 fragments were removed from each of the paired ovaries of ten goats. Ovarian fragments were kept in Petri dishes containing PBS for manipulation. One fragment (control) per ovarian pair was fixed in 4% paraformaldehyde for 12 h for routine histological studies. The remaining 14 fragments were cryopreserved (as described below) by using 0.5 M sucrose (Sigma, Poole, Dorset, UK), 1.0 DMSO (Merck, Darmstadt, Germany), or 1.0 EG (Sigma), alone or in combination (see experimental protocol in Fig. 1), before being fixed in 4% paraformaldehyde. Fixed ovarian fragments were dehydrated, embedded in paraffin wax, and serially sectioned at 5 μm. Every seventh section was mounted and stained with hematoxylin-eosin.

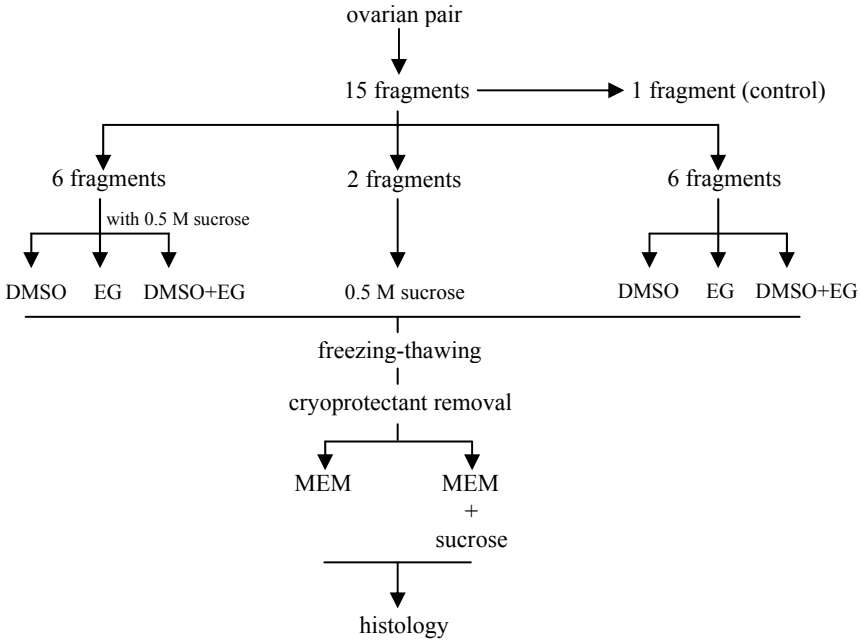

Fig. 1 Experimental protocol for experiment I (*DMSO* dimethyl sulfoxide, *EG* ethylene glycol, *MEM* minimum essential medium)

All sections were examined by using a light microscope (Olympus, Tokyo, Japan) at a magnification of ×400. Follicles were classified as preantral if they had

no antrum. This included oocytes surrounded by one layer of flattened and/or one or more layers of cuboidal granulosa cells. Only preantral follicles in which the oocyte nucleus could be observed in the section were counted. Follicular quality was evaluated based on the morphological integrity of the oocyte, granulosa cells, and basement membrane. Preantral follicles were histologically classified as (1) normal if they contained an intact oocyte and intact granulosa cells, (2) degenerate if they contained a pyknotic oocyte nucleus, shrunken ooplasm, and/or disorganized granulosa cells (e.g. enlargement in volume combined or not with the detachment from the basement membrane).

Experiment II: viability of cryopreserved preantral follicles

In a second experiment, the effect of cryopreservation in sucrose, DMSO, and EG on the viability of preantral follicles was studied (for protocol, see Fig. 2).

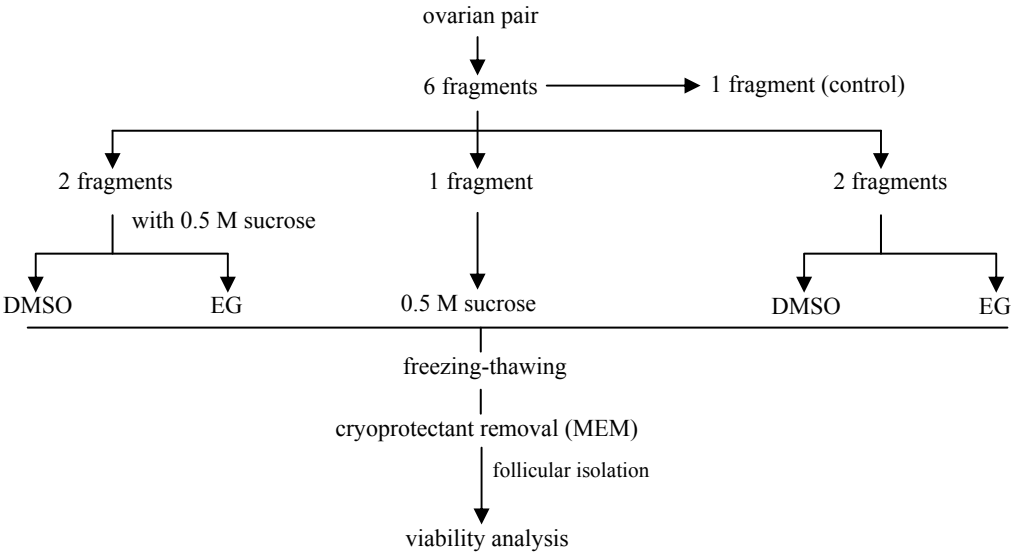

Fig. 2 Experimental protocol for experiment II (abbreviations as in Fig. 1)

After dissection of the ovaries from five goats, six fragments of 1 mm^3 were obtained from each ovarian pair. One fragment (control) was immediately submitted to follicular isolation as quickly as possible according to the procedure described below. The viability of morphologically normal preantral follicles was

analyzed by using live (calcein-AM) and dead (ethidium homodimer-1) markers (see below). Based on the results of experiment I, no further work was performed with thaw solutions containing sucrose, DMSO, and EG, and all cryopreserved material was diluted in the absence of sucrose. The fragments were cryopreserved in (1) 1.0 M DMSO, (2) 1.0 M EG, (3) 0.5 M sucrose, (4) 1.0 M DMSO + 0.5 M sucrose, and (5) 1.0 M EG + 0.5 M sucrose. After being thawed and the subsequent removal of the cryoprotectant with minimum essential medium (MEM), the preantral follicles were isolated and examined for viability.

Experiment III: effect of in vitro culture on the viability of cryopreserved preantral follicles

From the results of experiment II, we defined the experimental protocol of experiment III (Fig. 3) to assess the viability of cryopreserved preantral follicles after short-term culture in vitro.

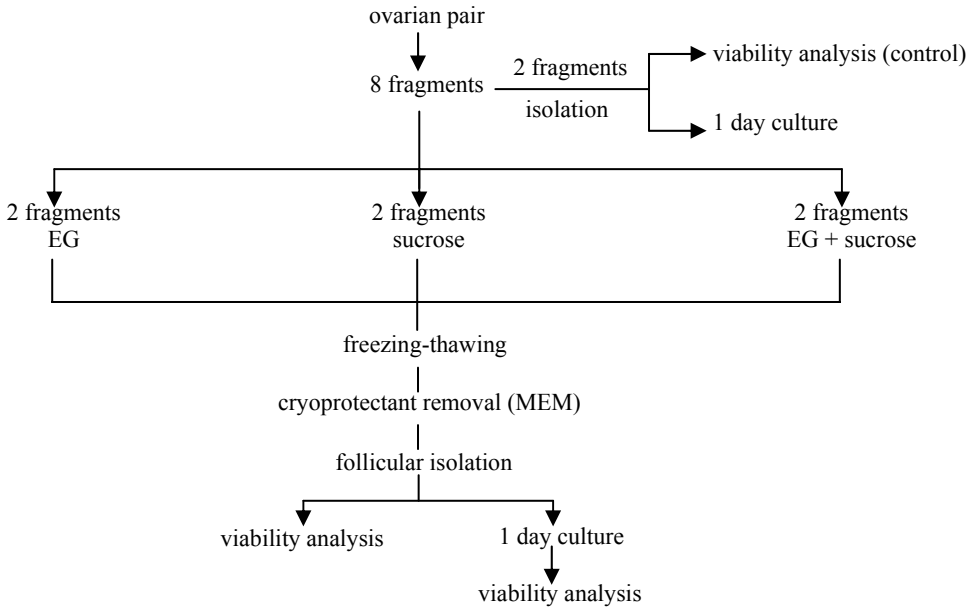

Fig. 3 Experimental protocol for experiment III (abbreviations as in Fig. 1)

After dissection of the ovaries from five goats, eight fragments of 1 mm^3 were obtained from each ovarian pair. Preantral follicles were isolated from two fragments (fresh control). Those that were morphologically normal were used

immediately for viability studies or were cultured in vitro for 24 h; viability was then assessed. The remaining six fragments were cryopreserved by using 0.5 M sucrose, 1.0 M EG, or 0.5 M sucrose + 1.0 M EG. After being thawed and following the removal of the cryoprotectant in MEM (Sigma), preantral follicles were mechanically isolated from the fragments; the morphologically normal follicles were submitted to viability analysis either immediately or after 24 h of culture in vitro.

Freeze and thaw procedures

Ovarian fragments were individually placed in straws (1.5 ml) and equilibrated in a controlled-rate programmable freezer (Planer Kryo 10 Series II, Cryotech Benelux, Schagen, The Netherlands) for 20 min at 20°C in 1 ml MEM (Sigma) supplemented with 0.5 M sucrose (Sigma), 1.0 M DMSO (Merck), or 1.0 M EG (Sigma), alone or in combination (Fig. 1). Straws were cooled at 2°C/min from 20°C to −7°C; ice-induction (seeding) was manually performed by touching the straws with forceps pre-cooled in liquid nitrogen. After seeding, the straws were held at this temperature (−7°C) for 10 min and then cooled at 0.3°C/min to −30°C and finally at 0.15°C/min to −33°C, after which the straws were plunged immediately into liquid nitrogen (−196°C) and stored for 1 week. When required, the straws were thawed in air for 1 min at room temperature (~25°C) and then immersed in a water bath at 37°C until the cryopreservation medium had completely melted. The cryoprotectant was then removed from the tissue at room temperature by a three-step equilibration (5 min each) in MEM with or without a supplement of 0.3 M sucrose. When sucrose was used, an additional wash was performed with MEM.

Assessment of oocyte and granulosa cells viability

Fresh and cryopreserved ovarian fragments were submitted to follicular isolation by application of a mechanical procedure for the isolation of caprine preantral follicles (Lucci et al. 1999). Briefly, the ovarian cortex was cut into small fragments with a tissue chopper (Meyvis, Gouda, The Netherlands). The ovarian fragments were then placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco) at room temperature (25°C) and then pipetted 40 times with a Pasteur pipette to release the preantral follicles. The suspension was successively filtered through 500-µm and 100-µm nylon-mesh filters, followed by

the isolation procedure within 10 min. Preantral follicles smaller than 100 µm were collected under a dissecting stereomicroscope (SZ-STS, Olympus, Tokyo, Japan) and transferred to HEPES-buffered M-199 + 1% bovine serum albumin (holding medium). Isolated follicles were incubated in the holding medium for 10 min at 37°C in a mixture of 4 µM calcein AM, 2 µM ethidium homodimer-1 (Molecular Probes Europe, Leiden, The Netherlands), and 10 µM Hoechst 33342 (Sigma) to detect esterase enzyme activity and membrane integrity and to enable the counting of the nuclei, respectively. After being labelled, stained follicles were washed three times in holding medium, mounted on a glass microscope slide in 5 µl antifade medium (Vectashield, Vector Labs, Burlingame, Calif.) to prevent photobleaching, and finally examined by epifluorescent microscopy (BH2-RFCA microscope, Olympus, Tokyo, Japan) equipped with a digital camera (Coolpix900, Nikon Instruments Europe, Badhoevedorp, The Netherlands). The emitted fluorescent signals of Hoechst, calcein, and ethidium homodimer were collected at 350 nm, 488 nm, and 568 nm, respectively. Oocytes and granulosa cells were classified as viable if ooplasm was stained positively with calcein, and chromatin was unlabeled with ethidium homodimer (Fig. 4). The percentages of viable granulosa cells were calculated in relation to the total number of Hoechst positive nuclei.

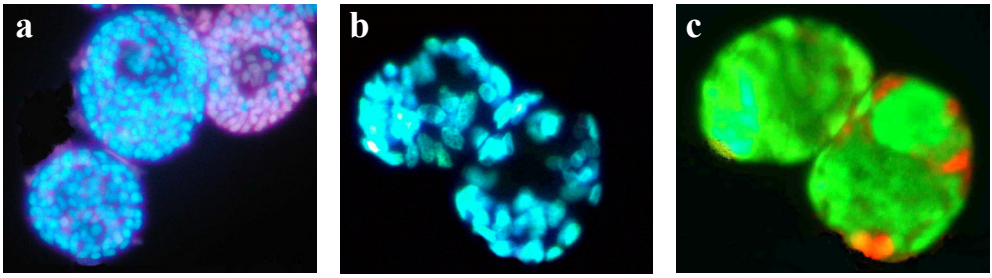

Fig. 4 Preantral caprine follicles stained with Hoechst (*blue*; general nuclear staining), ethidium homodimer-1 (*red*; stains nuclei of dead cells) and calcein-AM (*green*; stains cytoplasm of viable cells). **a, b** Follicles stained with Hoechst and ethidium homodimer-1. Absence of ethidium-staining follicles in **b** indicates their viability. **c** Same follicles as shown in **b**, but stained with calcein and ethidium homodimer-1, indicating presence of many viable and few dead cells, respectively. **a** ×200; **b, c** ×400.

In vitro culture

Preantral follicles (less than 100 µm in diameter) were cultured in 4-well culture dishes (25 follicles per well) in a 20 µl droplet of culture medium under

mineral oil at 39°C in a humidified atmosphere of 5% CO2 in-air (Nunc, Roskilde, Denmark). The culture medium consisted of alpha MEM (Sigma) supplemented with SPIT (5 ng/ml selenium, 110 µg/ml pyruvate, 10 µg/ml insulin, and 5.5 µg/ml transferrin; Sigma), 100 µg/ml penicillin/streptomycin (Gibco), 10% fetal calf serum (Gibco), 0.01 IU/ml human follicle-stimulating hormone (hFSH) recombinant (Organon, Oss, The Netherlands), and 10 ng/ml activin-A (R&D System Europe, Abingdon, UK).

Statistical analysis

In experiment I, preantral follicle quality was assessed morphologically by histology, and the mean percentage of normal preantral follicles between frozen-thawed and control fragments was compared by a one-way analysis of variance (ANOVA) and Tukey's test. The viability of oocytes and granulosa cells (for both experiments II and III) was analyzed by the chi-square test, and the numbers of total nuclei per follicle were compared by one-way ANOVA. In all cases, the statistical tests were performed by using Stat View for Windows, and the differences were considered significant when $P<0.05$.

Results

Experiment I: histology of cryopreserved preantral follicles

In total, 2229 preantral follicles were assessed by histology (approximately 150 follicles per treatment). Normal morphology was present in 77.5±3.5% of non-frozen controls (Table 1). The inclusion of sucrose in the freezing medium improved the post-thaw morphology of follicles in all groups (Table 1). The morphology of follicles cryopreserved in sucrose-containing media (with or without EG or DMSO) and thawed in sucrose-free media was not significantly different ($P>0.05$) from the controls (71.9±3.7% vs. 82.4±3.2%; Table 1). For ovarian fragments that were cryopreserved in DMSO only or in DMSO with EG but without sucrose, washes in medium containing sucrose were less damaging to follicle morphology than washes without sucrose (Table 1). Washes in medium supplemented with sucrose were detrimental to ovaries cryopreserved with EG or sucrose only.

Table 1 Mean percentages (± SEM) of histological normal preantral follicles (150 follicles/treatment) in control ovarian fragments and in fragments that were frozen-thawed (*FT*) and washed (*W*) in presence (+) or absence (-) of sucrose (*NA* not applicable).

Cryopreservation	Sucrose	DMSO	EG	DMSO + EG
FT- / W -	NA	27.6±1.6 *bA	38.6±6.5 *bA	30.0±5.3 *bA
FT+ / W -	78.5±3.4 aA	82.4±3.2 aA	71.9±3.7 aA,B	53.9±14.8 *aB
FT- / W +	NA	45.4±6.4 *bA	13.5±4.9 *cB	53.0±1.4 *aA
FT+ / W +	63.7±2.6 *bA	47.7±2.7 *bA	58.6±6.2 *bA	44.6±7.6 *aA
Control	77.5±3.5			

* Differs significantly from control (P<0.05)
Lowercase letters (*a-c*): values within columns are significantly different (P<0.05)
Uppercase letters (*A, B*): values within rows are significantly different (P<0.05)

Experiment II: viability of cryopreserved preantral follicles

Preantral follicles were mechanically isolated from cryopreserved ovarian fragments shortly after thawing, and a total of 603 preantral follicles was examined for their viability (evaluating oocytes and granulosa cells) by using a fluorescent labelling technique (approximately 100 follicles per treatment).

All cryopreservation treatments significantly (P<0.05) reduced the percentage of viable oocytes when compared to control values (98%; Fig. 5). The post-thaw viability of oocytes was reduced less by sucrose alone (86%) than by sucrose plus EG (76%), which in turn was significantly higher (P<0.05) than with sucrose plus DMSO (67%). Use of DMSO (60%) or EG (57%) as the only cryoprotectant significantly reduced the percentages of viable oocytes.

To evaluate the effects of the cryopreservation procedures on the viability of preantral follicles, the number of follicles with a viable oocyte enveloped by 90% or more viable granulosa cells was counted, and the percentage of viable follicles was calculated. As observed for oocytes, all cryopreservation treatments significantly (P<0.05) reduced the percentage of viable preantral follicles when compared with control values (92%; Fig. 5). The highest percentage of viable frozen-thawed preantral follicles was found after cryopreservation of ovarian tissue in a combination of sucrose and EG (72%). This percentage was significantly higher (P<0.05) than that of follicles that had been cryopreserved in sucrose only (62%), which in turn was higher (P<0.05) than that of follicles frozen-thawed in DMSO plus sucrose (50%). The percentages of viable preantral follicles were

significantly reduced when DMSO (43%) or EG (38%) were used as the sole cryoprotectant.

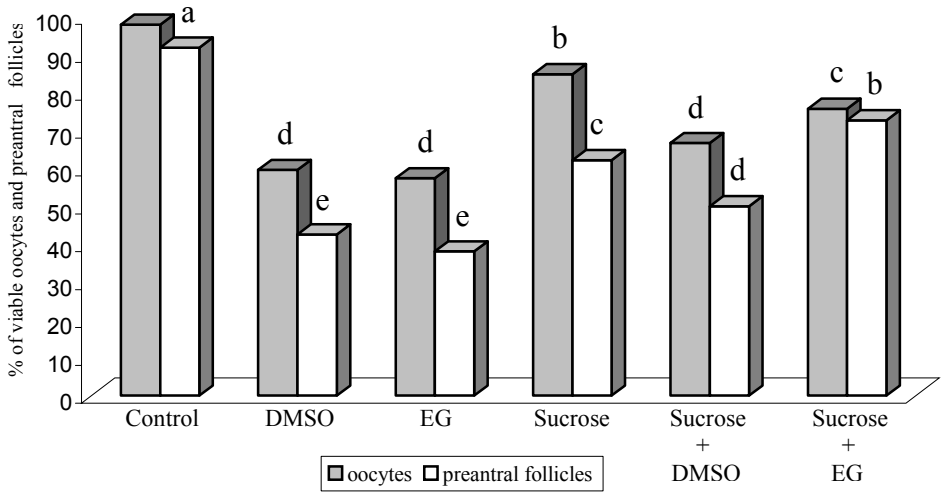

Fig. 5 Percentages of calcein-AM-positive and ethidium-homodimer-negative (viable) oocytes (*gray*) and preantral follicles (*white*), i.e. follicles with a viable oocyte surrounded by ≥ 90% viable granulosa cells, in control ovarian fragments and in fragments that were frozen-thawed (*a* – *e* values are significantly different at P<0.05). Abbreviations as in Fig. 1.

Experiment III: viability of cryopreserved preantral follicles after short-term in vitro culture

In total, viability was assessed for 423 preantral follicles (50–55 follicles per treatment). Table 2 shows the percentage of viable oocytes and preantral follicles (i.e. viable oocyte surrounded for > 90% of granulosa cells). Among the groups that were evaluated, EG plus sucrose had the highest viability immediately post-thaw (without additional culture) for both oocytes (75%) and preantral follicles (73%), but this was significantly lower than for non-frozen controls (96% and 92% viability for oocytes and preantral follicles, respectively; P<0.05). In vitro culture of isolated preantral follicles for 24 h decreased the viability of oocytes and preantral follicles, even in non frozen control follicles (96% versus 69% for oocytes; 92% versus 57% for preantral follicles; P<0.05). The viability percentages of oocytes cryopreserved in a mixture of sucrose and EG did not fall in culture (76% before culture versus 74% after culture).

Table 2 Percentages of viable oocytes and viable preantral follicles, with a viable oocyte surrounded by 90% or more viable granulosa cells, after isolation (fresh follicles) and freezing-thawing (*FT*), before and after 1 day culture (*n* = 50-55 follicles; *NA* not applicable; *EG* ethylene glycol).

Preantral follicles	Cryoprotectant	Time of culture			
		Before culture		After 1 day	
		oocytes	preantral follicles	oocytes	preantral follicles
Fresh	NA	96a,A	92a,A	69a,B	57a,B
FT	EG	59d,A	39d,A	44b,B	20b,B
	Sucrose	85b,A	64c,A	32c,B	11c,B
	EG + sucrose	76c,A	73b,A	74a,A	59a,B

Lowercase letters (*a, b*): values within columns (comparing oocytes or preantral follicles) are significantly different (P<0.05)
Uppercase letters (*A, B*): values within rows (oocytes or preantral follicles) are significantly different (P<0.05)

Discussion

The current study describes the viability of caprine ovarian preantral follicles, based on both follicular morphology and the intra follicular distribution of fluorescent markers for live versus dead cells (calcein AM and ethidium homodimer, respectively), after cryopreservation with sucrose, DMSO, or EG alone or as mixtures. We have shown that the viability of oocytes and their surrounding granulosa cells in caprine preantral follicles is preserved after freezing-thawing in a mixture of sucrose and EG, followed by washes in a medium without sucrose. Mandelbaum et al., (1988) have demonstrated, in human embryos, the beneficial effect of adding sugar (sucrose) to cryopreservation media and suggest that sucrose acts as an osmotic buffer against the stresses incurred by the cells during the addition and removal of penetrating cryoprotectant agents. However, in our studies, the beneficial effect has been observed only when sucrose is added to the cryopreservation media but when cryoprotectant removal is performed in MEM without sucrose. Nowshari et al. (2005) and Bianchi et al. (2005) have obtained good results with cryopreserved camel embryos and human oocytes, respectively, when cryoprotectant removal is carried out in medium without sucrose. Martinez-Madrid et al. (2004) have also obtained encouraging results after cryoprotectant removal from frozen-thawed human ovaries by a series of decreasing concentrations of sucrose (0.25 M, 0.1 M, and 0 M). To limit damage

occurring during the cryopreservation procedure, the amount of intracellular water must be reduced by an increasing dehydration process (Chen et al., 2004). This can be achieved successfully by adding a membrane non-permeating cryoprotectant such as sucrose to the freezing medium. Sucrose can be successfully used as an extracellular cryoprotectant for early staged mouse follicles, especially when it is combined with the intracellular cryoprotectant EG (Salehnia, 2002). For human follicles, a mixture of sucrose with DMSO appears to be the best cryoprotectant (Hovatta et al., 1996).With regard to human oocytes, Bianchi et al. (2005) have observed a high survival frequency (83%) when 0.3 M sucrose is added to 1.5 M propanediol to serve as the cryoprotectant. Furthermore, Chen et al. (2004) have used the same permeating cryoprotectant with 0.1 M sucrose and have observed a survival rate of 75%. Fabri et al. (2001) have shown the importance of adding a higher (0.5 M) concentration of sucrose in cryopreservation media to protect human oocytes during freezing. Our report is the first study that has employed sucrose during the freezing-thawing process of early staged goat ovarian follicles and markers of live versus dead cells to assess the viability of cryopreserved preantral follicles from domestic animals. Although we have noted the importance of the addition of sucrose to the cryopreservation medium, we have also observed that the washing steps (cryoprotectant removal) of thawed ovarian fragments must be performed in medium without sucrose, except when cryopreservation has been carried out by using only DMSO as the cryoprotectant or DMSO in combination with EG. In these last-mentioned cases, we speculate that slow re-hydration in the presence of sucrose is important. When ovarian fragments are cryopreserved in a solution containing sucrose, this compound is obviously present during the thawing and washing processes, facilitating the re-hydration of the cells at a suitable rate. However, if this procedure is so slow (as obtained when sucrose is added to the wash medium), the permeating cryoprotectants might remain in the tissue for a longer period and might have more toxic effects on the cells. Despite toxic effects, the rates of the introduction and removal of cryoprotectants can affect the outcome of a freeze preservation protocol. According to Pedro et al. (2005), cells can be injured by osmotic over-swelling during the removal of the cryoprotectant after warming, if the diffusion of the permeated cryoprotectant out of the cell is too slow. Thus, knowledge of the biophysical properties of the cryoprotectant loading process is crucial to ensure the viability and functionality of the tissue post-thaw (Devireddy 2005).

Cryoprotectants are compounds that moderate the normally lethal effect of ice formation and the build-up of extracellular salts (Newton et al., 1998). Newton et al. (1998) have studied the rate of penetration of four permeating cryoprotectants (glycerol, EG, DMSO, and propylene glycol) in human ovarian tissue and shown that, in this respect, EG and DMSO provide the optimal protection of cells. In a typical cryopreservation protocol, the tissue to be preserved is first loaded with cryoprotectants to alleviate cell damage from either dehydration or intracellular ice formation during the subsequent freezing process (Devireddy and Bischof, 2003). When cryoprotectants are added to a tissue section, the concentration differences between the vascular space and intracellular space induce intracellular water (solvent) to leave the cells and concomitantly permeable cryoprotectants (solute) to move into the cells. This coupled solvent-solute transport causes osmotic and chemical injury to the tissue cells (Devireddy, 2005). Studying caprine preantral follicles, Rodrigues and co-workers (2004a,b, 2005) have found that the use of DMSO or EG (at a concentration of 1.5 M) as cryoprotectants results in a normal follicular morphology (i.e. histology and ultrastructure). However, the cryopreservation procedure does not maintain the percentage of morphologically normal preantral follicles at the level of those found for fresh non-frozen preantral follicles. Furthermore, Rodrigues et al. (2004a,b, 2005) provide no information regarding the viability of the studied preantral follicles and have not investigated the effects of sucrose. In our study, both EG and sucrose appear to be important for maintaining the viability of preantral follicles. EG is more efficient than DMSO. Although the protective action of cryoprotectants is considered colligative, each agent has its own specific properties (Pedro et al., 2005). EG (62.07 kDa) has a lower molecular weight than DMSO (78.13 kDa), and this facilitates its permeation into the ovarian tissue.

In vitro culture is an essential step in the evaluation of the cryopreservation process on preantral follicles. Several authors (murine: Newton and Illingworth, 2001; caprine: Rodrigues et al., 2005) have reported the maintenance of morphology and viability as the major problem in short-term cultures of cryopreserved preantral follicles. In comparison with the present study, Rodrigues et al. (2005) have only used intracellular cryoprotectants and at a higher concentration (1.5 M), which might explain the different observed effects on the in vitro survival of caprine follicles. The currently used in vitro culture protocol must be improved because of the significant reduction of viable fresh preantral follicles after culture for 24 h. Nevertheless, when preantral follicles are cryopreserved in a

mixture of EG and sucrose, their viability is maintained at a similar level to that observed for cultured fresh follicles. Thus, the use of this cryoprotectant solution is suitable for the effective permeation of ovarian tissue during cryopreservation and, when followed by the adequate removal of the cryoprotectant, preantral follicles are able to survive after short-term in vitro culture as successfully as fresh follicles.

Thus, the viability of preantral follicles, as assessed by stains for live versus dead cells and by short-term in vitro culture, is maintained at control levels after cryopreservation in a mixture of sucrose and EG. Further studies are required to evaluate the viability of frozen-thawed preantral follicles after in vitro culture for longer periods.

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References

Amorim CA, Rodrigues APR, Rondina D, Gonçalves PBD, Figueiredo JR, Giorgetti A (2003a) Cryopreservation of ovine primordial follicles using dimethyl sulfoxide. Fertil Steril 79:682–686

Amorim CA, Rondina D, Rodrigues APR, Costa SHF, Goncalves PBD, Figueiredo JR, Giorgetti A (2003b) Isolated ovine primordial follicles cryopreserved in different concentrations of ethylene glycol. Theriogenology 60:735–742

Amorim CA, Rondina D, Rodrigues APR, Goncalves PBD, Figueiredo JR, Giorgetti A (2004) Cryopreservation of isolated ovine primordial follicles with propylene glycol and glycerol. Fertil Steril 81:735–740

Bianchi V, Coticchio G, Fava L, Flamigni C, Borini A (2005) Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. Hum Reprod 20:1078–1083

Capacchietti G, Cecconi S, Gioia L, Turriani M (2004) Effect of cryoprotectant agents on the potential development of sheep preantral follicles. Vet Res Commun 28:173–176

Cecconi S, Capacchietti G, Russo V, Berardinelli P, Mattioli M, Barboni B (2004) In vitro growth of preantral follicles isolated from cryopreserved ovine ovarian tissue. Biol Reprod 70:12–17

Chen ZJ, Li M, Li Y, Zhao LX, Tang R, Sheng Y, Gao X, Chang CH, Feng HL (2004) Effects of sucrose concentration on the developmental potential of human frozen-thawed oocytes at different stages of maturity. Hum Reprod 19:2345–2349

Cortvrindt RG, Smitz JEJ (2001) Fluorescent probes allow rapid and precise recording of follicle density and staging in human ovarian cortical biopsy samples. Fertil Steril 75:588–593

De Clerck LS, Bridts CH, Mertens AM, Moens MM, Stevens WJ (1994) Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. J Immunol Methods 172:115–124

Demirci B, Salle B, Frappart L, Franck M, Guerin JF, Lornage J (2002) Morphological alterations and DNA fragmentation in oocytes from primordial and primary follicles after freezingthawing of ovarian cortex in sheep. Fertil Steril 77:595–600

Devireddy RV (2005) Predicted permeability parameters of human ovarian tissue cells to various cryoprotectants and water. Mol Reprod Dev 70:333–343

Devireddy RV, Bischof JC(2003) Recent advances in cryobiology using calorimetry. In: Kakac S, Smirnov H, Mila MR (ed) Low temperature and cryogenic refrigeration. Kluwer, The Netherlands, pp 265–294

Fabri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C (2001) Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 16:411–416

Gook DA, Edgar DH, Borg J, Archer J, McBain JC (2005) Diagnostic assessment of the development potential of human cryopreserved ovarian tissue from multiple patients using xenografting. Hum Reprod 20:72–78

Hovatta O, Silye R, Krausz T, Abir R, Margara R, Trew G, Lass A, Winston RM (1996) Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. Hum Reprod 11:1268–1272

Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, Van Den Donk HA, Van Den Hurk R (1995) Effects of fetal bovine serum, FSH and 17 β-estradiol on the culture of bovine preantral follicles. Theriogenology 44:217–226

Jorio A, Mariana JC, Lahlou-Kassi A (1991) Development of the population of ovarian follicles during the prepubertal period in D'man and Timahdite sheep. Anim Reprod Sci 26:239–250

Liu RH, Sun QY, Li YH, Jiao LH, Wang WH (2003) Maturation of porcine oocytes after cooling at the germinal vesicle stage. Zygote 11:299–305

Lucci CM, Amorim CA, Bao SN, Figueiredo JR, Rodrigues APR, Silva JRV, Goncalves PBD (1999) Effect of the interval of serial sections of ovarian tissue in the tissue chopper on the number of isolated caprine preantral follicles. Anim Reprod Sci 56:39–49

Lucci CM, Kacinskis MA, Lopes LHR, Rumpf R, Bao SN (2004) Effect of different cryoprotectants on the structural preservation of follicles in frozen zebu bovine (Bos indicus) ovarian tissue. Theriogenology 61:1101–1114

Mandelbaum J, Junca AM, Plachot M, Alnot MO, Alvarez S, Debache C, Salat-Baroux J, Cohen J (1988) Human embryo cryopreservation, extrinsic and intrinsic parameters of success. Hum Reprod 2:709–715

Martinez-Madrid B, Dolmans MM, Van Langendonckt A, Defrere S, Donnez J (2004) Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. Fertil Steril 82:1390–1394

Newton H, Illingworth P (2001) In vitro growth of murine preantral follicles after isolation from cryopreserved ovarian tissue. Hum Reprod 16:423–429

Newton H, Fisher J, Arnold JRP, Pegg DE, Faddy MJ, Gosden RG (1998) Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. Hum Reprod 13:376–380

Nowshari MA, Ali SA, Saleem S (2005) Offspring resulting from transfer of cryopreserved embryos in camel (Camelus dromedarius). Theriogenology 63:2513–2522

Pedro PB, Yokoyama E, Zhu SE, Yoshida N, Valdez DM Jr, Tanaka M, Edashige K, Kasai M (2005) Permeability of mouse oocytes and embryos at various developmental stages to five cryoprotectants. J Reprod Dev 51:235–246

Poole CA, Brookes NH, Clover GM (1993) Keratocyte networks visualized in the living cornea using vital dyes. J Cell Sci 106:685–692

Rodrigues APR, Amorim CA, Costa SH, Matos MHT, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004a) Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology 61:1009–1024

Rodrigues APR, Amorim CA, Costa SH, Matos MHT, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004b) Cryopreservation of caprine ovarian tissue using dimethylsulphoxide and propanediol. Anim Reprod Sci 84:211–227

Rodrigues APR, Amorim CA, Costa SHF, Santos RR, Lucci CM, JF Nunes, Figueiredo JR (2005) Cryopreservation and short-term culture of isolated caprine primordial follicles. J Small Rum Res 56:103–111

Salehnia M (2002) Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. Exp Anim 51:509–512

Santos RR, Rodrigues APR, Costa SHF, Silva JRV, Matos MHT, Lucci CM, Bao SN, Van den Hurk R, Figueiredo JR (2006) Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. Anim Reprod Sci 91:249–263

Schotanus K, Hage WJ, Vanderstchele H, Van den Hurk R (1997) Effects of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. Theriogenology 48:471–483

Smith GD, Silva E, Silva CA (2004) Developmental consequences of cryopreservation of mammalian oocytes and embryos. RBM Online 9:171–178

Van den Hurk R, Spek ER, Hage WJ, Fair T, Ralph JH, Schotanus K (1998) Ultrastructure and viability of isolated bovine preantral follicles. Hum Reprod Up 4:833–841

Chapter 3

Vitrification of goat preantral follicles enclosed in ovarian tissue by using conventional and solid-surface vitrification methods

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Abstract

Caprine preantral follicles within ovarian fragments were exposed to or vitrified in the presence of sucrose, dimethyl sulfoxide (DMSO), ethylene glycol (EG), or various combinations thereof. The fragments were cryopreserved by using either a conventional (CV) or a solid-surface vitrification (SSV) protocol, and the cryoprotectants were removed by equilibrating vitrified ovarian fragments in "warming solution" consisting of minimum essential medium and heat-inactivated fetal calf serum (MEM^+) followed by washes in MEM^+ with or without sucrose. Histological analysis of follicle integrity showed that the percentages of normal follicles in ovarian fragments vitrified in sucrose mixed with EG and/or DMSO (CV method) or mixed with EG or DMSO (SSV method) followed by washes in MEM^+ plus sucrose were similar to those of controls (ovarian fragments fixed without previous vitrification). Unlike for MEM^+ (supplemented or unsupplemented by sucrose) and DMSO followed by washes in the absence of sucrose, the percentages of normal follicles found after exposure to cryoprotectant did not significantly differ from that found after vitrification, indicating that follicular degeneration was attributable to a toxic effect of cryoprotectants and not to the vitrification procedure. The viability of preantral follicles after the CV and SSV procedures was investigated by using calcein-AM and the ethidium-homodimer as "live" and "dead" markers, respectively. In both tested vitrification procedures, the highest percentages of viable follicles were observed when a mixture of sucrose and EG (70% for CV and 72% for SSV) was used. Preantral follicles were also vitrified (either by CV or SSV) in sucrose and EG and then cultured for 24 h, after which their viability was compared with that of cultured fresh and uncultured vitrified follicles. The viability of these follicles was maintained after SSV, but not after CV. Thus, the viability of caprine preantral follicles can be best preserved after SSV in a mixture of sucrose and EG, followed by washes in medium containing sucrose.

Keywords
Preantral follicles ♦ vitrification ♦ viability markers ♦ in vitro culture ♦ caprine

Introduction

Cryopreservation by vitrification has been successfully applied in ovarian tissue from mouse (Salehnia et al., 2002) and human (Isachenko et al., 2003) with minimal changes in morphology. Few reports have been published, however, on cryopreservation by vitrification of ovarian tissue of small ruminants such as sheep (Al-Aghbari and Menino, 2002). To our knowledge, no reports exist concerning this treatment for caprine ovarian tissue. There is a growing need for oocyte and early staged follicle banking with regard to economically important and endangered goat species, since goats are commercially interesting farm animals.

In general practice, ovarian cryopreservation is often carried out by the slow freezing of tissues in a variety of intracellular (permeating) cryoprotectants such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG) to which are sometimes added extracellular (non-permeating) cryoprotectants such as sucrose. Slow freezing is an expensive method because of the employment of sophisticated freeze control machines. In addition, this procedure can cause damage by intracellular ice crystal formation. Cryopreservation by vitrification, however, involves very rapid cooling (-2,500°C/min) in which solutions go directly from the aqueous phase to a solid amorphous phase without exposure to a crystalline state, hence avoiding intracellular ice crystal formation in which damage can occur (Rall and Fahy, 1985).

To achieve the rapid cooling of tissue necessary for vitrification, several methods employ high cryoprotectant concentrations, generally between 20% (Isachenko et al., 2003) and 40% (Salehnia et al., 2002). In addition, to achieve rapid and optimal freezing, small volumes of vitrification solution are necessary, which, together with the tissue that has to be vitrified, are cooled by immersion in liquid nitrogen (LN_2). Grids for electron microscopy (Martino et al., 1996), glass capillaries (Hochi et al., 1994), open-pulled plastic straws (Vajta et al., 1997), and a new freezing technique called solid-surface vitrification (SSV; Begin et al., 2003) have been used to reduce the volume of vitrification solution. This vitrification technique necessitates direct exposure of tissue to a solid surface cooled from −150°C to −180°C (Dinnyes et al., 2000).

The vitrification solutions used in our experiments contain permeating (EG or DMSO) or non-permeating (sucrose) cryoprotectants or mixtures of both.

Vitrification simplifies the process of cooling to a great extent and decreases chilling injury. One drawn back is, however, that cryoprotectants may induce toxicity, extracellular ice formation, and adverse osmotic effects. In order to assess the vitrification process, morphological studies are inadequate because the analysis of morphology is not always correlated with the viability or developmental competence of the follicles. Schotanus et al., (1997) were the first to show that viability analysis by using the fluorescent markers, calcein-AM and ethidium homodimer-1, can be employed as a tool to predict whether ovarian follicle cells are viable. This viability analysis has been used to evaluate the quality of isolated bovine (Schotanus et al., 1997; Van den Hurk et al., 1998), murine (Cortvrindt and Smitz, 2001), and human (Martinez-Madrid et al., 2004) preantral follicles.

The aim of this study has been to develop a vitrification procedure that optimally preserves the morphology, viability, and in vitro developmental capacity of caprine preantral follicles. Follicles have been vitrified according to two different procedures, viz., conventional vitrification (CV) and SSV, by using sucrose, DMSO, and EG, or mixtures thereof. Follicles frozen by these protocols have also been subsequently thawed, and some have been cultured for 24 h. The morphology of preantral follicles has been analyzed after staining with hematoxylin-eosin, and their viability has been investigated by using calcein-AM and the ethidium homodimer as “live” and “dead” markers, respectively.

Materials and Methods

Source and preparation of ovarian tissue

This study comprised three experimental conditions. For each experiment, ovaries from healthy adult mixed-breed goats (Capra hircus) were obtained at a local slaughterhouse (Monfoort, The Netherlands). The material was transported in thermos flasks at 30°C, within 1 h of slaughter, to the laboratory where the ovaries were trimmed from adhering tissue followed by a wash in 70% alcohol and two washes in phosphate-buffered saline (PBS). The cortex from each pair of ovaries was then removed and cut into small fragments (1 mm^3) that were then placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco, Paisley, UK) at room temperature (25°C). Pieces of 1 mm3 in size were used for all experiments. Selected ovarian fragments were randomly distributed over the experiments. All

fragments contained sufficient numbers (more than 30) of preantral follicles, and no obvious differences in the numbers of preantral follicles were observed.

Experimental design
Experiment I: histological analysis of vitrified-warmed ovarian tissue

CV method

After the dissection of 10 paired ovaries, 33 fragments of 1 mm^3 were collected from each pair. One fragment (control) was fixed in 4% paraformaldehyde for 12 h for routine histological examination. The remaining 32 fragments were submitted either to the toxicity test (16 fragments) or to the vitrification procedure (16 fragments). For toxicity testing and vitrification, minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL; MEM^+) was used as the holding medium supplemented with sucrose, DMSO, EG, or a mixture of these cryoprotectants, or unsupplemented. Subsequently, ovarian fragments were fixed in 4% paraformaldehyde and analyzed. To perform CV, two vitrification solutions (VS) were used. VS-I contained MEM^+ with or without 0.5 M sucrose, 20% EG and/or 20% DMSO and was prepared prior to use. Similarly, VS-II was composed of MEM^+ with or without 0.5 M sucrose to which 40% EG and/or 40% DMSO was added or omitted. For cryoprotectant removal, cryoprotectant diluent (CD) solutions were prepared prior to use. CD-I consisted of MEM^+ with 0.3 M sucrose, CD-II consisted of MEM^+ with 0.15 M sucrose, and CD-III consisted of MEM^+ only. Each fragment was initially kept at room temperature in the holding medium for ~10 min, followed by exposure to VS-I for 4 min. Subsequently, the fragments were exposed to VS-II for 1 min and transferred to straws containing this vitrification solution. Immediately after this equilibration period, 16 fragments were submitted to cryoprotectant removal and were fixed for histological analysis, whereas the remaining straws were immersed vertically into liquid nitrogen. These vitrified ovarian fragments were stored in liquid nitrogen (−196°C) for 1 week. The straws were thawed in air for 1 min at room temperature (~25°C) and then vertically immersed in CD-I solution at 37°C in a water bath for 5 min, followed by CD-II and CD-III each for 5 min or in three washes of CD-III each for 5 min. After removal of cryoprotectant, ovarian fragments were fixed for histological analysis (see experimental protocol at Fig. 1).

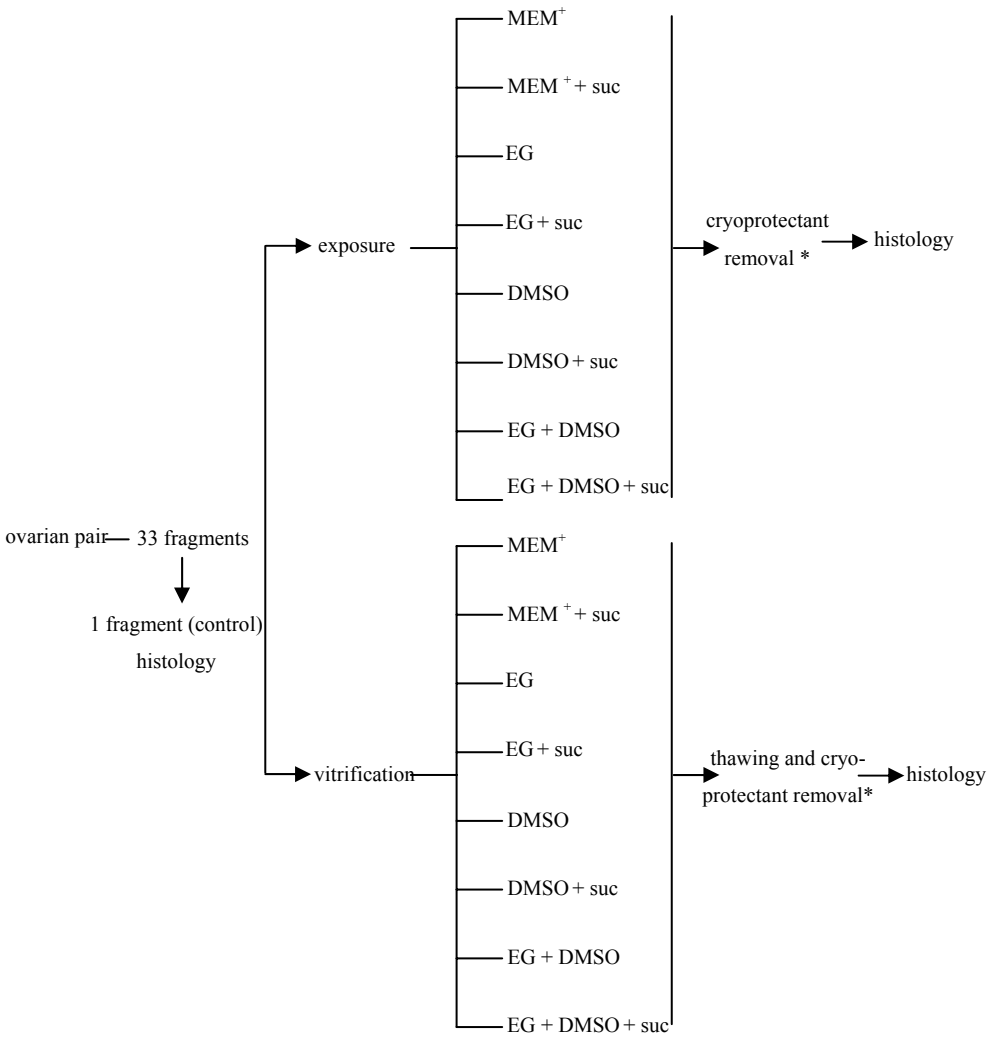

Fig. 1 Experimental protocol for experiment I using CV or SSV method (*DMSO* dimethyl sulfoxide, *EG* ethylene glycol, *MEM* minimum essential medium, *suc* Sucrose) * cryoprotectant removal was performed washing the tissue in three steps using MEM only or added by sucrose at two first steps (0.3 and 0.15 M)

SSV method

After dissection of 10 pair of ovaries, 33 fragments of 1 mm^3 were collected from each pair. As for CV (see Fig. 1), one fragment (control) was fixed

in 4% paraformaldehyde for 12 h for routine histological examination, and the remaining 32 fragments were submitted to the SSV procedure by using MEM^+ or MEM^+ supplemented with 0.5 M sucrose, 35% DMSO, 35% EG, or a combination of these cryoprotectants. Vitrification solution (VS) was prepared just prior to use. CD-I, -II, and -III were the same as those used for CV. Each fragment was initially kept at room temperature in the holding medium for ~10 min, followed by VS for 5 min. As for CV, 16 fragments were submitted to cryoprotectant removal and fixed for histological analysis, and the remaining 16 fragments were placed on a cold surface, consisting of a hollow cube of aluminium foil partially immersed in liquid nitrogen. The vitrified fragments were transferred into cryovials, by using nitrogen-cooled forceps, for storage in the liquid phase of a liquid nitrogen tank. Vitrified ovarian fragments were maintained in cryostorage for 1 week. The cryovials were treated as described above for straws and then fragments were immersed in a CD-I solution in a water bath at 37°C for 5 min followed by CD-II and CDIII for 5 min each or in three washes of CD-III each for 5 min. After cryoprotectant removal, ovarian fragments were fixed for histological analysis.

Histological analysis

The fixed ovarian tisues were dehydrated, embedded in paraffin wax, and serially sectioned at 5 μm. Every seventh section was mounted and stained with hematoxylin-eosin. All sections were examined by using a light microscope (Olympus, Tokyo, Japan) at a magnification of ×400. Follicles were classified as preantral if they had no antrum. This included oocytes surrounded by one layer of flattened and/or one or more layers of cuboidal granulosa cells. Only preantral follicles in which the oocyte nucleus could be observed in the section were counted. Follicular quality was evaluated based on the morphological integrity of the oocyte, granulosa cells, and basement membrane. Preantral follicles were histologically classified as (1) normal, when they contained an intact oocyte and intact granulosa cells, (2) degenerated, when they contained a pyknotic oocyte nuclei, shrunken ooplasm, and/or disorganized granulosa cells (e.g. enlargement in volume and/or detachment from the basement membrane).

Experiment II: viability of SSV or CV preantral follicles after being warmed

For each protocol, five ovarian pairs were used. From each ovarian pair, five fragments of 1 mm^3 were obtained. One fragment (control) was immediately

submitted to follicular isolation according to the procedure described below. The viability of oocytes and surrounding granulosa cells of preantral follicles was analyzed by using "live" (calcein-AM) and "dead" (ethidium homodimer-1) cell markers (see below). The remaining four fragments were subjected to the vitrification procedure (CV or SSV) by using the cryoprotectant solutions that showed the best results in experiment I. For CV, we used: (1) 0.5 M sucrose, (2) 20% DMSO + 0.5 M sucrose (VSI) followed by 40% DMSO + 0.5 M sucrose (VS-II), (3) 20% EG + 0.5 M sucrose (VS-I) followed by 40% EG + 0.5 M sucrose (VS-II), and (4) 20% DMSO + EG + 0.5 M sucrose (VS-I) followed by 40% DMSO + EG + 0.5 M sucrose (VS-II). For SSV, we used: (1) 0.5 M sucrose, (2) 35% DMSO + 0.5 M sucrose, (3) 35% EG + 0.5 M sucrose, and (4) 35% DMSO + EG + 0.5 M sucrose. After the thawing step, the cryoprotectant was removed by washing the fragments in CD-I, CD-II, or CD-III. The preantral follicles were then mechanically isolated from the fragments and submitted to viability analysis (Fig. 2).

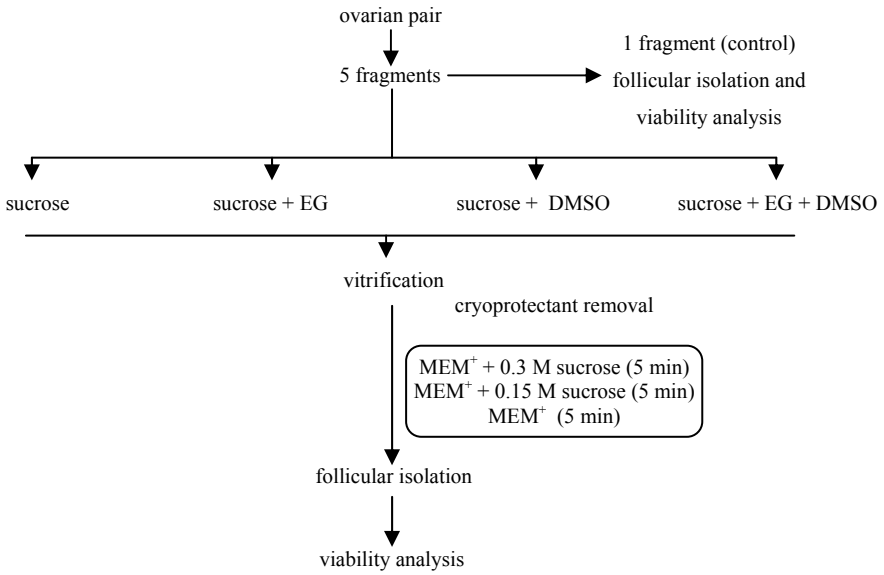

Fig. 2 Experimental protocol for experiment II (abbreviations as in Fig. 1)

Experiment III: effect of in vitro culture on the viability of vitrified-warmed preantral follicles

Experiment III was designed to assess the viability of vitrified preantral follicles (comparing both procedures) before and after short-term (24 h) in vitro culture. Six fragments of 1 mm^3 were obtained from each ovarian pair collected from five goats. Preantral follicles were isolated from two fragments (fresh control) and immediately used for viability assessment or were cultured in vitro for 24 h and subsequently examined for their viability. The remaining four fragments were subjected to CV or SSV with sucrose + EG as cryoprotectants in the same concentrations used for experiment II. After being thawed and the subsequent removal of the cryoprotectant, preantral follicles were mechanically isolated from the fragments, cultured in vitro for 24 h or used fresh, and subsequently submitted to viability analysis.

Assessment of oocyte and granulosa cells viability

Caprine preantral follicles were isolated from fresh and vitrified ovarian fragments by applying a mechanical procedure as described by Lucci et al., (1999). Briefly, the ovarian cortex was cut into small fragments by using a tissue chopper (Meyvis, Gouda, the Netherlands). The ovarian fragments were then placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco, Paisley, UK) at room temperature (~25°C) and pipetted 40 times with a Pasteur pipette to release the preantral follicles. The suspension was successively filtered through 500- and 100-µm nylon mesh filters. The isolation procedure was carried out within 10 min. Preantral follicles smaller than 100 µm were collected under a dissecting stereomicroscope (SZSTS, Olympus, Tokyo, Japan) and transferred to HEPES buffered M-199 + 1% bovine serum albumin (holding medium). Isolated follicles were incubated in the holding medium for 10 min at 37°C in a mixture of 4 µM calcein-AM, 2 µM ethidium homodimer-1 (Molecular Probes Europe, Leiden, The Netherlands) and 10 µM Hoechst 33342 (Sigma) to detect esterase enzyme activity and membrane integrity and to enable the nuclei to be counted, respectively. After being labelled, stained follicles were washed three times in holding medium, mounted on a glass microscope slide in 5 µl antifading medium (Vectashield, Vector Lab., Burlingame, Calif.) to prevent photobleaching, and finally examined by using an epifluorescence microscope (BH2-RFCA, Olympus, Tokyo, Japan) equipped with a digital camera (Coolpix900, Nikon Instruments Europe,

Badhoevedorp, The Netherlands). The emitted fluorescent signals of Hoechst, calcein-AM, and ethidium homodimer were collected at 350, 488, and 568 nm, respectively. Oocytes and granulosa cells were classified as viable if the ooplasm was stained positively with calcein-AM, and chromatin was not labelled with ethidium homodimer (Fig. 3). Percentages of viable granulosa cells were calculated in relation to the total number of Hoechst positive nuclei.

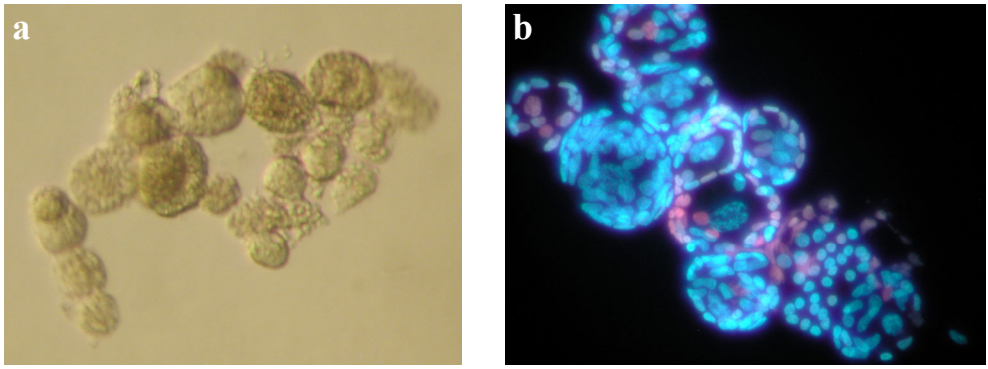

Fig. 3 Vitrified-warmed preantral caprine follicles before (**a**) and after (**b**) staining with Hoechst (*blue*; general nuclear staining), ethidium homodimer-1 (*red*; stains nuclei of dead cells). Absence of ethidium-staining in oocytes and follicular cells indicates their viability. ×400.

In vitro culture

Preantral follicles (less than 100 µm in diameter) were cultured in 4-well culture dishes (25 follicles per well) in a 20 µl droplet of culture medium under mineral oil at 39°C in a humidified atmosphere of 5% CO2 in-air (Nunc, Roskilde, Denmark). The culture medium consisted of alpha MEM (Sigma) supplemented with SPIT (5 ng/ml selenium, 110 µg/ml pyruvate, 10 µg/ml insulin, and 5.5 µg/ml transferrin; Sigma), 100 µg/ml penicillin/streptomycin (Gibco), 10% fetal calf serum (Gibco), 0.01 IU/ml human follicle-stimulating hormone (hFSH) recombinant (Organon, Oss, The Netherlands), and 10 ng/ml activin-A (R&D System Europe, Abingdon, UK).

Statistical analysis

In experiment I, preantral follicle quality was assessed morphological and histologically, and the mean percentage of normal preantral follicles in exposed, vitrified and control control fragments were compared by a one-way analysis of

variance (ANOVA) and Tukey's test. In experiments II and III, the viability of oocytes and granulosa cells was analyzed by the chi-square test, and the numbers of total nuclei per follicle were compared by one-way ANOVA. In all cases, the statistical tests were performed by using Stat View for Windows, and the differences were considered significant when $P<0.05$.

Results

Experiment I: histological analysis of vitrified-warmed ovarian tissue

In total, 9966 preantral follicles were histologically examined following exposure or vitrification-warming of caprine ovarian fragments (approximately 150 follicles per treatment in each procedure). When tissues were subjected to CV or SSV and subsequently washed in the absence of sucrose, degenerating preantral follicles generally showed nuclear pycnosis, cytoplasmic retraction, and coagulative necrosis. These follicles also exhibited an increased volume and contained numerous cytoplasmic vacuoles.

For both procedures, addition of sucrose to the washing medium of vitrified follicles significantly improved ($P<0.05$) follicle morphology in all treatments, except when ovarian fragments were vitrified in media containing DMSO without sucrose. Data from exposure (toxicity) tests compared with those of vitrification tests demonstrated that the percentages of normal preantral follicles did not significantly differ ($P>0.05$), except when the cryopreservation medium consisted of MEM^+ either supplemented or unsupplemented with sucrose. Similar results were observed when preantral follicles were vitrified in the presence of only DMSO and washed in the absence of sucrose after CV.

CV evaluation

The percentage of morphologically normal follicles in control ovarian fragments (84%) was not significantly different ($P>0.05$) from that observed after exposure to MEM^+ supplemented either with or without sucrose and followed by washes in sucrose (80%–86%) or without sucrose (Table 1). Similar percentages were found when DMSO (78%), EG (83%), or both (79%) were used in combination with sucrose and after cryoprotectant removal in the presence of

sucrose. Likewise, vitrification did not significantly alter the percentages of normal follicles when sucrose was used in combination with EG (82%), DMSO (80%), or DMSO + EG (78%). In contrast, vitrification of caprine ovarian tissue with sucrose alone as cryoprotectant resulted in a significantly ($P<0.05$) lower percentage (70%) of normal follicles.

Table 1 Mean percentages (± SEM) of histologically normal preantral follicles in control ovarian fragments and in fragments exposed and/or vitrified-warmed (*CV* conventional vitrification) in the presence (E/V^+) or absence (E/V^-) of sucrose and washed in the presence (W^+) or absence (W^-) of sucrose (150 follicles/treatment).

	MEM^+		DMSO		EG		DMSO + EG	
	exposure	CV	exposure	CV	exposure	CV	exposure	CV
E/V^-W^-	$86±3.1^{aAE}$	$4±3.5^{*dCF}$	$53±7.4^{*cBE}$	$41±3.2^{*cAF}$	$18±2.9^{*dDE}$	$13±2.6^{*dBE}$	$44±1.9^{*bCE}$	$45±4.2^{*bAE}$
E/V^+W^-	$81±4.2^{aAE}$	$22±2.6^{*cDF}$	$65±1.8^{*bBE}$	$64±5.2^{*bAE}$	$42±5.2^{*cCE}$	$35±4.8^{*cCE}$	$63±3.1^{*cBE}$	$59±5.7^{*cBE}$
E/V^-W^+	$80±3.8^{aAE}$	$39±4.3^{*bBF}$	$8±1.6^{*dCE}$	$8±2.7^{*dCE}$	$64±5.9^{*bBE}$	$52±6.7^{*bAE}$	$5±2.9^{*aCE}$	$5±1.8^{*aCE}$
E/V^+W^+	$81±2.5^{aAE}$	$70±1.4^{*aBF}$	$78±4.2^{aAE}$	$80±2.4^{aAE}$	$84±2.6^{aAE}$	$82±3.6^{aAE}$	$79±4.3^{dAE}$	$78±2.1^{dAE}$
Control	84±4.1							

***** Differs significantly from control; $^{a-d}$Values within columns are significantly different; $^{A-D}$Values within rows are significantly different among each cryopreservation medium when exposure or vitrification tests are compared; $^{E, F}$Values within rows are significantly different between exposure and vitrification tests ($P<0.05$)

SSV evaluation

As in the CV procedure, the percentage of morphologically normal preantral follicles did not change from control value (84%; $P>0.05$) when ovarian fragments were exposed to MEM^+ supplemented with sucrose (82%–85%) or without sucrose (Table 2). This also occurred when fragments were exposed to DMSO (85%) or EG (86%) in combination with sucrose and when cryoprotectant removal was performed in the presence of sucrose. Vitrification of ovarian fragments did not significantly influence ($P>0.05$) the percentage of normal follicles compared with that in non-frozen control fragments (87%) when sucrose was mixed with EG (79%) or DMSO (81%). Vitrification in a mixture of sucrose, EG, and DMSO or in sucrose alone, however, resulted in significantly lower ($P<0.05$) percentages of normal preantral follicles (65% and 74%, respectively).

Table 2 Mean percentages (± SEM) of histologically normal preantral follicles in control ovarian fragments and in fragments exposed and/or vitrified-warmed (*SSV* solid-surface vitrification) in the presence (E/V^+) or absence (E/V^-) of sucrose and washed in the presence (W^+) or absence (W^-) of sucrose (150 follicles/treatment).

	MEM^+		DMSO		EG		DMSO + EG	
	exposure	SSV	exposure	SSV	exposure	SSV	exposure	SSV
E/V^-W^-	83 ± 3.3^{aAE}	$6\pm4.8^{*dCF}$	$60\pm7.6^{*bBE}$	$68\pm2.3^{*bAE}$	$11\pm2.8^{*dCE}$	$11\pm3.4^{*dBE}$	$66\pm3.4^{*bBE}$	$66\pm3.6^{*aAE}$
E/V^+W^-	86 ± 2.6^{aAE}	$21\pm3.7^{*cCF}$	$55\pm5.5^{*bBE}$	$45\pm4.8^{*cAE}$	$23\pm6.3^{*cCE}$	$29\pm1.7^{*cBE}$	$54\pm3.9^{*cBE}$	$45\pm4.4^{*bAE}$
E/V^-W^+	83 ± 2.2^{aAE}	$43\pm2.8^{*bAF}$	$6\pm2.9^{*cCE}$	$8\pm3.6^{*dBE}$	$56\pm8.2^{*bBE}$	$38\pm6.9^{*bAE}$	$10\pm3.6^{*dCE}$	$6\pm1.8^{*cBE}$
E/V^+W^+	83 ± 2.5^{aAE}	$75\pm2.4^{*aBF}$	85 ± 3.6^{aAE}	82 ± 4.1^{aAE}	87 ± 4.8^{aAE}	80 ± 4.5^{aAE}	$70\pm4.2^{*aBE}$	$66\pm2.9^{*aCE}$
Control	88 ± 3.5							

***** Differs significantly from control; $^{a-d}$Values within columns are significantly different; $^{A-C}$Values within rows are significantly different among each cryopreservation medium when exposure or vitrification tests are compared; $^{E, F}$Values within rows are significantly different between exposure and vitrification tests ($P<0.05$)

Experiment II: viability of SSV or CV preantral follicles after being warmed

Preantral follicles were mechanically isolated from cryopreserved ovarian fragments shortly after being thawing, and 1237 preantral follicles were examined for their viability (approximately 100 follicles per treatment in each procedure).

CV evaluation

All CV treatments significantly ($P<0.05$) reduced the percentage of viable oocytes when compared with control values (91%; Fig. 4). The viability of vitrified oocytes was optimally maintained when sucrose was added to EG (82%) or to EG + DMSO (76%). The percentages were significantly higher ($P<0.05$) than those found after vitrification in sucrose + DMSO (64%), which in its turn was significantly higher ($P<0.05$) than that of oocytes vitrified in presence of sucrose only (45%).

With regard to viable follicles (follicles with a viable oocyte enveloped by 90% or more viable granulosa cells), as observed for oocytes, all conventional vitrification treatments significantly ($P<0.05$) reduced the percentage of viable preantral follicles when compared with control values (87%; Fig. 2). Again as shown for oocytes, the highest percentage of viable vitrified-warmed preantral follicles was found after vitrification of ovarian tissue in a mixture of sucrose and EG (70%). This percentage was significantly higher ($P<0.05$) than that of follicles

cryopreserved in sucrose + EG and DMSO (62%), which in its turn was higher (P<0.05) than that of follicles vitrified-warmed in DMSO + sucrose (50%). The lowest percentages of viable preantral follicles (12%) were found when only sucrose was used as cryoprotectant.

SSV evaluation

All SSV treatments significantly (P<0.05) reduced the percentage of viable oocytes when compared with control values (93%; Fig. 4). The viability of oocytes was optimally maintained when sucrose was added to EG (80%), DMSO (78%), or EG + DMSO (83%), the percentages being significantly higher (P<0.05) than the percentage obtained after vitrification in sucrose only (53%).

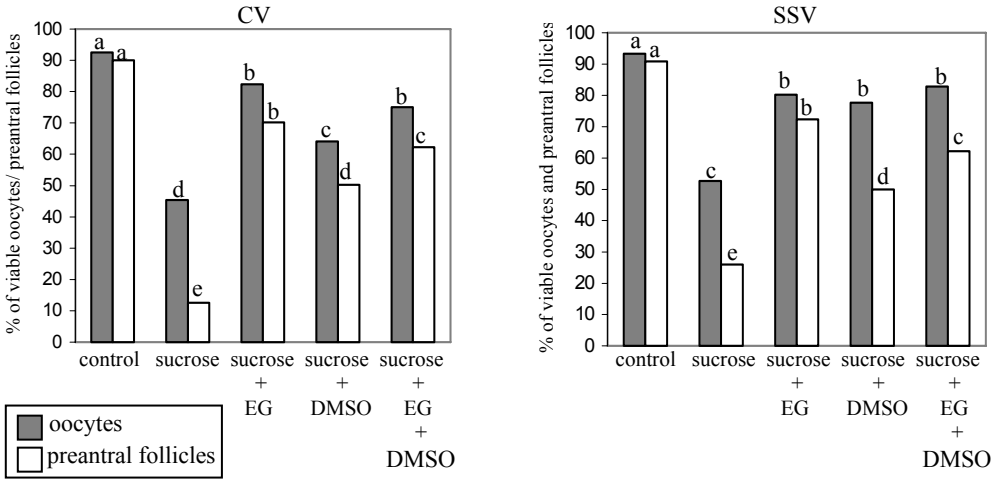

Fig. 4 Percentages of calcein-AM-positive and ethidium-homodimer-negative (viable) oocytes (filled bars) and preantral follicles (*open bars*), i.e. follicles with a viable oocyte surrounded by ≥ 90% viable granulosa cells, in control ovarian fragments and in fragments vitrified by the conventional and solid-surface procedures (*a–e* values are significantly different at P<0.05).

As observed for follicular oocytes, all vitrification treatments significantly (P<0.05) reduced the percentage of viable preantral follicles when compared with control values (91%; Fig. 2). Again as shown for oocytes, the highest percentage of viable vitrified-warmed preantral follicles was found after vitrification of ovarian tissue in a combination of sucrose and EG (72%). This percentage was significantly higher (P<0.05) than that of follicles that had been cryopreserved in sucrose + EG and DMSO (62%), which in its turn was higher (P<0.05) than that of

follicles vitrified-warmed in DMSO + sucrose (50%). Once more, low percentages of viable preantral follicles were found when sucrose only (26%) was used as cryoprotectant.

Experiment III: effect of in vitro culture on the viability of vitrified-warmed preantral follicles

In total, 512 preantral follicles were examined for their viability before and after short-term culture (24 h), i.e. more than 80 follicles per treatment. Table 3 shows the percentage of viable oocytes and preantral follicles (i.e. viable oocyte surrounded by >90% of granulosa cells).

Table 3 Percentages of viable oocytes and viable preantral follicles, with a viable oocyte surrounded by 90% or more viable granulosa cells, after isolation (fresh follicles) and freezing-thawing (*FT*), before and after 1 day culture (*n* = 50-55 follicles; *NA* not applicable; *EG* ethylene glycol).

Preantral follicles	Vitrification procedure	Viability percentages			
		Before culture		After 24 h	
		oocytes	preantral follicles	Oocytes	preantral follicles
Fresh	None	95^{aA}	90^{aA}	72^{aB}	65^{aB}
Vitrified	CV	81^{bA}	68^{bA}	61^{bB}	55^{bB}
	SSV	79^{bA}	69^{bA}	70^{aB}	$58a^{bB}$

$^{a-d}$Values within columns (comparing oocytes or preantral follicles) are significantly different; $^{A-C}$Values within rows (oocytes or preantral follicles) are significantly different ($P<0.05$)

In accord with the results observed in experiment II (Fig. 4), EG + sucrose yielded a high viability (without additional culture) of oocytes (81% for CV; 79% for SSV) and preantral follicles (68% for CV; 69% for SSV) and significantly lower percentages than in non-frozen controls (95% and 90% viability for oocytes and preantral follicles, respectively; $P<0.05$). The in vitro culture of isolated preantral follicles for 24 h, however, dramatically decreased their viability, both when they were cultured freshly (non-vitrified; 95% vs. 72% for oocytes; 90% vs 65% for preantral follicles; $P<0.05$) and after CV (81% vs. 61% for oocytes; 68% vs. 55% for preantral follicles; $P<0.05$) or SSV (79% vs. 70% for oocytes; 69% vs. 58% for preantral follicles; $P<0.05$). Nevertheless, SSV gave percentages of oocytes and preantral follicles similar to those observed in non-vitrified follicles.

Discussion

After the exposure of follicles to sucrose, DMSO, EG, or a mixture of these cryoprotectants or after their vitrification in the presence of these compounds by using CV or SSV, a combination of sucrose and EG appeared to be the best cryoprotectant for the preservation of follicular morphology and viability. This finding corroborated those of a previous study (Santos et al., 2006), in which we examined the effect of conventional cryopreservation (slow-cooling) on caprine preantral follicles. We showed the importance of the presence of both non-permeating (sucrose) and permeating (EG) cryoprotectants in a freezing solution for the successful preservation of follicular morphology and viability. Permeating cryoprotectants are organic solutes, which protect the structure and functioning of the intracellular organelles of the cells during cooling and warming (Bautista and Kanagawa 1998). Non-permeating cryoprotectants are macromolecules such as sugars whose role is to reduce ice crystal formation during freezing and that facilitate the dehydration of cells prior to cooling (Begin et al., 2003). When the results from the latter study are compared with the current investigation, the use of a mixture of sucrose and EG as cryoprotectants makes the vitrification of goat preantral follicles as successful as slow cryopreservation, since similar rates of viable preantral follicles have been obtained after both vitrification-warming procedures. Our results furthermore correspond with those obtained by Salehnia et al. (2002) who have shown the protective effect of vitrification with 40% EG + 0.5 M sucrose on the ultrastructure of murine preantral follicles.

Vitrification has important advantages above the conventional cryopreservation of tissues. The latter slow cooling method requires the use of a freeze-control machine, which enables rapid cooling to the temperature below the melting point of the extracellular solution (generally −7°C), at which point ice crystals form in the bathing medium. Dehydration continues slowly until the cells are immersed in liquid nitrogen, usually when a temperature below −30°C has been reached (Stachecki and Cohen, 2004). In contrast, vitrification is a relatively new freezing method that does not require the use of expensive equipment and involves exposure of the cells to high concentrations of cryoprotectant for brief periods of time. With this method, the formation of amorphous crystal-like structures rather than that of actual crystals is induced (Hovatta, 2005), probably because of the high osmolarity of the vitrification solution. Furthermore, during vitrification, cooling is so rapid that structural damage attributable to crystal formation does not occur, and

a glass-like solid structure forms instead of crystals (Stachecki and Cohen, 2004). A disadvantage of the vitrification procedure, however, is the necessity of high cryoprotectant concentrations, which can cause cell damage because of their toxicity. To limit toxic damage, it is necessary to use an optimum equilibration period. Exposure of ovarian tissue to vitrification media has been performed over periods ranging from 20 s (two steps of 10 s each; Salehnia, 2002) to 30 min (two steps of 15 min each; Rahimi et al., 2004). Based on preliminary studies in which equilibration times of 1–10 min have been tested (R.R. Santos, unpublished), we have used a 5-min cryoprotectant exposure. In the present study, vitrified ovarian tissue does not show a reduction of viability rates when compared with that only exposed to cryoprotectant agents, indicating the success of the vitrification procedure. Only CV with DMSO in the absence of sucrose and subsequent washes in the absence of sucrose results in a further decrease of the percentage of normal follicles compared with exposure only to DMSO. DMSO and EG are the most commonly used permeable cryoprotectants, DMSO presenting a higher rate of permeation than EG. However, DMSO is more toxic (Shaw et al., 2000) than EG. Similar observations have been obtained in this present investigation, since the use of DMSO in our exposure studies results in higher rates of follicular degeneration than when EG is used. After studying the effects of glycerol, EG, DMSO, and propylene glycol on the morphology of caprine preantral follicles, Rodrigues et al., (2004a,b) have concluded that DMSO and EG are the best cryoprotectants. However, these authors have not compared the morphological effects of DMSO with those caused by EG. A beneficial effect of EG during vitrification has also been shown with respect to the morphology of human ovarian tissue and follicles (Isachenko et al., 2003). The effect of both cryoprotectants on the follicular survival is improved when sucrose is added to cryopreservation media.

Several reports (Toth et al., 1994; Isachencko et al., 2003; Chen et al., 2004) have indicated a beneficial effect of supplementing the freezing medium with sucrose, which acts by facilitating the dehydration and rehydration of a cell at a secure rate. During cell dehydration by cryopreservation, a delicate balance must be maintained between the removal of free water that could form ice crystals and the removal of bound water, because excessive loss of bound water results in the loss of structural support to proteins and lipids (Wright et al., 2004). When sucrose is employed as a cryoprotectant, it may serve as a replacement for bound water on the membranes to diminish injury from dehydration stresses. Previously, Crowe et al. (1998) have postulated that sugars are useful in the stabilization of lipid

membranes and proteins when cells are dehydrated. Fabri et al. (2001) have also demonstrated the importance of 0.5 M sucrose in the protection of human oocytes during freezing.

In most studies on follicle cryopreservation, only visual observations of gross follicular morphology have been made. In order to improve freezing protocols significantly, information must be obtained with regard to the viability of the cells. Some authors have used trypan blue as a marker of the viability of sheep preantral follicles (Demirci et al., 2001; Amorim et al., 2003). However, trypan blue only detects membrane integrity and, in contrast to fluorescent markers such as ethidium homodimer and calcein-AM, does not give information regarding DNA integrity and cytoplasmic enzymatic activity, respectively. Calcein-AM is cleaved by esterase enzymes in the cytoplasm of living cells, and the cleavage product, a fluorescent compound, remains in the cytoplasm (De Clerck et al., 1994). Ethidium homodimer-1 is used to assess cell plasma membrane integrity, since it only binds to the DNA of cells with compromised membranes (Poole et al., 1993). Such fluorescent probes have been used to assess membrane integrity and cell viability after the cryopreservation of sperm (Pena et al., 2005) and of somatic cells such as Schwann cells (Decherchi et al., 1997). Recently, these fluorescent probes have been used to demonstrate the viability of goat preantral follicles after slow-cooling (Santos et al., 2006).

The in vitro culture of preantral follicles is an important tool to evaluate the success of follicular cryopreservation. Once preantral follicles are submitted to the cryopreservation procedure, dehydration and rehydration during cryoprotectant removal can cause changes in the morphology and viability of the follicles. To reacquire their normal morphology and to observe the effects of cryopreservation, equilibration in a warm medium is necessary; this can be achieved by short-term in vitro culture. The process of re-warming the cryopreserved tissue in a nutrient-rich environment allows the follicular cells to re-establish metabolic activity, normal cell volume, and cell-cell contacts (Paynter et al., 1999). In a previous study of a slow-cooling protocol to freeze goat preantral follicles, we have shown that the viability status of cryopreserved preantral follicles is more precisely determined after short-term in vitro culture than immediately after their being thawed (Santos et al., 2006). Although the currently used in vitro culture protocol must be improved because of the significant reduction of viable fresh preantral follicles after 24-h culture, the viability of preantral follicles vitrified by SSV is similar to

that of non-frozen follicles and is better maintained than that of follicles vitrified by CV. CV has previously been described for rabbit embryos (Vicente and Garcia-Ximenez, 1996; Naik et al., 2005) and has been used in our study with some modifications, whereas the alternative (SSV) technique has been applied for bovine oocytes (Dinnyes et al., 2000) and goat oocytes (Begin et al., 2003) derived from antral follicles. The currently observed superiority of the SSV procedure over CV might be attributable to the small volume of vitrification solution that we have used based on the study of Yeoman et al. (2005) who have shown that the minimizing of the volume of the vitrified sample helps to maximize cooling rates and improves vitrification of monkey ovarian tissue.

In conclusion, both SSV and CV of caprine preantral follicles in a mixture of sucrose and EG or sucrose and DMSO, followed by rinsing in medium with sucrose, can effectively be applied to maintain their morphology. However, to maintain the viability of vitrified and in vitro cultured preantral follicles, caprine ovarian tissue has to be vitrified using the SSV method in a mixture of sucrose and EG.

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References

Al-Aghbari AM, Menino AR (2002) Survival of oocytes recovered from vitrified sheep ovarian tissues. Anim Reprod Sci 71:101-110

Amorim CA, Rondina D, Rodrigues APR, Costa SHF, Goncalves PBD, Figueiredo JR, Giorgetti A (2003) Isolated ovine primodial follicles cryopreserved in different concentrations of ethylene glycol. Theriogenology 60:735-742

Bautista JA, Kanagawa H (1998) Current status of vitrification of embryos and oocytes in domestic animals: ethylene glycol as an emerging cryoprotectant of choice. Jpn J Vet Res 45:183-191

Begin I, Bhatia B, Baldassarre H, Dinnyes A, Keefer CL (2003) Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. Theriogenology 59:1839-1850

Chen ZJ, Li M, Zhao LX, Tang R, Sheng Y, Gao X, Chang CH, Feng HL (2004) Effects of sucrose concentration on the developmental potential of human frozen-thawed oocytes at different stages of maturity. Hum Reprod 19:2345-2349

Cortvrindt RG, Smitz JEJ (2001) Fluorescent probes allow rapid and precise recording of follicle density and staging in human ovarian cortical biopsy samples. Fertil Steril 75:588-593

Crowe JH, Carpenter JF, Crowe LM (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60:73-103

De Clerck LS, Bridts CH, Mertens AM, Moens MM, Stevens WJ (1994) Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. J Immunol Methods 172:115-124

Decherchi P, Cochard P, Gauthier P (1997) Dual staining assessment of Schwann cell viability within whole peripheral nerves using calcein-AM and ethidium homodimer. J Neurosci Methods 71:205-213

Demirci B, Lornage J, Salle B, Frappart L, Franck M, Guerin JF (2001) Follicular viability and morphology of sheep ovaries after exposure to cryoprotectant and cryopreservation with different freezing protocols. Fertil Steril 75:754-762

Dinnyes A, Dai Y, Jiang S, Yang X (2000) High developmental rates of vitrified bovine oocytes following parthenogenetic activation, and somatic cell nuclear transfer. Biol Reprod 63:513-518

Fabri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C (2001) Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 16:411-416

Hochi S, Fujimoto T, Braun J, Oguri N (1994) Pregnancies following transfer of equine embryos cryopreserved by vitrification. Theriogenology 42:483-488

Hovatta O (2005) Methods for cryopreservation of human ovarian tissue. Reprod Biomed Online 10:729–734 Isachenko E, Isachenko V, Ramihi G, Nawroth F (2003) Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. Eur J Obstet Gynecol Reprod Biol 108:186-193

Lucci CM, Amorim CA, Bao SN, Figueiredo JR, Rodrigues AP, Silva JR, Goncalves PB (1999) Effect of the interval of serial sections of ovarian tissue in the tissue chopper on the number of isolated caprine preantral follicles. Anim Reprod Sci 56:39-49

Martinez-Madrid B, Dolmans MM, Van Langendonckt A, Defrere S, Donnez J (2004) Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. Fertil Steril 82:1390-1394

Martino A, Pollard JW, Leibo SP (1996) Effect of chilling bovine oocytes on their developmental competence. Mol Reprod Dev 45:503-512

Naik BR, Rao BS, Vagdevi R, Gnanprakash M, Amarnath D, Rao VH (2005) Conventional slow freezing, vitrification and open pulled straw (OPS) vitrification of rabbit embryos. Anim Reprod Sci 86:329-338

Paynter SJ, Cooper A, Fuller BJ, Shaw RW (1999) Cryopreservation of bovine ovarian tissue: structural normality of follicles after thawing and culture in vitro. Cryobiology 38:301-309

Pena FJ, Saraiva F, Johannisson A, Walgren M, Rodriguez-Martinez H (2005) A new and simple method to evaluate early membrane changes in frozen boar spermatozoa. Int J Androl 28:107-114

Poole CA, Brookes NH, Clover GM (1993) Keratocyte networks visualized in the living cornea using vital dyes. J Cell Sci 106:685-692

Rahimi G, Isachenko E, Isachenko V, Sauer H, Wartenberg M, Tawadros S, Hescheler J, Mallmann P, Nawroth F (2004) Comparison of necrosis in human ovarian tissue after conventional slow freezing or vitrification and transplantation in ovariectomized SCID mice. RBM Online 9:187-193

Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at −196 degrees C by vitrification. Nature 24:387-402

Rodrigues AP, Amorim CA, Costa SH, Matos MH, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004a) Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology 61:1009-1024

Rodrigues AP, Amorim CA, Costa SH, Matos MH, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004b) Cryopreservation of caprine ovarian tissue using dimethylsulphoxide and propanediol. Anim Reprod Sci 84:211-227

Salehnia M (2002) Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. Exp Anim 51:509-512

Salehnia M, Abbasian ME, Rezazadeh VM (2002) Ultrastructure of follicles after vitrification of mouse ovarian tissue. Fertil Steril 78:644-645

Santos RR, Tharasanit T, Figueiredo JR, Van Haeften T, Van den Hurk R (2006) Improved preservation of caprine preantral follicle viability after cryopreservation in sucrose and ethylene glycol. Cell Tissue Res 325:523-531

Schotanus K, Hage WJ, Vanderstichele H, Van den Hurk R (1997) Effects of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. Theriogenology 48:471-483

Shaw J, Oranratnachai A, Trounson A (2000) Fundamental cryobiology of ammalian oocytes and ovarian tissue. Theriogenology 53:59-72

Stachecki JJ, Cohen J (2004) An overview of oocyte cryopreservation. RBM Online 9:152-163

Toth TL, Baka SG, Veeck LL (1994) Fertilization and in vitro development of cryopreserved human prophase I oocytes. Fertil Steril 61:891-894

Vajta G, Booth PJ, Holm P, Greve T, Callesen H (1997) Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. Cryo-letters 18:191–195

Van den Hurk R, Spek ER, Hage WJ, Fair T, Ralph JH, Schotanus K (1998) Ultrastructure and viability of isolated bovine preantral follicles. Hum Reprod Up 4:833-841

Vicente JS, Garcia-Ximenez F (1996) Direct transfer of vitrified embryos. Theriogenology 45:811-815

Wright DL, Eroglu A, Toner M, Toth TL (2004) Use of sugars in cryopreserving human oocytes. RBM Online 9:179-186

Yeoman RR, Wolf DP, Lee DM (2005) Coculture of monkey ovarian tissue increases survival after vitrification and slow-rate freezing. Fertil Steril 83:1248-1254

Chapter 4

Osmotic tolerance and freezability of isolated caprine early-staged follicles

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Submitted

Abstract

Isolated caprine early-staged follicles were submitted to osmotic tolerance tests (in the presence of cryoprotectants, such as sucrose and EG, or NaCl solutions), as well as exposed to and cryopreserved (by slow or rapid cooling) in MEM alone or MEM supplemented with sucrose, ethylene glycol (1.0 or 4.0 M), or both. When follicles were exposed to 1.5 M NaCl, only 2±2% of the follicles were viable, whereas 87±3% of the follicles were viable after exposure to 4.0 M EG. Regarding exposure time, the highest percentage of viable follicles were obtained when follicles were exposed for 10 min to 1.0 M EG + 0.5 M sucrose, while exposure for 60 seconds to 4.0 M EG + 0.5 M sucrose also maintained high percentage viability in follicles. Slow cooling in presence of 1.0 M EG + 0.5 M sucrose (75±6%), or rapid cooling in presence of 4.0 M EG + 0.5 M sucrose (71±5%) resulted in a significantly higher proportion of viable follicles than all other treatments ($P<0.05$). Twenty-four hour culture of frozen-thawed follicles was used to assess functional survival, and only slow frozen follicles showed viability rates similar to control follicles (64±3% vs. 69±4% respectively; $P>0.05$). Interestingly, the percentage of viable rapid cooled follicles (59±3%) were similar to those obtained after in vitro culture of conventional slow cooled follicles, however, significantly lower when compared to control. Thus, in addition to determining optimal concentrations of EG and sucrose for the successful cryopreservation of caprine early-staged follicles we report the development of rapid and slow cooling protocols.

Keywords
caprine ♦ follicles ♦ osmotic ♦ tolerance ♦ cryopreservation

Introduction

The major aim of our study was to establish and improve a cryopreservation method for early-staged caprine follicles. The factors which indirectly influence the cryo-survival of cells in general fall into two distinct categories: (i) the internal fixed properties inherent to the specific individual cells of the tissues of interest, and (ii) the external factors involved in all essential steps of the cryopreservation procedure (Aubard et al., 1994). Internal properties that should be considered include: (1) the geometry of individual cells (2) the permeability of individual cells and the entire follicle to water and cryoprotectants at various temperatures, (3) the age and the developmental stage of the follicle. External factors that should be considered include: (1) the way the material is transported and treated prior to cooling, (2) the composition of solutions, including the type and method of addition of the cryoprotectant, (3) the cooling rate, including holding time, and the final super-cooling and transfer to liquid nitrogen, (4) the thawing rate, and (5) the rate and method of cryoprotectant removal/dilution. In the present study we attempt to improve external factors in order to improve the method for storing early-staged caprine follicles.

Previously, our group has developed two cryopreservation protocols (conventional slow and rapid cooling) to store caprine ovarian fragments (Santos et al., 2006a; 2007a). Our rationale for this present study is to further improve these protocols to better preserve the gametes enclosed in early-staged follicles (primordial, primary and secondary follicles); the major source of oocytes in the ovary (Gougeon, 1993; Telfer, 2001). Improved storage of such follicles can be useful for endangered breeds, or can at a later stage be applied to rescue oocytes from human patients subjected to chemotherapy treatments for cancer. Furthermore, in contrast to the rodent, domestic ruminants are considered as the experimental models of choice for reproductive studies in human (Gandolfi et al., 2006). Humans and small ruminants possess ovaries and follicles of similar size, morphology and structure (Lucci et al., 2001). The length of folliculogenesis (e.g. time necessary for a primordial follicle to reach the pre-ovulatory stage) is also comparable (Baird et al., 2004).

Successful cryopreservation protocols developed for caprine ovarian tissue, (Santos et al., 2006a; 2007a), may not be suitable for isolated cells or groups of cells, such as follicles, the material used for cryopreservation in the current study.

In contrast to larger parts of ovarian tissues, follicles lack the protection of a connective tissue barrier. This absence is likely to influence the intrinsic properties of the tissue, particularly an increased permeability to cryoprotectants and water. Damage caused by cryoprotectants is often associated with shrinking and swelling of the cell in response to highly concentrated solutions (Mazur et al., 2000; Walters et al., 2005). During exposure to high concentrations of cryoprotectants (equilibrium phase), cells initially shrink as water flows out, but subsequently swell as water and cryoprotective agents enter to protects free water bonds and bi-layer cell membranes (Agca et al., 2002). Many authors claim that cryoprotectant solutions cause cell damage via hyper-osmotic changes (Agca et al., 2000; Mullen et al., 2004), while other authors hypothesize that cellular damage may be caused by cryoprotectant removal (Santos et al., 2007a) or by the composition of certain cryoprotectant solutions (Stachecki et al., 1998).

Since it is unclear whether the damage caused by cryoprotectants results from osmotic changes or interactions of the agents with cells, we studied both their osmotic and chemical effects. It is claimed that cryoprotectants cause cell damage because they are used in concentrations that exceed physiological osmolality levels (280 – 300 mOsmol/l). However, previous studies have shown that high concentrations of electrolytes are correlated with cellular damage (Lovelock, 1953), and cryoprotectants are non-ionic solutes. Thus, we wanted to compare these solutions with a solution easily dissociable into ions (NaCl).

As mentioned earlier, cryoprotectant removal and a return to isotonic conditions may cause cell damage (Santos et al., 2007a), as does temperature exposure (De la Cueva et al., 1997). Exposure of early-staged follicles to cryoprotectant solutions is often performed at 20^{o}C and, after freezing-thawing, cryoprotectant removal is performed to permit cells to return to isotonic conditions (Rodrigues et al., 2004a,b; Santos et al., 2006a,b; 2007a,b). It has been suggested however, that osmotic tolerance (i.e. the ability to survive exposure to high solute concentrations) is dependent on temperature, since less osmotic damage was observed at low temperatures such as 4^{o}C (Steponkus, 1984). Furthermore, it has been observed that sperm cells appear to maintain membrane integrity if they have not returned to isotonic conditions after exposure to cryoprotectants (Armitage and Mazur, 1984; Zawlodzka and Takamatsu, 2005). This can simply be due to the method of cryoprotectant removal (in gradual series or by direct exposure of cells to a cryoprotectant-free medium).

In addition, the viability of cryopreserved early-staged follicles may be further compromised after thawing and subsequent culture. We have demonstrated that 24 h in vitro culture of frozen-thawed caprine ovarian follicles is an essential step in the evaluation of the cryopreservation process (Santos et al., 2006a). In that study cryo-survival of early-staged follicles was similar in a sucrose containing medium irrespective of whether or not the cryoprotectant EG was present. After 24 h in vitro culture however, only follicles frozen-thawed in the presence of EG plus sucrose maintained a viability rate similar to that of non-exposed fresh control follicles.

Therefore, the aim of this study was to determine the influence of various external factors so as to improve cryopreservation of early-staged caprine follicles. The factors tested were: iso- and anisotonic solutions, (NaCl, sucrose and EG) at two different exposure temperatures (4 and 20^{o}C), the effect on viability of intra-cellular (EG) and extra-cellular (sucrose) cryoprotectants. These variables were compared using both a conventional slow freezing protocol and a rapid freezing protocol.

Materials and Methods

Source and preparation of ovarian tissue

This study is composed of four different experiments (detailed below). In each experiment, female reproductive organs from 5 adult (1 – 2 years old) mixed-breed goats (*Capra hircus*) were obtained at a local slaughterhouse (Montfoort). The ovarian pairs were transported in thermos flasks at 30°C to the laboratory within 1 h of slaughter. In the laboratory, adhering tissues were removed from the ovaries, which were subsequently washed once in 70% alcohol for 10 sec and twice in phosphate-buffered saline (PBS). The cortex from each pair of ovaries was then removed and cut into small fragments (1 mm^3). The fragments were placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco, Paisley, UK) at room temperature (RT), and then follicles were isolated (described below). The isolation procedure resulted in the recovery of a similar number of early-staged follicles from each ovarian pair (~ 20 – 25 follicles). Due to the time required to micromanipulate follicles, treatments were conducted on different days.

Follicle isolation

Caprine early-staged follicles (primordial, primary and secondary follicles) were isolated from ovarian fragments using a mechanical procedure described by Lucci et al. (1999a). In brief, the ovarian cortex from each animal was cut into small fragments with a tissue chopper (Meyvis, Gouda, the Netherlands). The ovarian fragments were then placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco) and subsequently pipetted 40 times with a Pasteur pipette to release the early-staged follicles. The suspension was successively filtered through 500 μm and 100 μm nylon-mesh filters. Only early-staged follicles smaller than 100 μm in diameter were used in our experiments and were collected under a stereomicroscope and transferred to HEPES-buffered M-199 + 1% bovine serum albumin (holding medium; Sigma, St. Louis, MI, USA).

Cryopreservation

Slow cooling

For conventional slow cooling, groups of approximately 25 early-staged follicles were placed in an eye-dish plate containing 1ml of cryopreservation media (see experiment 3) and then loaded into 1 ml Cassou straws (IMV, L'Aigle, France). Straws were first rinsed by aspirating freezing medium and expelling it immediately, followed by aspiration of freezing medium containing the early-staged follicles. Subsequently, straws were sealed with humidified polyvinylalcohol powder, placed into the controlled-rate programmable freezer (Planer Kryo 10 Series II, Cryotech Benelux, Schagen, the Netherlands) and incubated for 10 min at 20°C. Samples were then cooled at a rate of 2°C/min from 20°C to -7°C; ice crystal induction (seeding) was manually performed by touching the straws with forceps pre-cooled in liquid nitrogen. After seeding, the straws were held at -7°C for 10 min and then cooled at 0.3°C/min to -30°C and finally at 0.15°C/min to -33°C, after which the straws were immersed into liquid nitrogen (−196°C) and stored for 1 week.

When required, the straws were thawed in air for 1 min at RT and then immersed in a water bath at 37°C until the cryopreservation medium was

completely melted, after which the cryoprotectant was removed in three steps by incubating in MEM with 0.3, 0.15 and 0.0 M sucrose for 5 min each.

Rapid cooling

For rapid cooling, groups of approximately 25 early-staged follicles were placed in 1 ml straws containing the cryopreservation media, as described for slow cooling, and incubated at 20°C for 60 sec (see experiment 3). Immediately after the equilibration period, straws were immersed vertically into liquid nitrogen and stored in liquid nitrogen for 1 week. When required, the straws were thawed in air for 1 min at RT and then immersed in a water bath at 37°C until the cryopreservation medium had completely melted. Based on results from experiment 2, cryoprotectant was removed by washing 3 times for 5 min each in sucrose free MEM solution.

In vitro culture

In vitro culture was performed in 4-well culture dishes (Nunc, Roskilde, Denmark) (25 follicles per well) in a 20 µl droplet of culture medium under mineral oil at 39°C, in a humidified chamber with 5% CO_2 in air. The culture medium consisted of alpha MEM (Sigma) supplemented with SPIT (5 ng/ml selenium, 110 µg/ml pyruvate, 10 µg/ml insulin, and 5.5 µg/ml transferrin; Sigma), 100 µg/ml penicillin/streptomycin (Gibco), 10% fetal calf serum (Gibco), 0.01 IU/ml recombinant human follicle-stimulating hormone (hFSH; Organon, Oss, the Netherlands), and 10 ng/ml activin-A (R&D System Europe, Abingdon, UK).

Viability assessment

Trypan blue staining

Viability of follicles was evaluated by adding 5 µl of 0.4% trypan blue (Sigma) to each 100 µl of solution and incubating for 1 min (RT) as described by Jewgenow and co-workers (1998). Thereafter, follicles were examined using an inverted microscope and classified as non-viable when ≥ 10% of the cells were stained with trypan blue and viable when < 10% of the cells were stained with

trypan blue. As a positive control, follicles were treated with Triton X-100 for 3 minutes and stained with trypan blue (Fig. 1).

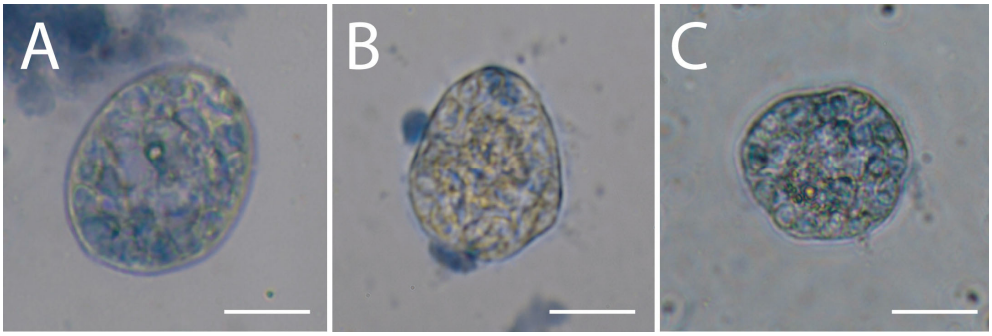

Fig. 1 Viability of goat early-staged follicles based on trypan blue staining (blue staining indicate non-viable cells). A: Positive control, Triton X-100 treated secondary follicle. B: Viable secondary follicle. C: Non-viable secondary follicle. Scale bars represent 50 µm.

Ethidium/Calcein staining

Follicles were incubated in the holding medium for 10 min at 37°C in a mixture of 4 µM calcein AM, 2 µM ethidium homodimer-1 (Molecular Probes Europe, Leiden, The Netherlands), and 10 µM Hoechst 33342 (Sigma). Stained follicles were washed three times in holding medium, mounted on a glass microscope slide in 5 µl antifade medium (Vectashield, Vector Labs, Burlingame, CA, USA) to prevent photobleaching, and finally examined with an epifluorescent microscope (BH2-RFCA, Olympus, Tokyo, Japan). Hoechst, calcein and ethidium homodimer were excited at 350 nm, 488 nm, and 568 nm, respectively. Corresponding probe emission was collected at 450 nm, 530 nm, and 620 nm (all band pass filters). Oocytes and granulosa cells were classified as viable if the ooplasm was stained positively with calcein, and chromatin was unlabeled with ethidium homodimer. Percentage of viable granulosa cells were calculated in relation to the total number of Hoechst positive nuclei (Fig. 2). Follicles were considered viable, when a viable oocyte was surrounded by ≥ 90% of viable granulosa cells (Santos et al., 2006a; 2007a).

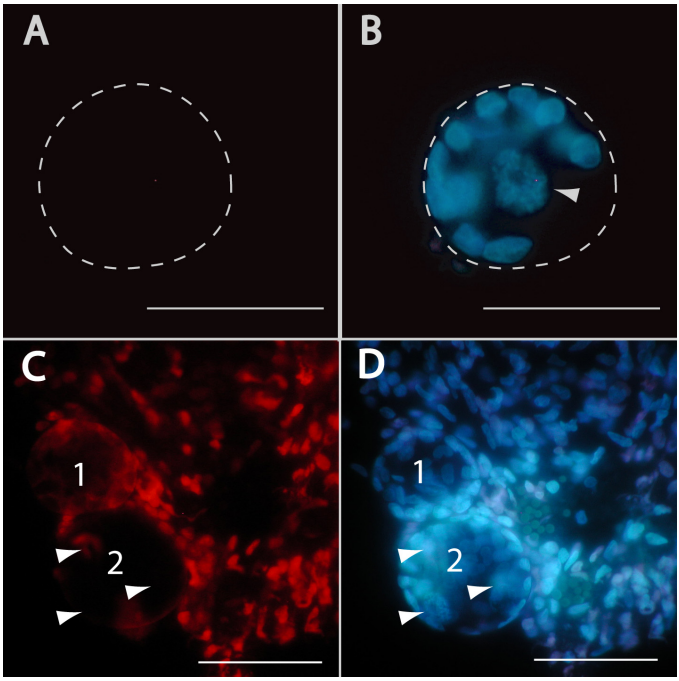

Fig. 2 Viability of goat early-staged follicles based on staining with Hoechst (blue staining represents all nuclei) and ethidium homodimer-1 (red staining represents nuclei from dead cells). A, B: Representative image of a viable primordial follicle. C, D: Representative image of two non-viable primordial follicles. Follicle 1 contains a viable oocyte, whereas, less than 90% of granulosa cells are viable. Follicle 2 contains three oocytes* of which one is deteriorated and less than 90% of granulosa cells are viable. Arrows indicate oocytes. Scale bars represent 30 μm. *Polyovular healthy early-staged (primordial, primary and secondary) follicles are commonly observed in goats (Lucci et al., 1999b).

Experiment 1. Osmotic tolerance of early-staged follicles to NaCl, sucrose and EG

To evaluate osmotic tolerance, caprine early-staged follicles were submitted to hypertonic conditions caused by intra- (1.0 or 4.0 M EG) and extracellular (0.5 M sucrose) cryoprotectant solutions or, 0.015, 0.15 or 1.5 M NaCl. To show the effect of cryoprotectant removal, early-staged follicles exposed to EG and sucrose were immediately evaluated with trypan blue, or washed in MEM or MEM supplemented with a series of gradually decreasing sucrose concentrations (0.3, 0.15 and 0 M), and then stained. As controls, follicles were evaluated immediately after isolation, and follicles were exposed to a 0.9% (0.15M) NaCl solution.

Groups of 20 early-staged follicles were subjected to each osmotic treatment with solutions of different osmolalities (28, 282 and 2800 mOsm/l of NaCl, 0.015, 0,15 and 1,5 M, respectively; 792 mOsm/l of sucrose, 0.5 M; 2726 and 5242 mOsm/l of EG, 1.0 and 4.0 M, respectively) and one isotonic solution

(0.9% NaCl, 286 mOsm/l). Exposure was performed in 100 µl drops of the solutions for 10 min at 4 or 20°C (Fig. 3). After exposure, follicles were analysed using trypan blue staining. In the sucrose and EG treatments, follicles were analysed either immediately or after washing in MEM (Sigma) alone or in MEM supplemented with 0.3, 0.15 and 0 M sucrose as described above.

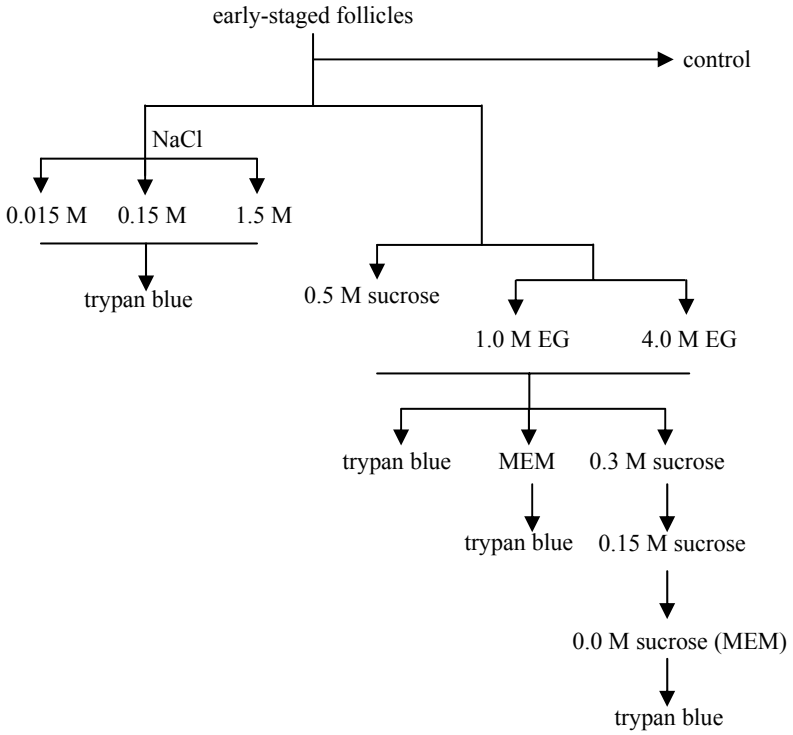

Fig. 3 Experiment 1: Osmotic tolerance of early-staged follicles to NaCl, sucrose and EG

Experiment 2. Effect of dose and exposure length to cryoprotectants on early-staged follicle viability prior to freezing

Sixteen aliquots (each containing about 25 isolated early-staged follicles <100 µm) of follicular suspension (1 ml) were placed in straws and incubated at 20°C for 1, 5, 10 or 20 min in 1 ml MEM with or without 0.5 M sucrose, 1.0 M EG or both (Fig. 4). Another sixteen aliquots were incubated for 15, 30, 60 or 120 sec in 1 ml MEM (Sigma) with or without 0.5 M sucrose, 4.0 M ethylene glycol or

both (Fig. 4). After incubation, the follicles from each group were washed in three steps (5 min each), to remove cryoprotectant, based on the best results from the experiment 1. After cryoprotectant removal, aliquots of follicles were analysed by trypan blue staining.

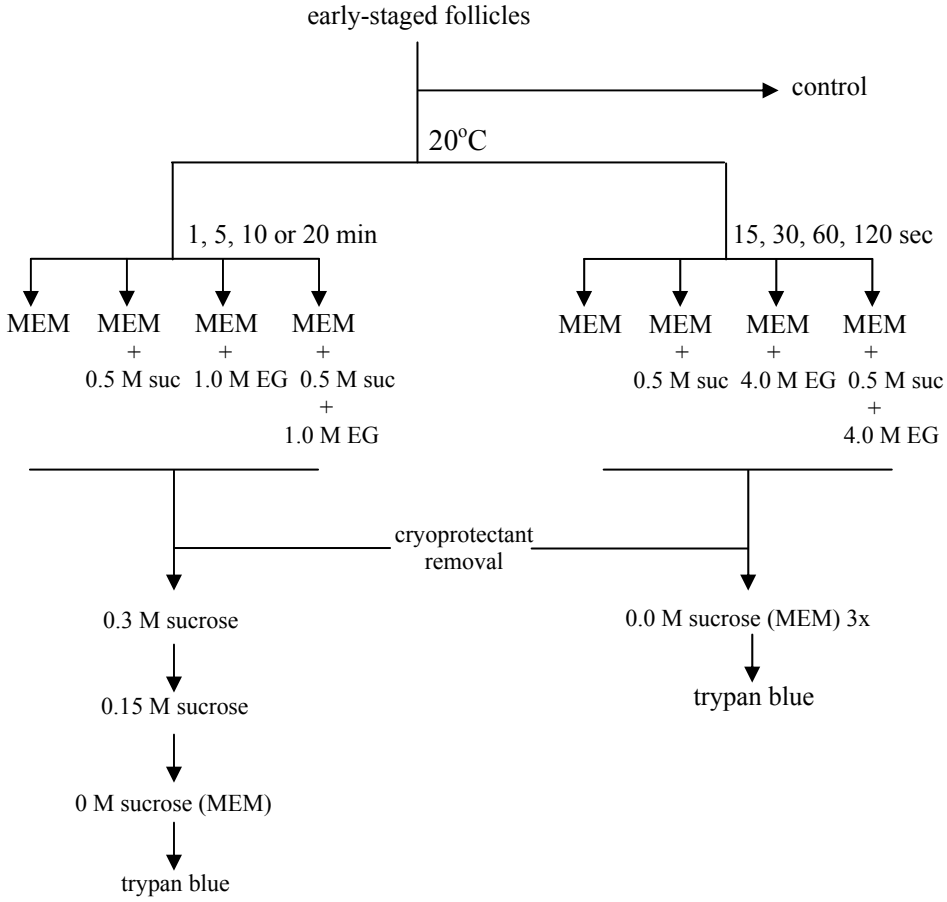

Fig. 4 Experiment 2: Dose and duration dependency of early-staged follicles exposed to cryoprotectants prior to freezing

Experiment 3. Comparison between slow and rapid

Follicles (20 per treatment) were conventionally cryopreserved (slow cooling) in MEM, MEM plus 0.5 M sucrose, MEM plus 1.0 M EG or MEM

supplemented with both 0.5 M sucrose and 1.0 M EG. Rapid cooling of follicles was performed in MEM, MEM plus 0.5 M sucrose, MEM plus 4.0 M EG or MEM supplemented with both 0.5 M sucrose and 4.0 M EG (based on optimal conditions observed in experiment 2). All cryopreservation procedures were performed in the absence of serum, and the duration of exposure of follicles to cryoprotectant agents before freezing was based on optimal results from the experiment 2 (10 min for slow and 60 sec for rapid cooling). After a week, samples were thawed, cryoprotectant was removed and early-staged follicles were evaluated using ethidium homodimer and calcein-AM as viability markers (Fig. 5).

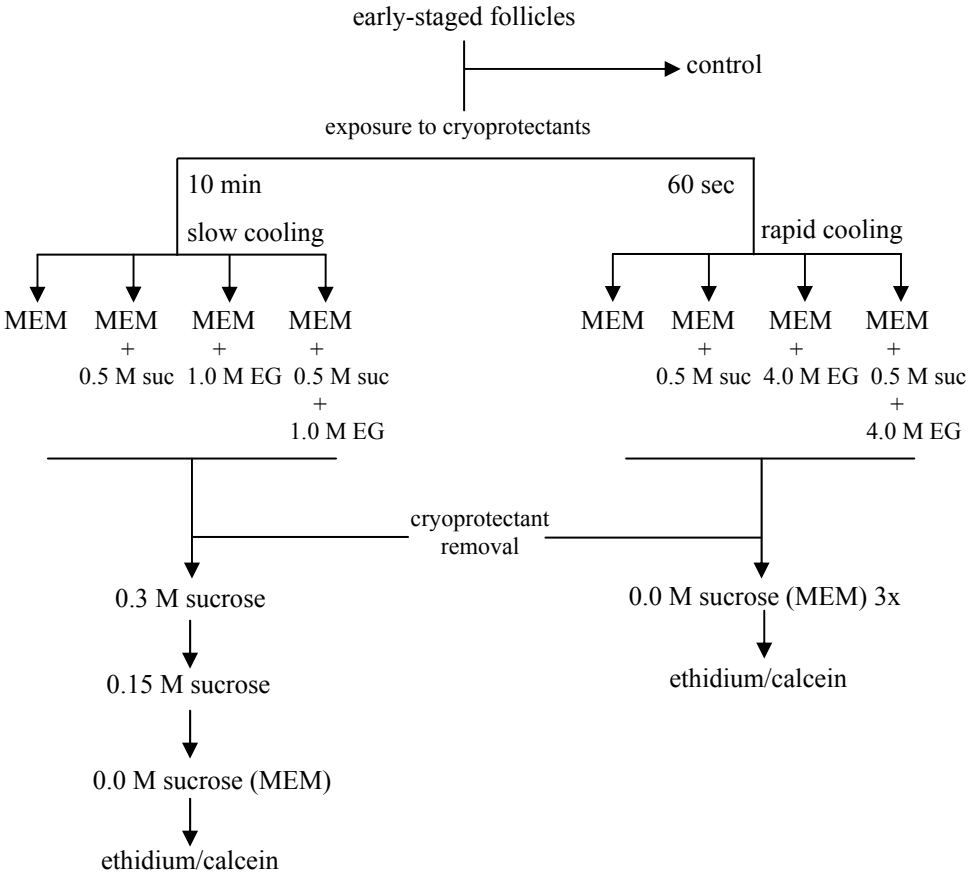

Fig. 5 Experiment 3: Comparison between slow and rapid cooling of early-staged follicles

Experiment 4. In vitro culture of early-staged follicles

To compare the best results obtained from both freezing procedures (see experiment 3), fresh (immediately isolated from fresh ovaries), slow or rapid cryopreserved early-staged follicles (25 follicles per treatment) were evaluated immediately or after in vitro cultured for 24 h using ethidium homodimer and calcein-AM viability markers (Fig. 6).

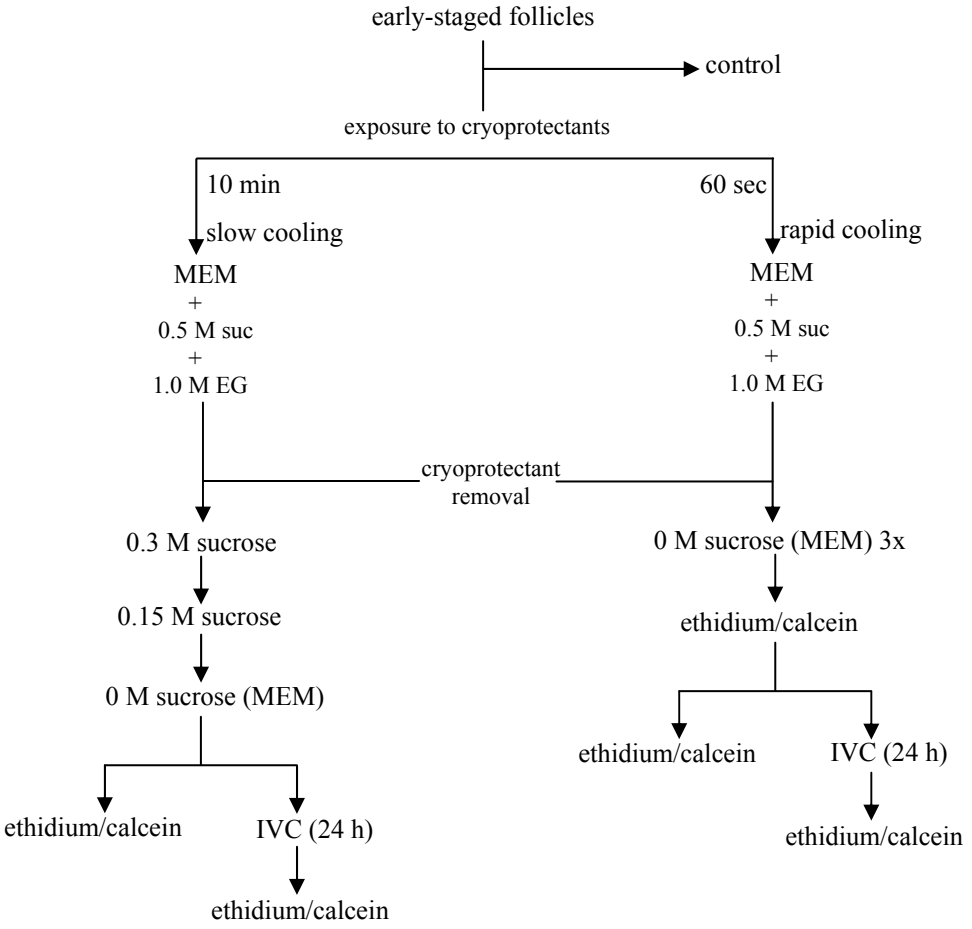

Fig. 6 Experiment 4: In vitro culture of early-staged follicles

Statistical analysis

The mean percentage of viable early-staged follicles and viability of oocytes and granulosa cells were compared by a one-way analysis of variance (ANOVA) and Tukey's test using Statview for Windows. Differences were considered significant when $P \leq 0.05$.

Results

Experiment 1. Osmotic tolerance of early-staged follicles to NaCl, sucrose and EG

Early-staged follicles were assessed for viability by trypan blue. At both 4 and 20°C, follicles were spherical under isotonic conditions (0.15 M NaCl), whereas they shrunk after exposure to a high osmolality of NaCl (1.5 M NaCl), or to 0.5 M sucrose, 1.0 M and 4.0 M EG. Conversely, follicles became swollen in hypotonic NaCl solution (0.015 M NaCl). In contrast to cells incubated in NaCl (1.5 M) or sucrose (0.5 M), cells maintained in EG regained their volume to some extent during exposure (Fig. 7).

After exposure to 0.015 M NaCl at 4 and 20°C, 42% and 50% of follicles respectively, did not stain with trypan blue. However, after exposure to 1.5 M NaCl at both temperatures only 2% of follicles were unstained, indicating that shrinkage resulted in a dramatic loss of membrane integrity in hypertonic compared to hypotonic conditions (Fig. 8a). In contrast, hypertonic solutions of sucrose or EG did not cause the same deleterious effect as highly concentrated NaCl solution (1.5 M). exposing Follicles exposed to 0.5 M sucrose (4°C) or 1.0 M EG (4 and 20°C), had a higher percentage of viable follicles when cryoprotectant was gradually removed by step-wise washing in MEM supplemented with decreasing sucrose concentrations, than when washed in MEM alone (Fig. 8b). In contrast, follicles exposed to 4.0 M EG at both temperatures, were damaged less when cryoprotectant was removed by MEM only. There was no significant difference in percentage viability of early-staged follicles between exposure temperatures, except in 4.0 M EG in which viability was higher at 20°C (Fig. 8b).

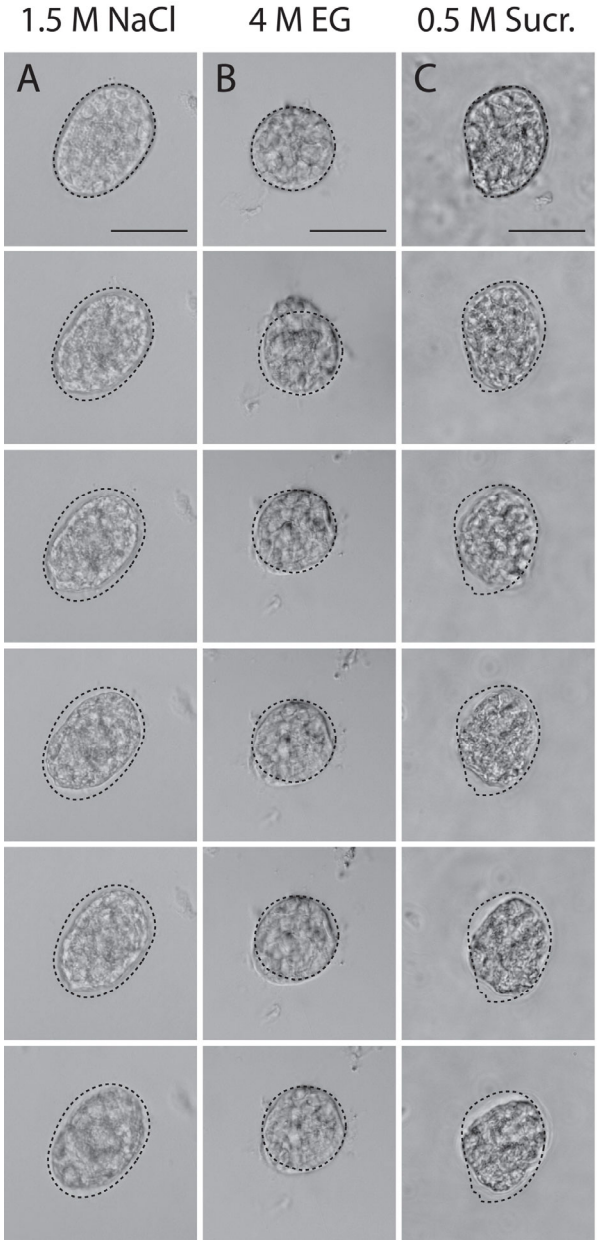

Fig. 7 Changes in follicular volume imposed during hyper-osmotic treatments. Images indicate changes in volume of primary follicles compared to the original volume (0 sec) during exposure to 1.5 M NaCl (A), 4.0 M EG (B), and 0.5 M Sucrose (C). Only the follicle exposed to 4.0 M EG regained its original volume. Scale bars represent 40 μm.

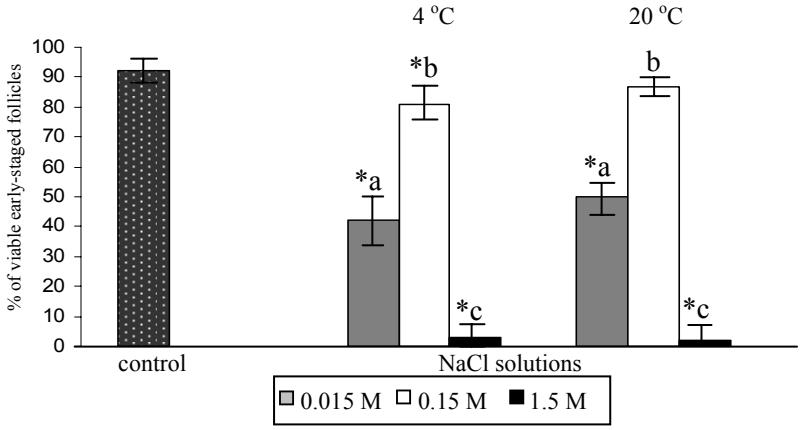

Fig. 8a Viability based on trypan blue staining of isolated caprine early-staged follicles exposed to NaCl solutions (Experiment 1). * differs significantly from control (P<0.05); Values with different letters (a,b,c) differ significantly among the NaCl solution concentrations (0.09, 0.9 and 9%) within the same temperature (P<0.05).

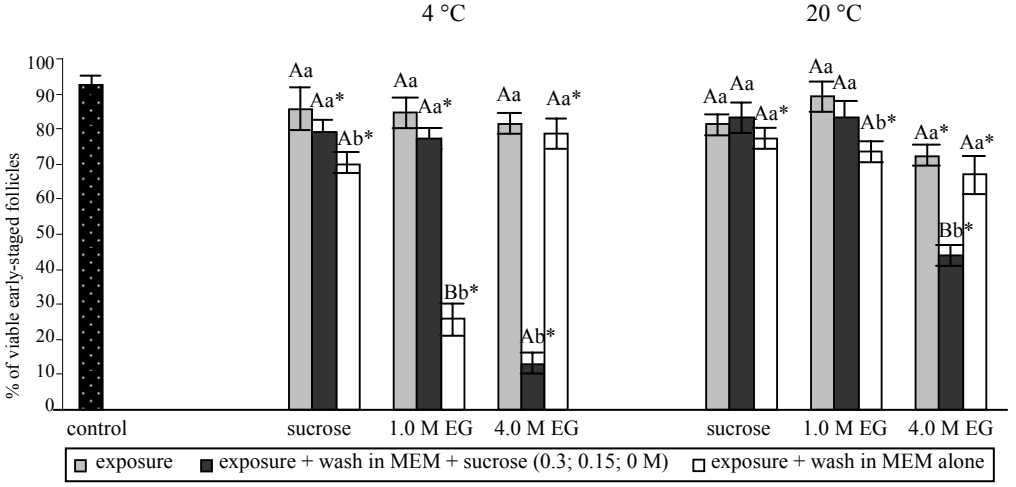

Fig. 8b Viability based on trypan blue staining of isolated caprine early-staged follicles exposed to sucrose (0.5 M), or EG (1.0 or 4.0 M) (Experiment 1). * differs significantly from control (P<0.05); Values with different lower-case letters (a,b) differ significantly among the effects of exposure only, or washing treatments after exposure to the cryoprotectant solutions (MEM alone or MEM supplemented with a series of gradually decreasing sucrose concentrations) (P<0.05); Values with different upper-case letters (A,B) differ significantly between 4 and 20°C using the same cryoprotectant solution (P<0.05).

Experiment 2. Effect of dose and exposure length to cryoprotectants on early-staged follicle viability prior to freezing

Based on results obtained in the first series of experiments above, a second series of experiments were performed to determine the optimal duration of follicle exposure to cryoprotectants using sucrose and EG. Thirty-four groups of early-staged follicles were stained with trypan blue and analysed after exposure to MEM or cryoprotectant.

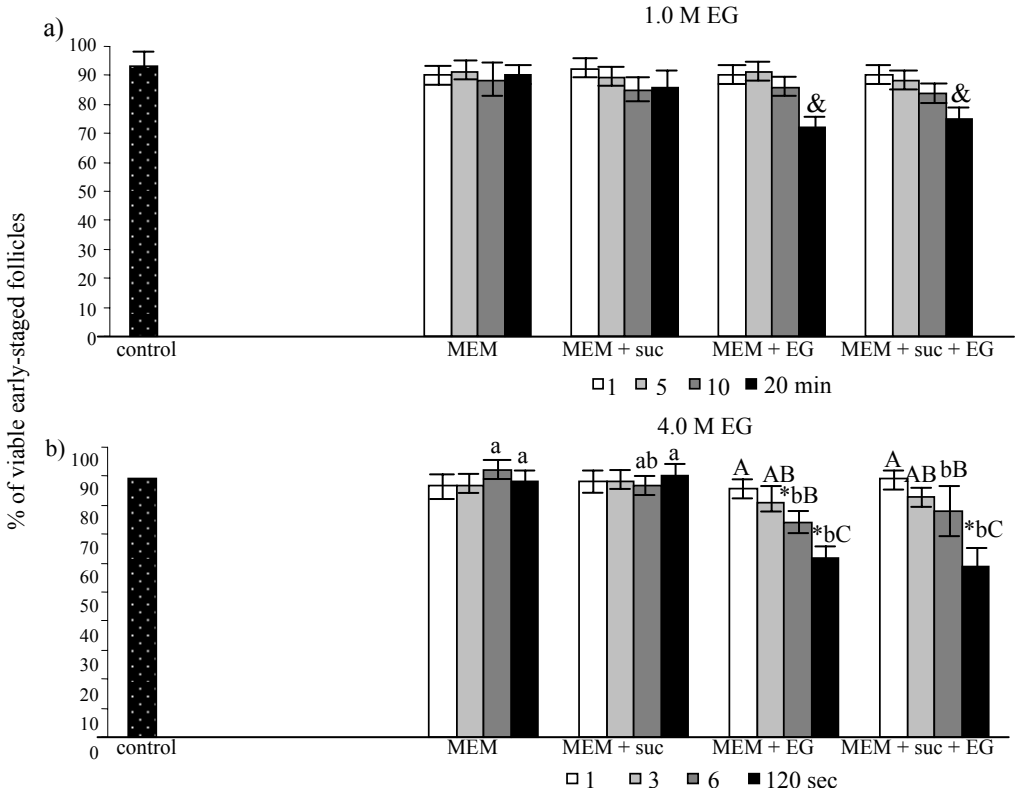

Fig. 9a,b Viability based on trypan blue staining of isolated caprine early-staged follicles exposed to MEM, sucrose (0.5 M), or EG 1.0 (a) or 4.0 M (b) (Experiment 2). & differs significantly from control as well as from other treatments ($P<0.05$); * differs significantly from control ($P<0.05$); Values with different lower-case letters (a,b) differ significantly among treatments within the same exposure time ($P<0.05$); Values with different upper-case letters (A,B) differ significantly between exposure times in the same cryoprotectant ($P<0.05$).

Among non-exposed control follicles, 93% were viable (trypan blue negative). Exposure of follicles to MEM supplemented with 0.5 M sucrose did not affect their viability. This was also observed when follicles were exposed to 1.0 M EG or 1.0 M EG supplemented with 0.5 M sucrose for til 10 min. Longer exposure (20 min) to 1.0 M EG with or without 0.5 M sucrose, resulted in a significant decrease in the percentage of viable follicles (Fig. 9a).

Viability of control group follicles and those exposed to MEM with or without 0.5 M sucrose and 4.0 M EG, is shown in Figure 5c. The majority of non-exposed controls were viable (89%). Exposure to MEM or MEM supplemented with 0.5 M sucrose for 120 sec, did not affect follicular viability. Viability was also similar to controls when follicles were exposed to MEM supplemented with 4.0 M EG or 4.0 M EG plus 0.5 M sucrose for 60 sec. Longer EG exposure (120 sec), resulted in a decreased percentage of viable follicles. Compared to 1.0 M EG, the window of optimal cryoprotectant exposure was dramatically reduced when 4.0 M EG was used (Fig. 9a,b).

Experiment 3. Comparison between slow and rapid cooling

Using the results from the experiment 2, we then assessed the effect of slow versus rapid cooled methods of cryopreservation on the viability of early-staged follicles. Viability was evaluated using ethidium homodimer and calcein-AM. All freezing treatments significantly reduced the percentages of viable early-staged follicles when compared to controls, except when conventionally cryopreserved (slow cooling) in a mixture of EG and sucrose (Fig. 10a,b). The highest percentage of viable conventionally cryopreserved follicles were obtained when early-staged follicles were slowly frozen in MEM containing 0.5 M sucrose and 1.0 M EG, while most rapid cooled follicles appeared viable when early-staged follicles were frozen in MEM containing 4.0 M EG (with or without sucrose). Both these most optimal cryopreservation methods resulted in similar follicular survival rates (Fig. 10a,b).

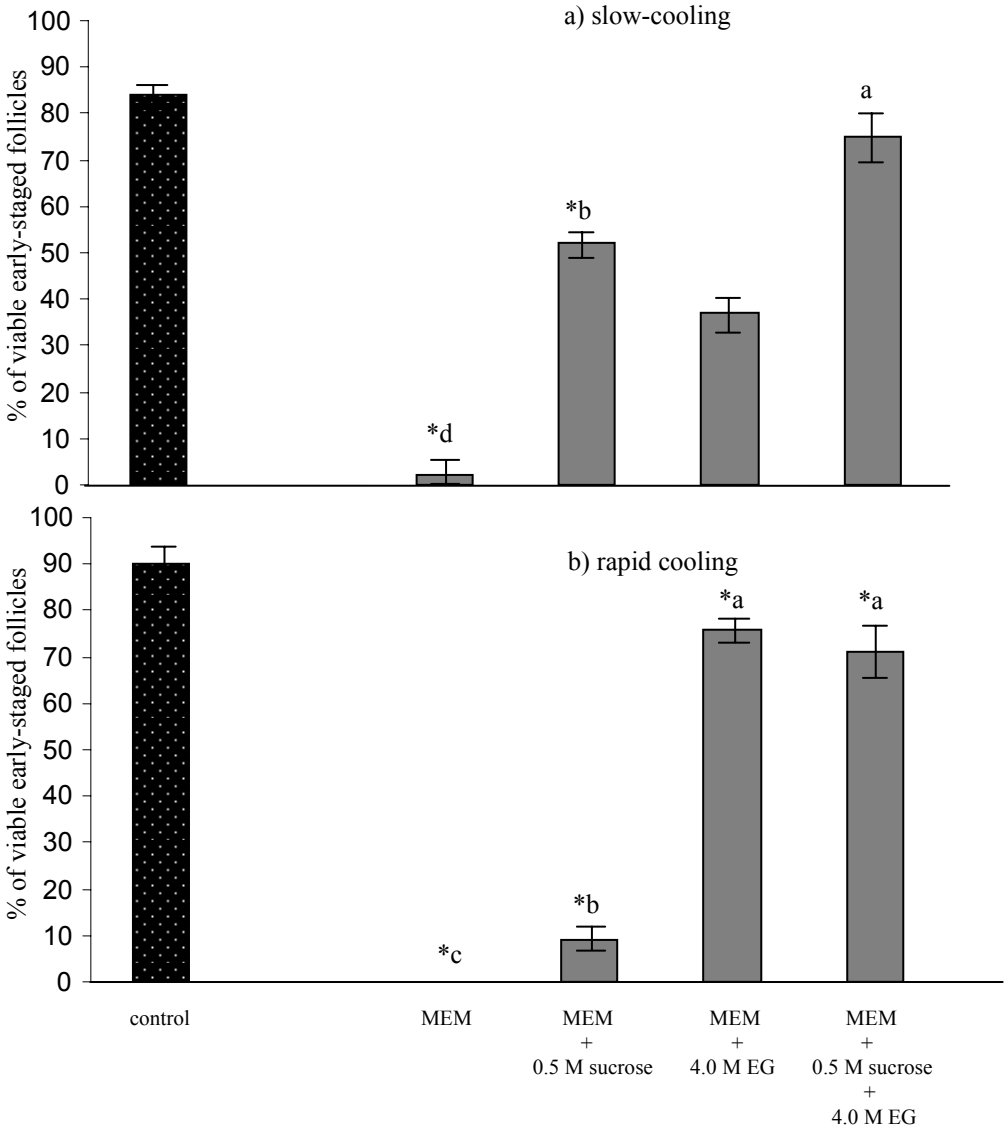

Fig. 10a,b Viability based on ethidium-homodimer and calcein-AM labeling of isolated caprine early-staged follicles after conventional (slow cooling) (a) and rapid cooling (b) (Experiment 3). Viable follicles had a viable oocyte surrounded by ≥ 90% viable granulosa cells. * differs significantly from control ($P<0.05$); Values with different letters (a,b,c,d) differ significantly among the treatments ($P<0.05$).

Experiment 4. In vitro culture of early-staged follicles

Based on the results of experiment 3, isolated follicles were in vitro cultured for 24 h before (fresh), or after freeze-thawing using the best slow and rapid cooling conditions. In vitro culture of isolated early-staged follicles for 24 h decreased the viability of non-frozen (control) follicles and their oocytes. After culture, the percentage of viable oocytes and follicles among conventionally cryopreserved (slow cooling) early-staged follicles did not significantly differ from those of fresh follicles. When compared to fresh follicles, the percentage of viable oocytes and follicles among rapidly cooled early-staged follicles decreased slightly but significantly during culture, whereas, no changes were detected for slowly cooled follicles (Fig. 11).

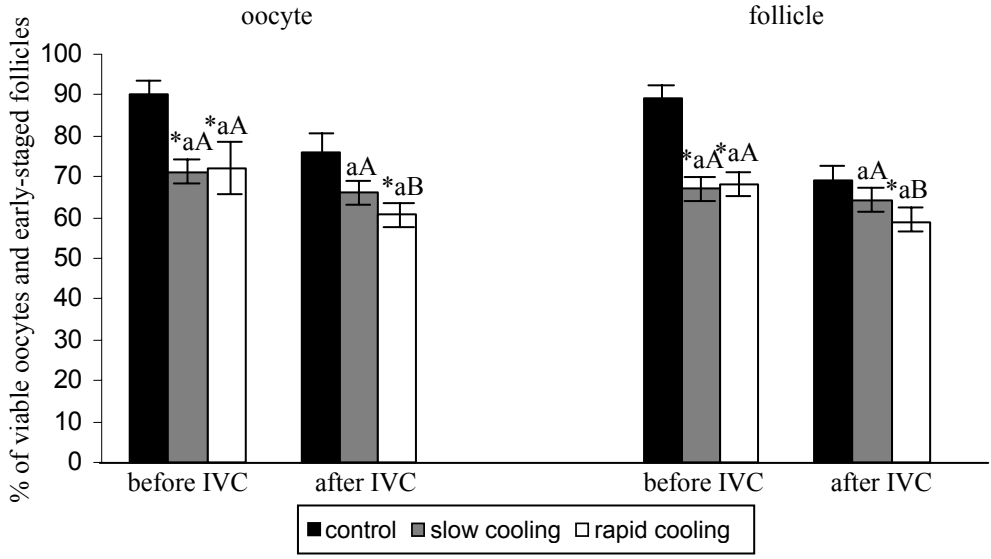

Fig. 11 Percentage of fresh and frozen-thawed viable oocytes and early-staged follicles before and after in vitro culture for 24 h (Experiment 4). Viable follicles had a viable oocyte surrounded by ≥ 90% viable granulosa cells. * differs significantly from control ($P<0.05$); Values with different lower-case letters (a,b) differ significantly between cryopreservation procedures before or after culture within the same tissue ($P<0.05$); Values with different upper-case letters (A,B) differ significantly before and after in vitro culture for 24 h within the same freezing procedure ($P<0.05$).

Discussion

In this paper we described methods to improve the preservation of caprine early-staged follicles. In this regard, we tested the effect of different osmotic pressures on those follicles; the effect of slow and rapid freezing in MEM supplemented with cryoprotectants such as sucrose, ethylene glycol or their mixture; and the effect of instant versus stepwise removal of these cryoprotectants.

As results show, exposure to hypotonic and hypertonic concentrations of NaCl reduced the viability of early-staged follicles, both at 4 and 20°C. We found that more damage is caused by high sodium concentrations than by high solute concentrations per se or by exposure temperature. Mouse zygotes exposed to high osmolar solutions (1200–2000 mOsm) of PBS supplemented with choline chloride remained intact after cooling and re-warming, whereas PBS supplemented with sodium chloride was lethal to the embryos subjected to the same regime (Toner et al., 1993). Therefore, although rarely stated, high solute concentrations are not necessarily damaging (Stachecki et al., 1998). Besides simple diffusion, eukaryotic cells have several mechanisms by which they counteract changes in osmolality. One of them, the Na^+/K^+ pump, plays an important role in the maintenance of intracellular osmotic conditions (Darnell et al., 1986).

Although EG is known by its low toxicity when used in cryopreservation procedures of caprine and bovine ovarian tissue (Santos et al., 2006a, 2007a; Celestino et al., 2007), other cryoprotectants such as DMSO better preserves the ultra-structure of sheep early-staged follicles when compared to EG (Santos et al., 2006b). This illustrates that it is fundamental to understand the chemical pathways of cryoprotectant-cell interaction to explain specific differences. Our findings show that, apart from exposure to EG or sucrose, loss of follicular viability was also caused by the procedure of removing cryoprotectant; which leads to damage (Karlsson & Toner, 1996). The optimal way to reacquire isotonic equilibrium after exposure was obtained by washing samples in solutions containing decreasing concentrations of sucrose (except when incubating follicles in 4.0 M EG). The high concentration of cryoprotectant (making it essential to avoid toxic effects to remove the cryoprotectant as quickly as possible by direct wash in MEM alone) or prolonged exposure to anisotonic condition might cause viability loss. Thus, it is possible that EG is more toxic under osmotic stress. In an additional experiment, we studied the effect on early-staged follicles of the cryoprotectant exposure, since

loss of viability in follicles can occur prior to freezing (Rodrigues et al., 2004a,b). Early-staged follicles cannot be exposed to the toxic EG for extended period of time (>10 min for 1.0 M EG and >60 sec for 4.0 M EG). Changes in membrane stability increased permeability of membranes for water and cations, leading to a higher susceptibility for damage (Crowe et al., 1990). To control these changes, exposure to cryoprotectant solutions must be improved, by using a carbohydrate such as sucrose (an extra-cellular cryoprotectant) which is usually used as an osmotic buffer to help stabilize the membrane.

In our study a simple medium (serum-free single intra-cellular cryoprotectant, EG, supplemented with an extra-cellular cryoprotectant, sucrose) was used instead of a mixture of two or several intra-cellular cryoprotective compounds. This approach has already been successful in slow-cooling studies with caprine early-staged follicles enclosed in ovarian tissue (Santos et al., 2006a). Human embryos have been cryopreserved in ethylene glycol-sugar based solutions (Ohta et al., 1996). Although EG is not the most optimal glass former (Fahy et al., 2004), it is the most indicated cryoprotectant to the cryopreservation of goat and human ovarian follicles. Furthermore, sucrose has been often successfully used in cryopreservation procedures (Chung et al., 2000; Kuleshova and Lopata, 2002).

Slow and rapid cooled follicles in a mixture of EG and sucrose had similar viability. However, after culturing only slow cooling resulted in viability similar to those of fresh follicles, while rapid cooling lead to a marginal but significant decrease in viability. Maintaining morphology and viability is a major problem in the vitro culture of early-staged follicles from large mammals (Santos et al., 2007b). Although in the present study rapid cooling was not as efficient as slow cooling, the obtained results represent a hope for the preservation of genetic material from endangered breed animals. The fact that these animals may be encountered dead and far from laboratories equipped with freezing machines, illustrate the importance of a simple freezing with no need of sophisticated equipments.

Our findings on osmotic tolerance, cryoprotectant removal, duration of cryoprotectant exposure, slow vs. rapid cooling, and post-thaw culture of isolated goat early-staged follicles show that the most suitable protocol for post-thaw survival involves exposure to MEM supplemented with 1.0 M EG and 0.5 M sucrose for 10 min, followed by slow-cooling and subsequent cryoprotectant

removal using sucrose in a series of decreasing concentrations (0.3, 0.15 and 0 M). It should be noted however that in our experiments, frozen-thawed follicles were analysed in vitro by 24 hour culture and although this has helped to improve the freeze-thawing protocol, indeed the in vivo survival and development of follicles frozen-thawed with the described methods demands further study.

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References

Agca Y, Liu J, Rutledge JJ, Critser ES, Critser JK (2000) Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol Reprod Dev 55:2112–2119

Agca Y, Gilmore J, Byers M, Woods EJ, Liu J, Critser JK (2002) Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. Biol Reprod 67:1493–1501

Armitage WJ, Mazur P (1984) Osmotic tolerance of human granulocytes. Am J Physiol 247:373-381

Aubard Y, Teissier MP, Baudet JH (1994) Cryopreservation of the ovary and ovarian tissue. Rev Fr Gynecol Obstet 89:192-197

Baird DT, Campbell B, Souza C, Telfer E (2004) Long-term ovarian function in sheep after ovariectomy and autotransplantation of cryopreserved cortical strips. Eur J Obstet Gyn R B 113:55–59

Celestino JJ, Santos RR, Lopes CA, Martins FS, Matos MH, Melo MA, Báo SN, Rodrigues AP, Silva JR, Figueiredo JR (2007) Preservation of bovine preantral follicle viability and ultra-structure after cooling and freezing of ovarian tissue. Anim Reprod Sci, epub ahead of print

Chung HM, Hong SW, Lim JM, Lee SH, Cha WT, Ko JJ, Han SY, Choi DH, Cha KY (2000) In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. Fertil Steril 73:545-551

Crowe JH, Carpenter JF, Crowe LM, Anchordoguy TJ (1990) Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27:219–231

Darnell J, Lodis H, Baltimore D (1986) Active transport of ions and ATP hydrolysis. In: Darnell J, Lodis H, Baltinore D (ed) Molecular Cell Biology. Scientific American Books, New York, pp 625-628

De la Cueva FI, Pujol MR, Rigau T, Bonet S, Miro J, Briz M, Rodriguez-Gill JE (1997) Resistance to osmotic stress of horse spermatozoa: The role of ionic pumps and their relationship to cryopreservation success. Theriogenology 48:947-968

Fahy GM, Wowk B, Wu J and Paynter S (2004) Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology 48:22-35

Gandolfi F, Paffoni A, Brambilla EP, Bonetti S, Brevini TAL, Ragni G (2006) Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. Fertil Steril 85:1150-1156

Gougeon A (1993) Dynamics of human follicular growth: a morphologic perspective. In: Adashi EY, Leung PCK (ed) The ovary. Raven Press, New York, pp 21–39

Jewgenow K, Penfold LM, Meyer HH, Wildt DE (1998) Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. J Reprod Fert. 112:39-47

Karlsson JOM, Toner M (1996) Long-term storage of tissues by cryopreservation: critical issues. Biomed 17:243-256

Kuleshova LL, Lopata A (2002) Vitrification can be more favorable than slow cooling. Fertil Steril 78:449-454

Lovelock JE (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochem Biophys Acta 11:28-36

Lucci CM, Amorim CA, Bao SN, Figueiredo JR, Rodrigues APR, Silva JRV, Gonçalves PBD (1999a) Effect of the interval of serial sections of ovarian tissue in the tissue chopper on the number of isolated caprine preantral follicles. Anim Reprod Sci 56:39-49

Lucci CM, Amorim CA, Rodrigues AP, Figueiredo JR, Báo SN, Silva JR, Gonçalves PB (1999b) Study of preantral follicle population in situ and after mechanical isolation from caprine ovaries at different reproductive stages. Anim Reprod Sci 56:223-236

Lucci CM, Silva JRV, Carvalho FCA, Figueiredo JR, Bao SN (2001) Light microscopical and ultrastructural characterization of goat preantral follicles. Small Rum Res 41:61-69

Mazur P, Katkov II, Katkova N, Critser JK (2000) The enhancement of the ability of mouse sperm to survive freezing and thawing by the use of high concentrations of glycerol and the presence of an *Escherichia coli* membrane preparation (oxyrase) to lower the oxygen concentration. Cryobiology 40:187–209

Mullen SF, Agca Y, Broermann DC, Jenkins CL, Johnson CA, Critser JK (2004) The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. Hum Reprod 19:1148–1154

Ohta N, Nohara M, Kojimahara T (1996) Ultrarapid freezing of human embryos by vitrification method. Jpn J Fertil Steril 41:276–279

Rodrigues AP, Amorim CA, Costa SH, Matos MHT, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004a) Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology 61:1009-1024

Rodrigues AP, Amorim CA, Costa SH, Matos MHT, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004b) Cryopreservation of caprine ovarian tissue using dimethyl sulfoxide and propanediol. Anim Reprod Sci 84:211-227

Santos RR, Tharasanit T, Figueiredo JR, Van Haeften T, Van den Hurk R (2006a) Preservation of caprine preantral follicles viability after cryopreservation in sucrose and ethylene glycol. Cell Tissue Res 325:523-531

Santos RR, Rodrigues APR, Costa SHF, Silva JRV, Matos MHT, Lucci CM, Bao SN, Van den Hurk R, Figueiredo JR (2006b) Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. Anim Reprod Sci 91:249-263

Santos RR, Tharasanit T, Van Haeften T, Figueiredo JR, Silva JRV, Van den Hurk R (2007a) Vitrification of goat preantral follicles enclosed in ovarian tissue by using conventional and solid-surface vitrification methods. Cell Tissue Res 327:167-176

Santos RR, Van den Hurk R, Rodrigues APR, Costa SHF, Martins FS, Matos MHT, Celestino JJH, Figueiredo JR (2007b) Effect of cryopreservation on viability, activation and growth of in situ and isolated ovine early-staged follicles. Anim Reprod Sci 99:53-64

Stachecki JJ, Cohen J, Willadsen SM (1998) Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. Cryobiology 37:346-354

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Ann Rev Plant Physiol 35:543–584

Telfer EE (2001) In vitro development of pig preantral follicles. Reprod 58:81–90

Toner M, Cravalho EG, Stachecki J, Fitzgerald T, Tompkins RG, Yarmush ML, Armant DR (1993) Nonequilibrium freezing of one-cell mouse embryos: Membrane integrity and developmental potential. Biophys J 64:1908–1921

Walters EM, Men H, Agca Y, Mullen SF, Critser ES, Critser JK (2005) Osmotic tolerance of mouse spermatozoa from various genetic backgrounds: Acrosome integrity, membrane integrity, and maintenance of motility. Cryobiology 50:193-205

Zawlodzka S, Takamatsu H (2005) Osmotic injury of PC-3 cells by hypertonic NaCl solutions at temperatures above 0^{o}C. Cryobiology 50:58-70

Chapter 5

Ovarian re-modulation, endocrine function restoration, and complete follicular development from frozen-thawed, auto-transplanted caprine ovarian cortex

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In preparation

Abstract

Fresh or frozen-thawed ovarian cortical fragments were auto-transplanted onto the uterus in bilaterally ovariectomized goats and recovered at various time points (from 7 to 118 days after transplantation), for histological analysis. Thrice weekly, blood was collected for plasma estrogen and progesterone determination. Early-staged follicles survived cryopreservation, but only primordial follicles presented normal morphology after transplantation. Re-vascularization and re-modulation of transplanted fragments was observed within 1 week and 1 month post-transplantation, respectively. Pre-ovulatory follicles or corpora lutea were found in three (60%) and four (80%) from five of the goats that received fresh and frozen-thawed grafts, respectively. The average time required to complete follicular development was 71,8 ± 13,9 and 71,2 ± 7,9 days in fresh and frozen-thawed grafts, respectively, which was accompanied by estrous behavior and increased plasma estrogen concentrations. In three from five animals with fresh as well as frozen-thawed transplanted tissue, a rise in estrogen concentrations was followed by a rise in plasma progesterone concentration, indicative of ovulation and corpus luteum activity. The present study shows the formation of a well-vascularised ovarian-like structure and recovery of gonadal activity after cryopreservation and auto-transplantation of caprine ovarian cortical tissue.

Keywords
goat ovary ♦ cryopreservation ♦ transplantation ♦ folliculogenesis ♦ steroids

Introduction

Cryopreservation of ovarian tissue has been advocated to preserve female gametes, particularly those in primordial follicles (Shaw et al., 2000; Santos et al., 2006a; Courbière et al., 2007). However, a germ plasm bank per se is not a guarantee for the propagation of genes, unless stored oocytes enclosed in ovarian follicles are able to grow, mature, and can be fertilized. Complete in vitro development of follicles from large mammals is currently not feasible, because biochemical mechanisms involved in the initial folliculogenesis are largely unknown. In addition, such an in vitro procedure takes months in an artificial environment (CO_2 chamber), and would include frequent handling and risks of abnormal oocyte development or, after fertilization, abnormal embryos. Thus, in vivo culture (transplantation) is a more suitable method to acquire and to study follicular development after freezing-thawing of ovarian tissue.

There are less invasive and more invasive methods to perform transplantation of ovarian tissue. Less-invasive methods are carried out subcutaneously, like transplantation to the forearm (Oktay et al., 2001), or the dorsal lumbal region (Hernandez-Fonseca et al., 2005, Israely et al., 2006), while more invasive methods include transplantation into the abdomen e.g. under the kidney capsule (Gosden et al., 1994; Bosch et al., 2004), onto the uterus (Aubard et al., 1999), onto a remaining ovary (Donnez et al., 2004), or at its original position (Bedaiwy et al., 2003; Bordes et al., 2005). Transplantation can be performed with complete ovaries (Bedaiwy et al., 2003) or part of an ovary (Gosden et al., 1994; Aubard et al., 1999; Oktay et al., 2001; Donnez et al., 2004; Bordes et al., 2005; Hernandez-Fonseca et al., 2005; Israely et al., 2006). Although, compared to transplantation into the abdomen, subcutaneous transplantation can be performed relatively easy, this is not a preferred method, because a) estrogen replacement may be necessary (Oktay et al., 2001), b) grafts are absorbed (Hernandez-Fonseca et al., 2005), c) several days before transplantation a wound must be created to allow formation of granulation tissue facilitating tissue re-vascularization (Israely et al., 2006), d) ovarian tissue is exposed to higher pressure than when located in the abdomen, and e) for aesthetic reasons.

Tissue re-vascularization and development of follicular antral cavities have been observed when ovarian fragments are transplanted under the kidney capsule (Gosden et al., 1994; Bosch et al., 2004). However, the relatively low number and

small size of transplanted ovarian cortical pieces per host (Gosden et al., 1994; Bosch et al., 2004), and the pressure that the kidney capsule exerts on the grafts might limit the number and the expansion of growing follicles, respectively (Gosden et al., 1994). Ovarian tissue can also be transplanted to its original position by grafting fragments inside a remaining ovary (human: Donnez et al., 2004) or directly to the ovarian hilus (sheep: Salle et al., 2002; Bordes et al., 2005) as ovarian tissue can be better re-vascularized if transplanted to a well-irrigated site. In both cases, pregnancies and offspring have been reported, but the success of such transplantation in woman could not be repeated (Donnez et al., 2006). Possibly, the ovulated oocytes originated from remaining ovarian tissue. In the experiments of Donnez and co-workers (2004), gonadotropic hormones were administrated to keep the female cycling, but such a treatment increases the risk for breast cancer (Magnusson et al., 1999; Eden, 2003). In experiments with sheep, malformation (1 from 3) and high mortality (2 from 5) of newborns were observed, when vitrified-warmed hemi-ovaries were auto-transplanted (Salle et al., 2002; Bordes et al., 2005).

Insufficient re-vascularization can be avoided by performing micro-anastomosis at transplantation of whole frozen-thawed ovaries (Jeremias et al., 2002; Arav et al., 2005). Although ovarian cryopreservation is relatively successful in juvenile mice, where the ovary has a spongy structure and that has a relatively high number of primordial follicles, efficient procedures must be developed for adult women and livestock animals (Imhof et al., 2006). Frozen-thawed ovine ovarian fragments transplanted onto the uterus displayed follicular growth reaching antrum formation within 13 weeks after transplantation (Aubard et al., 1999). However, PMSG injection was required to induce estrous and more than 95% of the follicles failed to survive. Nevertheless, this latter method seems useful for proper evaluation of an ovarian cryopreservation procedure, if complete ovariectomy is performed and tissue function is recovered without gonadotropic hormone administration.

In this study we have used an adequate experimental animal model, the goat, with ovarian and folliculogenic characteristics similar to those of women. Goats were completely ovariectomized before transplantation of frozen-thawed ovarian tissue was performed. We observed development of ovulatory follicles, formation of corpora lutea, and ovarian endocrine activity restoration without administration of gonadotropic hormones.

Materials and Methods

The Institutional Animal Care and Use Committee of Utrecht University approved this study. Twenty-six adult, non-pregnant normal cycling goats (Saanen and Alpine, 55–80 kg) were included in this study (between October 2006 and April 2007). Goats were divided into three groups: (i) controls (n=5): without surgery, (ii) fresh transplantation (n=11): goats were completely ovariectomized, where after 15 small cortical pieces (~1 mm^3) were immediately grafted onto the uterus, and (iii) frozen-thawed transplantation (n=10): ovarian tissue was completely removed, 15 small cortical (~1mm^3) fragments were cryopreserved, thawed and then auto-transplanted two 2 weeks after the first surgical procedure.

Ovariectomy, auto-transplantation, and euthanasia

Prior to surgery, goats were pre-medicated with medetomidine (Domitor®, Novartis Animal Health, Ontario, Canada) (20 μg/kg), and buprenorfin (Temgesic®, Schering-Plough, Utrecht, The Netherlands) (20 μg/kg). Anesthesia was intravenously induced by ketamine (Narketan®, Vetoquinol, Zurich, Switzerland) (5 mg/kg). Goats were orotracheally intubated with a silicone-cuffed tube (9 mm id, Cook®, Chicago, IL, USA) and anesthesia was maintained through inhalation of isoflurane (Isoflo®, AST pharma, Oudewater, The Netherlands). Isoflurane concentration was kept between 0.6 and 1.0%, goats were breathing spontaneously. A ventral midline skin incision was made to perform ovariectomy. Both ovaries were dissected from the oviduct and blood vessels were ligated. For transplantation of fresh ovarian tissue, 15 cortical fragments (~ 1 mm^3) were sutured together and grafted with prolene 5/0 onto the curvature minor of the uterus (left side). The same procedure was performed for frozen-thawed ovarian tissue, except that in a first procedure only one ovary was removed for fragmentation and freezing, while the other one was completely removed 2 weeks prior to grafting of the cryopreserved ovarian fragments. The effect of anesthesia was reversed with atipamezole (Antisedan®, Novartis Animal Health) (0.5-1 mg/kg) at the end of surgery. Flunixine meglumine (Bedozane®, Schering-Plough) (1 mg/kg) was administered for post-operative analgesia. Euthanasia was performed by intravenous administration of pentobarbital natrium (Euthanasate®, Apharmo, Arnhem, The Netherlands) (0.5 ml/kg).

Freezing and Thawing

The cryopreservation procedure has been described previously (Santos et al., 2006a). Briefly, ovarian fragments were individually placed in 1 ml Cassou straws (IMV, L'Aigle, France) and equilibrated in a controlled-rate programmable freezer (Planer Kryo 10 Series II, Cryotech Benelux, Schagen, The Netherlands) for 20 min at 20°C in Minimum Essential Medium (MEM; Sigma, St. Louis, MI, USA) added by 0.5 M sucrose (Sigma) and 1.0 M ethylene glycol (Sigma). Straws were cooled at 2°C/min from 20°C to -7°C; ice-induction (seeding) was manually performed by touching the straws with forceps pre-cooled in liquid nitrogen. After seeding, straws were held at -7°C for 10 min, cooled at 0.3°C/min to -30°C, after which the straws were immersed immediately into liquid nitrogen (-196°C) and stored for 2 weeks in liquid nitrogen. Before transplantation, the straws were thawed in air for 1 min at room temperature and then immersed in a water bath at 37°C until the cryopreservation medium had completely melted. The cryoprotectant was then removed from the tissue at room temperature by a three-step equilibration (5 min each) in MEM.

Light control

Goats are seasonal breeders, ovarian cycling starting in the autumn due to the decrease in daylight. Because this study was performed during winter and spring (the period in which goats are usually in anestrous), and one of our aims was to detect hormonal activity after transplantation, all animals were housed in a light controlled room (light 8 hours per day), using a described procedure (Chemineau et al., 1986).

Sexual behavior

Sexual behavior was evaluated daily, from the start of the experiment in control animals, and from day 60 post-transplantation onwards in the experimental animals. Heat detection was performed as previously described for goats (Billings and Katz, 1997, 1999). Briefly, one buck was introduced into the females' pen for 30 min and goats were screened on the occurrence of attractivity, proceptivity and receptivity.

Hormonal assays

Heparinized blood samples (jugular vein puncture) were collected thrice weekly, immediately centrifuged and plasma stored at -20^{o}C for hormone analysis. Concentrations of progesterone, estrogen and testosterone were determined by a solid-phase RIA method (Coat-A-Count TKPG, TKE and Total Testosterone, respectively; Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer as validated for progesterone and estrogen (Dieleman and Bevers, 1987), and for testosterone (Buijtels et al., 2006).

Histological Analysis

Follicular quality

To evaluate follicular morphology and development, immediately after collection, ovarian tissue from control and auto-transplanted goats was fixed in 10% formalin in PBS (Gibco, Paisley, UK) for 24 h, dehydrated in ethanol, clarified with xylene, and embedded in paraffin wax. Serial sections (5 μm) of ovarian tissue were cut and every seventh section was mounted on glass slides and stained with haematoxylin-eosin. Early-staged follicles were defined as primordial (follicles with an oocyte surrounded by one flattened layer pre-granulosa cells), intermediate (follicles with an oocyte surrounded by one layer of flattened and cuboidal granulosa cells), primary (follicles with an oocyte surrounded by one layer of cuboidal granulosa cells) or secondary (follicles with an oocyte surrounded by two or more layers of cuboidal granulosa cells). To avoid counting a follicle more than once, follicles were counted only in the sections where their oocyte nucleus was observed. Follicular quality was evaluated based on the morphological integrity of the oocyte, granulosa cells, and basement membrane, where after the percentages of normal and degenerated early-staged follicles were determined. Early-staged follicles were classified as (i) normal, when they contained an intact oocyte and intact granulosa cells, (ii) degenerated, when they contained a pyknotic oocyte nucleus, shrunken ooplasm or disorganized granulosa cells (e.g. when granulosa cells were enlarged in volume or detached from the basement membrane).

Follicular density

Quantitative analysis was conducted by determining the mean cortex area and counting the follicles in the 5th, 15th, and 25th histological sections of each ovary following the method used by Santos et al. (2006b) for estimation of buffalo early-staged follicles density (follicles/mm^2 of cortex). Only follicles with visible oocyte nuclei were counted.

Statistical analysis

The percentages of normal early-staged follicles were compared by a one-way analysis of variance (ANOVA) and Tukey's test. Mean values of follicular density per mm^2 were compared by pairwise Student's t test and ANOVA. In all cases, the statistical tests were performed by using Statview for Windows, and the differences were considered significant when $P \leq 0.05$.

Results

Ovarian tissue from goats that received fresh or frozen-thawed transplanted cortical fragments were processed for histo-morphological evaluation at various time points (from 7 up to 118 days) after surgery. Ovaries collected from control animals had histo-morphologically normal follicles (Fig. 1A). Cryopreserved ovarian cortical tissue fragments had a morphology similar to that of the controls, except that only early-staged follicles (primordial up to and including secondary follicles), had survived the freezing-thawing (Fig. 1B). To evaluate the transplantation procedure, grafted fresh ovarian tissue was recovered one week after surgery. In this transplant, blood vessels with erythrocytes were present (Fig. 1C), indicative of re-vascularization, while follicles that had survived the transplantation procedure were all primordial (Fig 1D,E).

One month post-grafting, in three from five animals that had received fresh ovarian cortical fragments and four from five goats with transplanted frozen-thawed cortical tissue, ovarian fragments had formed a single compact ovarian-like structure (Fig. 2A), with developing follicles (histo-morphologically normal intermediate follicles; Fig. 2B). In the frozen-thawed transplanted ovarian tissue of one of the five animals, follicles were absent, while the degenerating tissue

contained areas with numerous large, luteinized cells (Fig. 2C). In one animal that received fresh ovarian tissue and one that received frozen-thawed ovarian tissue, most of the ovarian fragments were lost within one month of grafting.

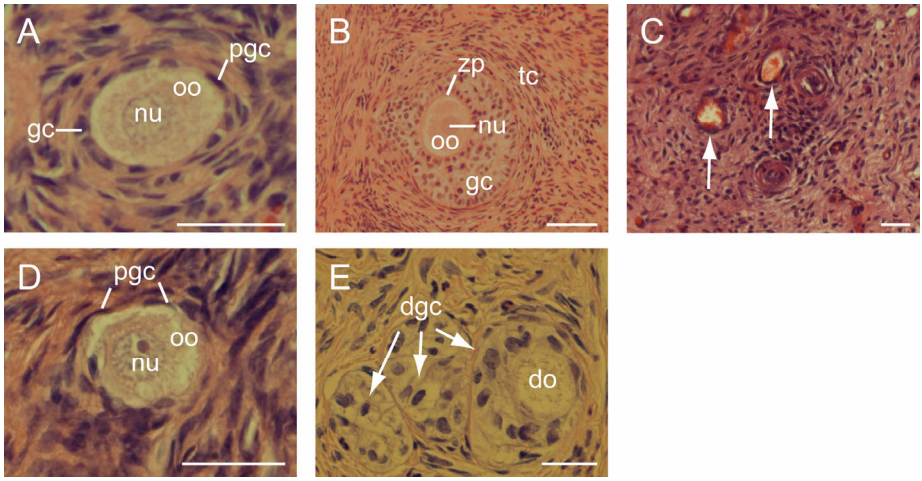

Fig. 1 Histological images of ovarian tissue (control, fresh and frozen-thawed) before and after transplantation. A: Intermediate follicle from control ovarian tissue. B: Secondary follicle with intact central nucleus immediately after freezing-thawing. C: Re-vascularized (arrows) fresh ovarian tissue one week after transplantation. D: Survival of a primordial follicle in fresh ovarian tissue one week after transplantation. E: Three degenerated developing follicles in ovarian tissue one week after transplantation. Note the absence of a nucleus in two of these follicles and the disorganization of granulosa cells. pgc: pre-granulosa cells; gc: granulosa cells; dgc: degenerated granulosa cells; oo: oocyte; do: degenerated oocyte; nu: nucleus; zp: zona pellucida; tc: theca cells. Scale bars represent 30 μm.

Fresh and frozen-thawed ovarian auto-grafts from ten other animals were collected between 86 and 116 days after grafting. In 80% (4 out of 5) of the animals that received frozen-thawed grafts and 60% (3 out of 5) of animals that received fresh ovarian fragments, tissue recovered appeared as a re-modulated ovary and contained, besides numerous early-staged follicles, degenerating antral follicles (Fig. 2D), intact pre-ovulatory follicles (two from fresh and one from frozen-thawed transplants) (Fig. 2E), from which cumulus-oocyte complexes were collected (Fig 2F). Depending on the time of collection, well-vascularized (one from fresh and one from frozen-thawed transplants) (Fig. 2G,H) or poorly vascularized (one from fresh and one from frozen-thawed transplants) (Fig 2I,J) corpora lutea, were also observed.

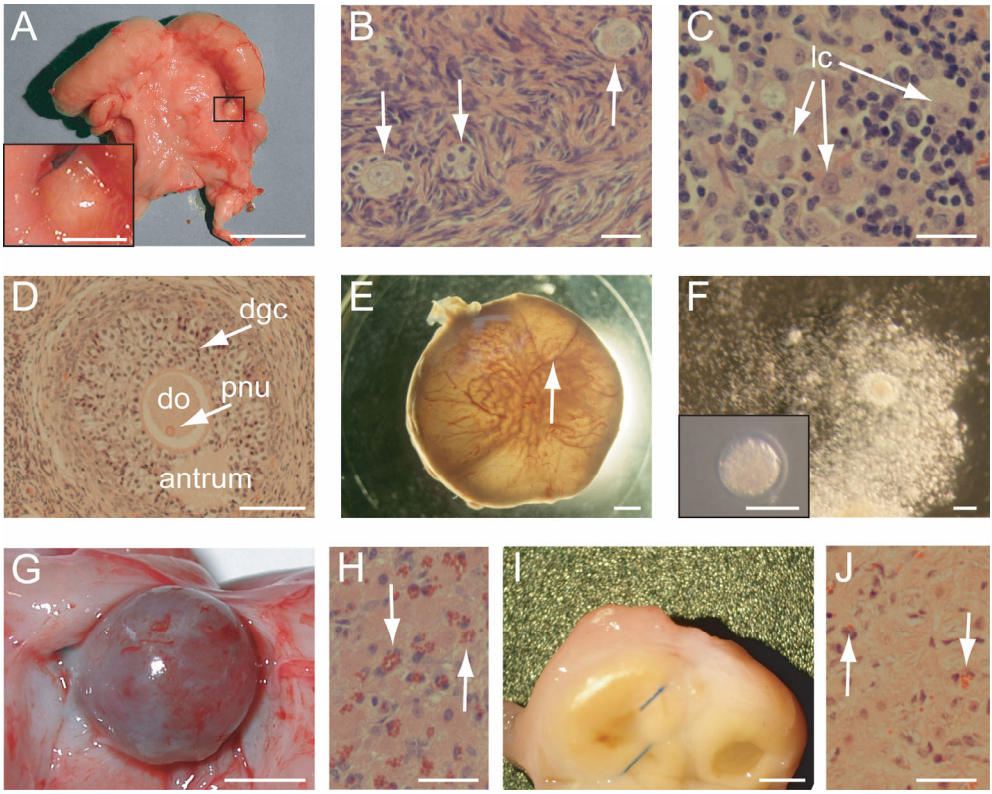

Fig. 2 Macroscopic and microscopic images of frozen-thawed transplanted ovarian tissue. A: Uterus with frozen-thawed ovarian tissue one month post-grafting forming a compact ovarian-like structure (inset shows higher magnification), B: in which primordial ovarian follicles start to develop to intermediate stages, indicative of activation (arrows indicate intermediate follicles). C: Numerous luteinized cells in degenerated transplanted frozen-thawed ovarian tissue one month after transplantation. D: Atretic small antral follicle at 86 days after transplantation (note the degenerating peripherally located oocyte nucleus and atretic granulosa cells) E: Macroscopic view of a pre-ovulatory follicle at three months after transplantation (arrow indicates blood vessels). F: A mature cumulus-oocyte-complex collected from a pre-ovulatory follicle (inset shows a higher magnification of the denuded oocyte). G: Macroscopic view of an active corpus luteum and its microscopical image (H) showing well vascularized tissue. I: Longitudinal section of re-modulated ovarian tissue with two corpora lutea, that are poorly vascularized (J) (arrows in H and J indicate erythrocytes). Scale bars: A: 5 cm (inset: 1cm); B: 30 µm; C: 30 µm; D: 100 µm; E: 1 mm; F: 100 µm; G: 1 cm; H: 30 µm; I: 1 mm; J: 30 µm.

Follicular quality of early-staged follicles after transplantation was evaluated by classifying them as normal or degenerated. Percentages of normal

follicles were not influenced by the period post-transplantation collection (data not shown), and data were grouped. A significant decrease in the percentages of normal early-staged follicles was observed in goats after transplantation when compared to that in ovaries of control animals (Fig. 3).

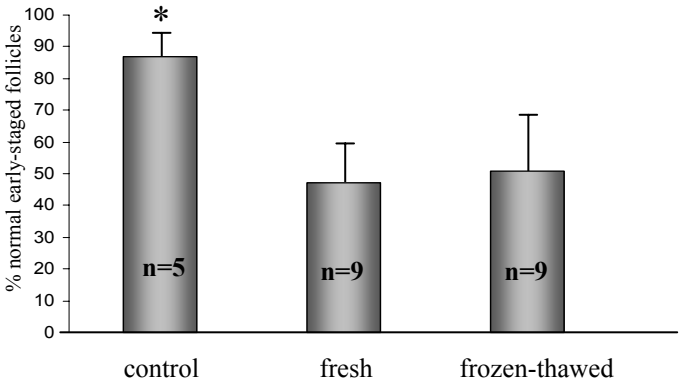

Figure 3. Mean percentages (Mean ± SD) of normal caprine early-staged follicles in control ovarian tissue and after. * Differs significantly.

To evaluate follicular loss independently of survival rates, follicular density was determined. The mean density of follicles was $14,1 \pm 3,7/mm^2$ in control ovarian tissue and had significantly decreased to $8,2 \pm 2,1/mm^2$ and $7,5 \pm 3,6/mm^2$ in fresh and frozen-thawed transplanted tissue, respectively. Differences between the latter two transplantation groups were not statistically ($P>0.05$) significant.

In all ten goats from which ovarian tissue was collected at least 86 days after transplantation, estrous behavior was first detected at days $71,8 \pm 13,9$ and $71,2 \pm 7,9$ after transplantation of fresh and frozen-thawed ovarian tissue, respectively. Estrous behaviour was accompanied by an increase in plasma estrogen concentration, although estrogen levels were about 2-fold lower compared with those of control animals (Fig. 4).

In two of the animals with fresh transplanted tissue and two animals with frozen-thawed transplanted tissue, the rise in estrogen concentration was not followed by a rise in plasma progesterone concentration, corresponding with the absence of corpora lutea. Testosterone concentrations remained at control levels,

i.e. lower than 0.1 ng/ml. All control animals were cycling during the whole experiment.

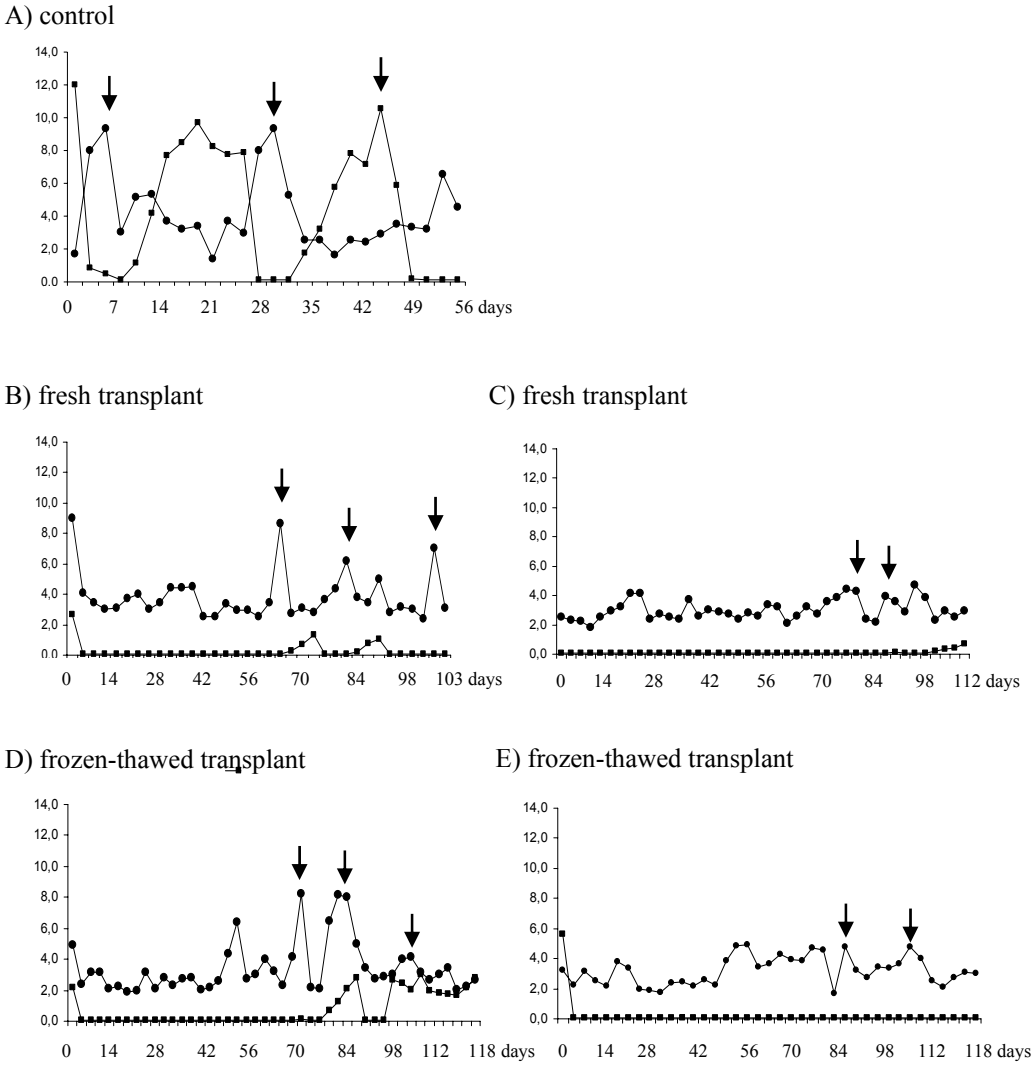

Figure 4. Representative hormone profiles (progesterone ∎ - ng/ml and estrogen ● - pg/ml) of A: control goat, B,C: goats that received fresh transplantation, and D,E: goats that received frozen-thawed ovarian tissue. Arrows indicate estrous behavior.

Discussion

In this study, we demonstrate complete follicular development and recovery of endocrine function after cryopreservation and auto-transplantation of small fragments of ovarian tissue in bilaterally ovariectomized goats. Re-vascularization of the grafted tissue is crucial for cell survival after transplantation of ovarian tissue (Israely et al., 2004). Within one week of transplantation re-vascularization occured, and only primordial follicles retained adequate histo-morphology (Aubard et al., 1999; this study). Although primordial follicles can survive for years in absence of nearby located blood vessels (Van den Hurk et al., 1997; Van den Hurk and Zhao, 2005), systemic factors supplied by neo-vascularization are essential for their activation and subsequent follicular development. After freezing-thawing of ovarian tissue with the current protocol, viable early-staged follicles, from primordial up to and including secondary, could be identified, indicating that any atresia of developing early-staged follicles was due to hypoxia suffered by the tissue before complete re-vascularization, but not by the cryopreservation method itself.

Between two and three months after transplantation of fresh or cryopreserved ovarian tissue, we observed antral and pre-ovulatory follicles and corpora lutea in re-modulated ovarian-like structures, indicating the ability of cryopreserved primordial follicles to completely develop until the ovulatory stage and to ovulate. This was accompanied by increased levels of serum estradiol. Recovery of endocrine function has been reported in mice after transplantation of cryopreserved ovarian tissue. However, in most of the studies the whole ovarian tissue was grafted (Gunasena et al., 1997), sometimes in hemi-ovariectomized recipients (Salehnia, 2002), instead of only a minimal part of the ovary in ovariectomized recipients as has been done in the present study. Furthermore, the current study demonstrates that, after adequate re-vascularization, ovarian tissue can reacquire its architecture, indicating that gonadal function can be recovered even when only a small fraction (~30%) of one ovary is transplanted. Transplantation of small fragments of an ovary opposed to complete ovaries facilitates re-vascularization, and therefore better survival and function of tissues.

To accentuate the different morphological observations, we analysed the hormonal profiles and behaviour of the animals at pre- and post-transplantation. Estrous behaviour, i.e. attractivity, procepitivity and receptivity, was observed in

all animals having transplants, but was not always, accompanied by ovulation and formation of corpora lutea. Two goats from each transplantation group presented such estrous behaviour in presence of only moderately increased serum estrogen levels and absence of a subsequent rise in progesterone levels. These findings corresponded with the absence of pre-ovulatory follicles and corpora lutea in these animals. The absence of a rise in plasma testosterone levels in these goats indicates that anovulation was not caused by hyperandrogenism, but more likely to too low estradiol levels, which apparently are sufficient to stimulate estrous behaviour, but insufficient to support finalization of folliculogenesis and subsequent ovulation.

In fresh or frozen-thawed transplants, about 50% of early-staged follicles had not developed, but instead underwent atresia. Moreover, follicular density appeared significantly decreased, probably because of the removal of follicles that were degenerated. It has been suggested that cryopreservation negatively influences follicular development, particularly by inhibiting the formation of primary from primordial follicles (Choi et al., 2007). However, we observed such an effect not only in frozen-thawed transplants, but also in fresh transplants, indicating that it is not the cryopreservation method, but the surgical procedure or the amount of tissue grafted that damages early-staged follicles. Apart from re-vascularization, other causes may determine follicle survival and development in transplants, such as a lack of adequate (neo)innervation (Van den Hurk et al., 1997) and a disturbance in the balance between inhibitory and stimulatory biochemical factors of local origin (Van den Hurk et al., 2000; Van den Hurk and Zhao, 2005). Despite the reduction in viable follicles in ovarian tissue transplants, we consider the followed freezing-thawing and transplantation procedures to be successful, since the main ovarian functions, development of early-staged follicles to ovulatory stages and adequate production of estrogens were recovered.

Duration of folliculogenesis, i.e. the development of a primordial follicle up to the pre-ovulatory stage, is determined by the first rise in serum estradiol level after transplantation, and appeared around 70 days in the goats used in the present studies, thus similar to that reported for adult women (Gougeon, 1985). In cows and sheep, folliculogenesis takes at least 105 (Van den Hurk et al., 2000) and 115 days (Bordes et al., 2005), respectively. Furthermore, in early developmental stages, mean diameters of goat oocytes and follicles are more similar to those of man than to those from other livestock animals (Lucci et al., 2001).

Our observations show ovarian tissue re-modulation and recovery of gonadal activity, i.e. acquisition of complete follicular development enabling augmentation of estrogen secretion as well as estrous behaviour, after cryopreservation and auto-transplantation of a small part of caprine ovarian tissue. Furthermore, folliculogenesis in adult goats was completed within a similar time frame as observed in women.

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References

Arav A, Revel A, Nathan Y, Bor A, Gacitua H, Yavin S, Gavish Z, Uri M, Elami A (2005) Oocyte recovery, embryo development and ovarian function after cryopreservation and transplantation of whole sheep ovary. Hum Reprod 20:3554-3559

Aubard Y, Piver P, Cogni Y, Fermeaux V, Poulin N, Driancourt MA (1999) Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum Reprod 14:2149-2154

Bedaiwy MA, Jeremias E, Gurunluoglu R, Hussein MR, Siemianow M, Biscotti C, Falcone T (2003) Restoration of ovarian function after autotransplantation of intact frozen/thawed sheep ovaries with microvascular anastomosis. Fertil Steril 79:594-602

Billings HJ, Katz LS (1997) Progesterone facilitation and inhibition of estrogen-induced sexual behavior in the female goat. Horm Behav 31:47-53

Billings HJ, Katz LS (1999) Facilitation of sexual behavior in French-alpine goats treated with intravaginal progesterone-releasing devices and estrogen. J Anim Sci 77:2073-2078

Bordes A, Lornage J, Demirci B, Franck M, Courbière B, Guerin JF, Salle B (2005) Normal gestations and live births after orthotopic autograft of vitrified-warmed hemi-ovaries into ewes. Hum Reprod 20:2745-2748

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

Buijtels JJCWM, Beijerink NJ, Kooistra HS, Dieleman SJ, Okkens AC (2006) Effects of gonadotrophin releasing hormone administration on the pituitary-ovarian axis in anoestrous vs ovariectomized bitches. Reprod Dom Anim 41:555-561

Chemineau P, Normant E, Ravault JP, Thimonier J (1986) Induction and persistence of pituitary and ovarian activity in the out-of-season lactating dairy goat after a treatment combining a skeleton photoperiod, melatonin and the male effect. J Reprod Fertil 78:497-504

Choi J, Lee JY, Lee E, Yoon BK, Bae D, Choi D (2007) Cryopreservation of the mouse ovary inhibits the onset of primordial follicle development. Cryobiology 54:55-62

Courbière B, Provansal M, Saias-Magnan J, Guillemain C, Noizet A, Grillo JM, Gamerre M (2007) What are at present the real hopes of pregnancy after ovarian cryopreservation? Gynecol Obstet Fertil (epub ahead for printing)

Dieleman SJ, Bevers MM (1987) Effects of monoclonal antibody against PMSG administred shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. J Reprod Fertil 81:533-542

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, Van Langendonckt A (2004) Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 364:1405-1410

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, Van Langendonckt A (2006) Restoration of ovarian function after orthotopic (intraovarian and periovarian) transplantation of cryopreserved ovarian tissue in a woman treated by bone marrow transplantation for sickle cell anaemia: case report. Hum Reprod 21:183-188

Eden J (2003) Progestins and breast cancer. Am J Obstet Gynecol 188:1123-1131

Gosden RG, Boulton MI, Grant K, Webb R (1994) Follicular development from ovarian xenografts in SCID mice. J Reprod Fertil 101:619-623

Gougeon A (1985) Origin and growth of the preovulatory follicle(s) in spontaneous and stimulated cycles. In: Testart J and Friedman R (ed) Human in vivo fertilisation, Insem Symposium, 24, Amsterdam, Elsevier

Gunasena KT, Vilines PM, Critser ES, Critser JK (1997) Live births after autologys transplant of cryopreserved mouse ovaries. Hum Reprod 12:101-106

Hernandez-Fonseca H, Bosch P, Miller D, Massey JB, Brackett BG (2005) Time course of follicular development after bovine ovarian tissue transplantation in male non-obese diabetic severe combined immunodeficient mice. Fertil Steril 83:1180-1187

Imhof M, Bergmeister H, Lipovac M, Rudas M, Hofstetter G, Huber J (2006) Orthotopic microvascular reanastomosis of whole cryopreserved ovine ovaries resulting in pregnancy and live birth. Fertil Steril 85:1208-1215

Israely T, Dafni H, Nevo N, Tsafriri A, Neeman M (2004) Angiogenesis in ectopic ovarian xenotransplantation: multiparameter characterization of the neovasculature by dynamic contrast-enhanced MRI. Magn Reson Med 52:741-750

Israely T, Nevo N, Harmelin A, Neeman M, Tsafriri A (2006) Reducing ischaemic damage in rodent ovarian xenografts transplanted into granulation tissue. Hum Reprod 21:1368-1379

Jeremias E, Bedaiwy MA, Gurunluoglu R, Biscotti CV, Siemionow M, Falcone T (2002) Heterotopic autotransplantation of the ovary with microvascular anastomosis: a novel surgical technique. Fertil Steril 77:1278-1282

Lucci CM, Silva JR, Carvalho CA, Figueiredo JR, Bao SN (2001) Light microscopical and ultrastructural characterization of goat preantral follicles. Small Rumin Res 41:61-69

Magnusson C, Baron JA, Correia N, Bergstrom R, Adami HO, Persson I (1999) Breast cancer risk following long-term oestrogen-progestins-replacement therapy. Int J Cancer 81:339-344

Oktay K, Economos K, Kan M, Rucinski J, Veeck L, Rosenwaks Z (2001) Endocrine function and oocyte retrieval after autologus transplantation of ovarian cortical strips to the forearm. JAMA 26:1490-1493

Salehnia M (2002) Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. Exp Anim 51:509-512

Salle B, Demirci B, Franck M, Rudigoz RC, Guerin JF, Lornage J (2002) Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. Fertil Steril 77:403-408

Santos RR, Tharasanit T, Figueiredo JR, Van Haeften T, Van den Hurk R (2006a) Preservation of caprine preantral follicles viability after cryopreservation in sucrose and ethylene glycol. Cell Tissue Res 325:523-531

Santos SSD, Biondi FC, Cordeiro MS, Miranda MS, Dantas JK, Figueiredo JR, Ohashi OM (2006b) Isolation, follicular density, and culture of preantral follicles of buffalo fetuses of different ages. Anim Reprod Sci 95:1-15

Shaw JM, Oranratnachai A, Trounson AO (2000) Fundamental cryobiology of mammalian oocytes and ovarian tissue. Theriogenology 53:59-72

Van den Hurk R, Bevers MM, Beckers JF (1997) In-vivo and in-vitro development of preantral follicles. Theriogenology 47:73-82

Van den Hurk R, Abir R, Telfer EE, Bevers MM (2000) Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. Hum Reprod Up 6:457-474

Van den Hurk R, Zhao J (2005) Formation of mammalian oocytes and their growth differentiation and maturation within ovarian follicles. Theriogenology 63:1717-1751

Chapter 6

Summarising discussion

Freezing or vitrification of ovarian tissue is a promising technique to rescue animal species and breeds from extinction and to restore fertility of women after cancer treatment by preserving a major source of genetic material: thousands to millions of immature oocytes enclosed in early-staged follicles. The preservation of ovaries implies that, apart from oocytes, also somatic cells are preserved, offering a proper physiological environment for oocyte growth and maturation through their production of local growth factors and hormones, thus without external interference.

Human ovaries or those from closely related species such as non-human primates are the best study objects to develop an efficient protocol for human ovarian tissue preservation. However, especially for primates, the ethical aspects of experiments are extensively and thoroughly discussed and weighed by Institutional Animal Care and Use Committees (IACUC), and when experiments are approved the sample sizes are always minimal. Once ovarian biopsies from women that have been submitted to surgery because of reproductive disorders are attainable more study material could be available. For biomedical studies, many protocols are designed and tested in laboratory animals such as mice and rats. The mouse, however, is not the preferred model for ovary cryopreservation studies as murine ovaries are spongy, facilitating cryoprotectant permeation and removal, which is completely different from the fibrous human ovarian tissue. In addition, human folliculogenesis takes 10 weeks, which is much longer than that the 3 weeks required in mice. A more suitable experimental animal model for human ovarian tissue preservation should present an ovarian structure, follicular development, and duration of folliculogenesis similar to those in women. Therefore, as is shown in this thesis, the goat is a favourable experimental model for ovarian cryopreservation for the human.

In this final chapter of the thesis, the main results are discussed, focusing on the development of efficient cryopreservation protocols for ovarian tissue and early-staged follicles, and transplantation of frozen-thawed ovarian tissue. Finally, some concluding remarks and future perspectives regarding cryopreservation and subsequent transplantation of ovarian tissue are given.

Establishment of a cryopreservation protocol for ovarian tissue

Establishment of cryopreservation protocols starts by choosing suitable cryoprotective agents. For this purpose, various intracellular cryoprotectants such as propanediol, ethylene glycol (EG), dimethyl sulfoxide (DMSO) and glycerol have been compared (Newton et al., 1996 – human; Jewgenow et al., 1998 – feline; Rodrigues et al., 2004a,b – caprine; Santos et al., 2006, 2007 - ovine) in concentrations ranging from 0.5 (Amorim et al., 2003a,b) to 3.0 M (Rodrigues et al., 2004a,b). However, several aspects need further attention, such as (i) the dimension of ovarian fragments, (ii) the volume of the cryoprotectant solution, (iii) the addition of extra-cellular cryoprotectant, and (iv) efficient methods to evaluate follicular quality.

In mice, ovarian tissue cryopreservation has been successfully developed. However, as mentioned before, because of structural differences (e.g. spongy ovaries in mice vs. fibrous ovaries in domestic animals and humans), it is not possible to cryopreserve the whole ovary (Imhof et al., 2006) from large mammals with the same success obtained with ovaries from mice (Harp et al., 1994). Indeed, cryopreservation of caprine ovarian slices with similar dimensions (3x3x1 mm) as a complete murine ovary was not successful (Rodrigues et al., 2004a,b). Nevertheless, the fibrous architecture of ovaries from large mammals does not imply that the concentration or volume of cryoprotectants needs to be higher than those routinely used for murine ovaries. Caprine ovarian tissue is successfully cryopreserved when fragmented in pieces of ~ 1mm^3 and exposed to a low volume (frozen in 1 ml or vitrified in 0.5 ml) of a combination of ethylene glycol and sucrose (**Chapters 2 and 3**). Yeoman et al. (2005) demonstrated that minimising the volume of vitrified samples helps to maximise cooling rates and improves vitrification of monkey ovarian tissue.

To validate a cryopreservation protocol, efficient evaluation methods are necessary. Caprine early-staged follicles were successfully cryopreserved in a combination of sucrose and EG, and normal follicular morphology (i.e. histology) was maintained similar to that of control follicles (**Chapters 2 and 3**). Because evaluation of histological morphology is not sufficient to assure follicular quality (Schotanus et al., 1997; Van den Hurk et al., 1998; Santos et al., 2006), viability markers were used to confirm the obtained results. The commonly used procedure for evaluation of cellular viability, membrane integrity, of cryopreserved ovarian

follicles is staining with trypan blue (Rodrigues et al., 2005 - caprine; Santos et al., 2007 - ovine; Fauque et al., 2007 - human). Although results can be obtained with this technique within several minutes, use of trypan blue does not allow (i) distinction between damage in the oocyte or in the granulosa cells, (ii) to determine the proportion of live/dead granulosa cells per follicle, and (iii) evaluation of cytoplasmic and nuclear integrity. Thus, fluorescent markers such as calcein-AM and ethidium homodimer-1 have additionally been used to assess the viability of cryopreserved early-staged follicles (**Chapters 2, 3 and 4**). Detection of esterase enzyme activity with calcein-AM and assessment of plasma membrane integrity with ethidium homodimer-1 has previously been performed to detect viability of bovine fresh (non-cryopreserved) early-staged follicles (Schotanus et al., 1997; Van den Hurk et al., 1998). In addition, by using these fluorescent markers, it is possible to distinguish between live and dead cells and to establish the ratio of viable granulosa cells per viable oocyte to consider follicular viability (**Chapters 2, 3 and 4**). Cellular viability after cryopreservation, however, does not guarantee follicular development. In vitro culture of cryopreserved early-staged follicles is another important tool to evaluate freezing/thawing and vitrification/warming procedures. Several follicular impairments caused by cryopreservation, such as granulosa cell-oocyte junction damage (Cecconi et al., 2004), basement membrane disruption (Santos et al., 2007) and reduction or delay in granulosa cell proliferation (Cortvrindt et al., 1996), are better detected after in vitro culture of follicles than immediately after their thawing. Thus, cryopreserved follicles may show a significant decline in quality after having them in vitro cultured for 24 h (Paynter et al., 1999; Santos et al., 2007).

Follicular damage by osmotic stress

It has been suggested that hyper and hypotonic stress can adversely affect cryo-resistance of cells (Meryman, 1968; Mazur et al., 1970; Pegg and Diaper, 1988). However, most of the conclusions over freezing damage were based on results with erythrocytes, which cannot be compared to complex structures such as ovarian follicles. Recently, Men and co-workers (2005) have demonstrated osmotic stress of in vitro produced blastocysts exposed to high concentrations of sucrose (2400 mOsm). In contrast, in **Chapter 4** it has been demonstrated that high concentrations of cryoprotectants, e.g. 4.0 M EG (5242 mOsm), are not responsible for osmotic damage. Possibly, in vitro produced embryos are more sensitive than early-staged follicles when submitted to cryoprotectants. Also use of only sucrose

(an extra-cellular cryoprotectant) to determine osmotic tolerance may increase the concentrations of electrolytes in cells. In addition, differences in permeability can determine the limit of dehydration and concentration of ions into the cell (Fahy, 2007), and when early-staged follicles were exposed to an ionic solution of 9% NaCl (2800 mOsm), most of the cells (92%) had lost their viability (**Chapter 4**). Ions can cause cellular damage (Lovelock, 1953) and cryoprotectants are non-ionic solutes. Furthermore, eukaryotic cells have several mechanisms besides that of simple diffusion by which they react against changes in osmolality such as Na^{+}/K^{+}-ATPase, to maintain intracellular osmotic conditions (Darnell et al., 1986). Similar to results described in **Chapter 4**, high concentrations of NaCl (1200–2000 mOsm) were toxic for embryos (Stachecki et al., 1998). It is concluded that biophysical and biochemical effects of cryopreservation can be cell or tissue specific.

Follicular development after cryopreservation

The aim of cryopreservation of gametes is storage for an undetermined time without viability impairment, followed by further development after thawing/warming. Despite the success obtained with the complete in vitro development of cryopreserved early-staged murine follicles (Eppig and O'Brien, 1996) - including development of frozen-thawed follicles followed by successful fertilization, embryo development (Smitz and Cortvrindt, 1998) and healthy offspring (Liu et al., 2001; De la Peña et al., 2002) - similar success is difficult to achieve with large mammals. Nevertheless, many studies have improved the in vitro culture of large mammalian early-staged follicles, and resulted in their activation and growth (Fortune et al., 1998; Silva et al., 2004, 2006; Gigli et al., 2006). For instance, testosterone supplementation during culture resulted in development of primary stage follicles to secondary follicles (Yang and Fortune, 2006; Santos, Van den Hurk and Roelen, data not published). When large secondary follicles were cultured in vitro, antrum formation (Gutierrez et al., 2000), oocyte maturation, fertilisation of oocytes and subsequent embryonic development have been accomplished (Wu and Tian, 2007). However, complete development and differentiation of follicles from primates and livestock animals, from primordial up to and including the pre-ovulatory stage, requires months of in vitro culture (Van den Hurk et al., 1999). Furthermore, adequate knowledge is necessary regarding the many factors involved in each step of follicular development, including follicular activation, granulosa cell proliferation and differentiation, oocyte growth, differentiation and maturation, and ovulation (for

reviews, see Van den Hurk et al., 2000; Van den Hurk and Zhao, 2005). Especially, more knowledge is needed regarding the factors that control the development and differentiation of the earliest follicle stages (see Smitz and Cortvrindt, 2002).

Because of the absence of an in vitro protocol for culture of early-staged follicles from large mammals and the importance of obtaining complete follicular development after cryopreservation, transplantation of ovarian tissue appears a plausible method. In **Chapter 5**, complete follicular development of early-staged goat follicles is demonstrated after freezing-thawing of ovarian tissue fragments and their re-implantation in ovariectomised donor animals. Thereby, ovarian cortical fragments re-modulated into a new ovarian-like organ, from which blood supply and endocrine function (oestradiol and progesterone production) were restored. These observations demonstrate that is not necessary to freeze a complete ovary and subsequently perform re-anastomosis of blood vessels to recover ovarian structure and function. Currently, in case of cancer treatment, only fragments of the ovary can be cryopreserved, while the remaining ovary is maintained. Presumably, because part of the original ovary remained in situ, a pregnancy could be obtained after transplantation of frozen-thawed ovarian fragments in the remaining ovary of a patient, followed by hormonal treatment (Donnez et al., 2004). To properly evaluate a cryopreservation procedure, frozen/vitrified tissue must be transplanted to a thoroughly ovariectomised recipient to show the ability of cryopreserved tissue to produce hormones by itself and to exhibit normal follicular development. However, because cryopreservation still is in an experimental phase, complete ovariectomy in human patients is not advisable, reinforcing the importance of a proper experimental model such as livestock mammals. Cryopreservation of human ovarian tissue is better compared experimentally with domestic ruminants such as cattle (Gandolfi et al., 2006), sheep (Baird et al., 1999) and goats (this thesis) than with mice. Furthermore, when comparing the duration of folliculogenesis in adult women (~70 days - Gougeon, 1996), cows (~105 days - Van den Hurk et al., 2000), ewes (~115 days - Bordes et al., 2005) and goats (~70 days - **Chapter 5**), goats are preferred for development of safe cryopreservation and transplantation methods to be applied to man. Although healthy offspring (2 lambs) was obtained in sheep after cryopreservation of ovarian tissue, birth of a malformed lamb raised caution for the use of ovarian cryopreservation in man (Bordes et al., 2005).

Concluding remarks and perspectives

Ovarian cryopreservation has been performed not only experimentally in laboratory and livestock animals, but is also applied in women after cancer treatment. Successful results, including normal offspring, have been reported in human after transplantation of frozen-thawed tissue (Donnez et al., 2004). However, this latter observation has not been successfully repeated yet. In the last three years, studies with women have re-evaluated cryopreservation and transplantation procedures. Furthermore, although live birth has been obtained in livestock animals, unwanted results such as malformed offspring has also been described. These unfortunate observations include also a warning for researchers to be cautious with the data thus far presented on the freezing and transplantation of human ovarian tissue and indicate that results are not standardized to guarantee success of a specific procedure.

It can be argued that abnormal follicular development in frozen-thawed ovarian tissue is not necessarily caused by the cryopreservation per se, but might be due to the surgical procedure as has been shown in **Chapter 5**. Indeed, immediately after thawing, cells appear to be viable and able to develop. However, in the studies thus far done, DNA integrity or activation of genes involved in malformations have not been evaluated. Even when cells are not affected genetically by freezing/vitrification, efficient transplantation of ovarian fragments or the whole ovarian tissue needs to be further studied and developed, before the method can be safely applied on a large scale. Thus, before clinical application, there are remaining points to be evaluated for cryopreservation and transplantation. For example, although because of the absence of DNA damage, early-staged follicles have a higher survival rate after cryopreservation than mature oocytes, this does not mean that cooling cannot affect DNA by activating genes related to diseases and malformations. Thus, more experiments should be performed in adequate animal models, such as the goat, until data can be satisfactorily repeated and normal offspring can be guaranteed.

Although cryopreservation of ovarian tissue should not be promoted to postpone childbearing in healthy women, such a technique may be used in the future for safeguarding maternity for women requiring chemo- or radiotherapy. In addition, it may in the near future be an important technique for the preservation of endangered breeds and species.

References

Amorim CA, Rodrigues APR, Rondina D, Gonçalves PBD, Figueiredo JR, Giorgetti A (2003a) Cryopreservation of ovine primordial follicles using dimethyl sulfoxide. Fert Steril 79:682-686

Amorim CA, Rondina D, Rodrigues APR, Costa SHF, Goncalves PBD, Figueiredo JR, Giorgetti A (2003b) Isolated ovine primordial follicles cryopreserved in different concentrations of ethylene glycol. Theriogenology 60:735-742

Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG (1999) Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196^{o}C. Endocrinology 140:462-471

Bordes A, Lornage J, Demirci B, Franck M, Courbiere B, Guerin JF, Salle B (2005) Normal gestations and live births after orthotopic autograft of vitrified-warmed hemi-ovaries into ewes. Hum Reprod 20:2745-2748

Cecconi S, Capacchietti G, Russo V, Berardinelli P, Mattioli M, Barboni B (2004) In vitro growth of preantral follicles isolated from cryopreserved ovine ovarian tissue. Biol Reprod 70:12-17

Cortvrindt R, Smitz J, Van Steirteghem AC (1996) A morphological and functional study of the effect of slow freezing followed by complete in-vitro maturation of primary mouse ovarian follicles. Hum Reprod 11:2648-2655

Darnell J, Lodis H, Baltimore D (1986) Active transport of ions and ATP hydrolysis. In: Darnell J, Lodis H, Baltinore D (ed) Molecular Cell Biology. pp 625-628. Scientific American Books, New York

De la Peña EC, Takahashi Y, Katagiri S, Atabay EC, Nagano M (2002) Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles. Reproduction 123:593-600

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, Van Langendonckt A (2004) Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 364:1405-1410

Eppig JJ, O'Brien MJ (1996) Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 54:197-207

Fahy GM (2007) Theoretical considerations for oocyte cryopreservation by freezing. RBM Online 14:709-714

Fauque P, Ban Amor A, Joanne C, Agnani G, Bresson JL, Roux C (2007) Use of trypan blue staining to assess the quality of ovarian cryopreservation. Fertil Steril 87:1200-1207

Fortune JE, Kito S, Wandji SA, Srsen V (1998) Activation of bovine and baboon primordial follicles in vitro. Theriogenology. 49:441-449

Gandolfi F, Paffoni A, Brambilla EP, Bonetti S, Brevini TAL, Ragni G (2006) Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. Fertil Steril 85:1150-1156

Gigli DA, Byrd DD, Fortune JE (2006) Effects of oxygen tension and supplements to the culture medium on activation and development of bovine follicles in vitro. Theriogenology 66:344-353

Gougeon A (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17:121-155

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Web R (2000) Growth and antrum formation of bovine preantral follicles in long-term culture in vitro. Biol Reprod 62:1322-1328

Harp R, Leibach J, Black J, Keldahl C, Karow A (1994) Cryopreservation of murine ovarian tissue. Cryobiology 31:336-343

Imhof M, Bergmeister H, Lipovac M, Rudas M, Hofstetter G, Huber J (2006) Orthotopic microvascular reanastomosis of whole cryopreserved ovine ovaries resulting in pregnancy and live birth. Fertil Steril 85:1208-1215

Jewgenow K, Penfold LM, Meyer HH, Wildt DE (1998) Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. J Reprod Fertil 112:39-47

Liu J, Van der Elst J, Van den Broecke R, Chont M (2001) Live offspring by in vitro fertilization of oocytes from cryopreserved primordial mouse follicles after sequential in vivo transplantation and in vitro maturation. Biol Reprod 64:171-178

Lovelock JE (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochem Biophys Acta 11:28-36

Mazur P (1970) Cryobiology: the freezing of biological systems. Science 168:939-949

Men H, Agca Y, Mullen SF, Critser ES, Critser JK (2005) Osmotic tolerance of in vitro produced porcine blastocysts assessed by their morphological integrity and cellular actin filament organization. Cryobiology 51:119-129

Meryman HT (1968) Modified model for the mechanism of freezing injury in erythrocytes. Nature 218:333-336

Newton H, Aubard Y, Rutherford A, Sharma V, Gosden R (1996) Low temperature storage and grafting of human ovarian tissue. Hum Reprod 11:1487-1491

Paynter SJ, Cooper A, Fuller BJ, Shaw RW (1999) Cryopreservation of bovine ovarian tissue: structural normality of follicles after thawing and culture in vitro. Cryobiology 38:301-309

Pegg DE, Diaper MP (1988) On the mechanism of injury to slowly frozen erythrocytes. Biophysical J 54:471-488

Rodrigues AP, Amorim CA, Costa SH, Matos MHT, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004a) Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology 61:1009-1024

Rodrigues AP, Amorim CA, Costa SH, Matos MHT, Santos RR, Lucci CM, Bao SN, Ohashi OM, Figueiredo JR (2004b) Cryopreservation of caprine ovarian tissue using dimethyl sulfoxide and propanediol. Anim Reprod Sci 84:211-227

Rodrigues APR, Amorim CA, Costa SHF, Santos RR, Lucci CM, JF Nunes, Figueiredo JR (2005) Cryopreservation and short-term culture of isolated caprine primordial follicles. J Small Rum Res 56:103-111

Santos RR, Rodrigues APR, Costa SHF, Silva JRV, Matos MHT, Lucci CM, Bao SN, Van den Hurk R, Figueiredo JR (2006) Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. Anim Reprod Sci 91:249-263

Santos RR, Van den Hurk R, Rodrigues APR, Costa SHF, Martins FS, Matos MHT, Celestino JJH, Figueiredo JR (2007) Effect of cryopreservation on viability, activation and growth of in situ and isolated ovine early-stage follicles. Anim Reprod Sci 99:53-64

Schotanus K, Hage WJ, Vanderstichele H, Van den Hurk, R (1997) Effects of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. Theriogenology 48:471-483

Silva JRV, Van den Hurk R, Van Tol HT, Roelen BAJ, Figueiredo JR (2004) Gene expression and protein localisation for activin-A, follistatin and activin receptors in goat ovaries. J Endocrinol 183:405-415

Silva JRV, Tharasanit T, Taverne MA, Van der Weijden GC, Santos RR, Figueiredo JR, Van den Hurk R (2006) The activin-follistatin system and in vitro early follicle development in goats. J Endocrinol 189:113-125

Smitz JE, Cortvrindt R (1998) Follicle culture after ovarian cryostage. Maturitas 30:171-179

Smitz JE, Cortvrindt RG (2002) The earliest stages of folliculogenesis in vitro. Reproduction 123:185-202

Stachecki JJ, Cohen J, Willadsen SM (1998) Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. Cryobiology 37:346-354

Van den Hurk R, Spek ER, Hage WJ, Fair T, Ralph JH, Schotanus K (1998) Ultrastructure and viability of isolated bovine preantral follicles. Hum Reprod Up 4:833-841

Van den Hurk R, Bevers MM, Dieleman SJ (1999) Folliculogenesis and oocyte development in mammals (livestock animals). In: Comparative endocrinology and reproduction, Joy KP, Krishna A, Haldar C (ed) pp.296-312. Narosa Publishing House, New Delhi, India

Van den Hurk R, Abir R, Telfer EE, Bevers MM (2000) Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. Hum Reprod Up 6:457-474

Van den Hurk R, Zhao J (2005) Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology 63:1717-1751

Wu J, Tian Q (2007) Role of follicle stimulating hormone and epidermal growth factor in the development of porcine preantral follicle in vitro. Zygote 15:233-240

Yang MY, Fortune JE (2006) Testosterone stimulates primary to secondary follicle transition in bovine follicles in vitro. Biol Reprod 75:924-932

Yeoman RR, Wolf DP, Lee DM (2005) Coculture of monkey ovarian tissue increases survival after vitrification and slow-rate freezing. Fertil Steril 83:1248-1254

Samenvatting

Het invriezen van eierstokken is een veelbelovend middel om diersoorten en –rassen voor uitsterven te behoeden, en om de vruchtbaarheid te behouden na een behandeling voor kanker. Immers dankzij bevriezing kunnen theoretisch duizenden tot miljoenen onrijpe follikels gedurende lange tijd worden bewaard zonder dat de daarin aanwezige eicellen het bevruchtend vermogen zouden verliezen. Essentieel daarbij is dat de integriteit van de onrijpe eicellen en overige follikelcellen in de ingevroren eierstokken intact blijven ook na ontdooiing voor eventuele in vitro of in vivo rijping van de eicellen die vervolgens -na bevruchting- zouden kunnen leiden tot nageslacht.

Dit proefschrift richt zich op de stapsgewijze invloed en verbetering van invriesprotocollen voor eierstokken en onrijpe follikels. Daarnaast wordt er aandacht besteed aan de mogelijkheid om bevroren/ontdooid eierstokweefsel te plaatsen in steriel gemaakte geiten met als doel om vanuit dit teruggeplaatste eierstokweefsel weer reproductieve en cyclische geiten te genereren. Mogelijk kan de geit dienen als experimenteel diermodel voor soortgelijke toepassingen bij de mens.

De ontwikkeling van een invriesmethode voor eierstokken begint bij het kiezen van i) het meest efficiënte antivries middel ii) de grootte van de blokjes gesneden eierstokmateriaal iii) het volume van de invriesoplossing iv) de toevoeging van een eventuele extracellulair antivries middel en v) efficiënte methoden om de follikelkwaliteit te beoordelen. Gebaseerd op deze parameters werd weefsel van geiteneierstokken succesvol ingevroren. Corticale eierstok blokjes van ~1 mm^3 werden blootgesteld aan een klein volume (1 ml bij langzame en 0.5 ml bij snelle bevriezing) van een combinatie van ethyleenglycol en sucrose. Succes van invriezen was in dit geval gebaseerd op een kwaliteitsbeoordeling na een zogenaamde vitaliteitskleuringen (**Hoofdstukken 2 en 3**). Het overleven van een follikel na invriezen en ondooiing garandeert echter nog niet dat dit materiaal vervolgens de capaciteit heeft behouden om verder te ontwikkelen ten behoeve van de eicelrijping. Een significante kwaliteitsdaling werd waargenomen van ingevroren/ontdooide follikels na 24 uur in vitro gerijpt te zijn.

Na het invriezen/ontdooien bleken follikels niet in staat om even levensvatbaar te blijven als verse (controle) follikels, waarschijnlijk door de giftige

werking van de antivries middelen. Deze kunnen ook osmotische stress veroorzaken omdat ze een verhoging van de osmolaliteit induceren. In **Hoofdstuk 4** wordt aangetoond dat een hoge concentratie van antivriesmiddelen zoals 4.0 M EG (5242 mOsm) niet verantwoordelijk zijn voor osmotische schade. Bij blootstelling van jonge stadium follikels aan een ionenconcentratie van 9% NaCl (2800 mOsm) ging echter wel het merendeel (92%) van de cellen dood. Dit toont aan dat ionen de celschade veroorzaken in tegenstelling tot niet-ionogene antivries middelen.

Het doel van het invriezen van vroeg stadium follikels is de opslag van dit materiaal voor een onbepaalde tijd zonder dat de levensvatbaarheid verloren gaat. Tevens maakt dit materiaal het mogelijk om de complete follikel ontwikkeling en eicelrijping na ontdooiing te volgen. Hoewel er bij de muis succes is behaald met ingevroren vroeg stadium follikels, die na ontdooiing bevrucht konden worden en waar van gezonde nakomelingen uit voortkwamen, is gebleken dat eenzelfde resultaat moeilijk te verkrijgen is bij grotere zoogdieren.

Door dit gebrek aan resultaat alsmede het belang om complete follikelontwikkeling te verkrijgen na cryopreservatie is bestudeerd of de transplantatie van eierstokweefsel doeltreffender is bij grote zoogdieren. In **Hoofdstuk 5** wordt een succesvolle diepvries bewaarmethode beschreven waarbij na ontdooiing van de eierstokweefselblokjes in vitro een volledige follikelontwikkeling (van primordiaal tot pre-ovulatoir) te zien gaven. Deze eierstokweefselblokjes bleken goed te kunnen worden teruggeplaatst in geiten waaruit de eierstokken waren verwijderd. De teruggeplaatste corticale eierstok weefselblokjes vormden in deze geiten een nieuw ovarium-achtig orgaan waarin bloedaanvoer en hormoonproductie (oestradiol en progesteron) en de endocriene as met het hypofyse/hypothalamus systeem (GnRH,LH en FSH) hersteld werden zodat duidelijk oestrus gedrag en cycliciteit waarneembaar werden. Een aantal geiten worden thans in het tweede paarseizoen getest op cycliciteit en vruchtbaarheid.

Follikels van een vroeg stadium lijken onmiddellijk na ontdooien levensvatbaar en in staat om te ontwikkelen tot pre-ovulatoire follikels. Een volgende stap voordat invriezen van eierstokken in de kliniek toegepast kan worden bestaat uit het evalueren van enkele vooralsnog onbekende facetten van invriezen, ontdooien en transplantatie. Hoewel minimale DNA-schade en een hoog

overlevingspercentage van vroege stadium follikels na ontdooiing zijn waargenomen garandeert dat niet er geen invloeden zijn op transcriptie van genen betrokken bij ziekte of abnormale ontwikkeling. Daarnaast zijn meer experimenten in adequate diermodellen, zoals de geit, nodig die zullen leiden tot herhaalbare resultaten en wellicht meer garanties kunnen bieden op normale nakomelingen. Het lijkt niet wenselijk ingevroren eierstokweefsel te bewaren om de vruchtbare leeftijd van vrouwen te verhogen. Echter na verder ontwikkeling kan deze techniek in de toekomst gebruikt worden om de eierstokweefsel van vrouwen te bewaren die een chemotherapie of bestraling behoeven. Deze vrouwen zouden na genezing en terugplaatsing van het ingevroren onbehandelde eierstokweefsel vruchtbaarheid kunnen terugkrijgen. Daarnaast kan deze techniek ook belangrijke zijn om bedreigde diersoorten en rassen te behoeden voor uitsterven.

Resumo

A congelação ou vitrificação de tecido ovariano consistem em técnicas promissoras para a preservação de espécies e raças animais em via de extinção, bem como para a restauração da fertilidade de mulheres após tratamento de câncer, através da preservação de milhares a milhões de oócitos imaturos inclusos em folículos pré-antrais. A presente Tese destina-se ao desenvolvimento de protocolos eficientes de criopreservação do tecido ovariano e folículos pré-antrais isolados caprinos, bem como o transplante de tecido ovariano caprino congelado-descongelado como um possível modelo para humanos.

O estabelecimento de protocolos de criopreservação inicia-se pela escolha (i) dos agents crioprotetores mais eficientes, (ii) da dimensão dos fragmentos ovarianos, (iii) do volume da solução crioprotetora, (iv) da adição de crioprotetores extra-celulares, e (v) de métodos eficientes para a avaliação da qualidade folicular. Baseado nos supracitados parâmetros, tecido ovariano caprino foi criopreservado com sucesso através da utilização de pequenos fragmentos (~1mm^3) de córtex ovariano expostos a um reduzido volume (congelado em 1 ml ou vitrificado em 0.5 ml) de uma combinação de etileno glicol e sacarose, de acordo com análises da viabilidade folicular utilizando marcadores fluorescentes (**Capítulos 2 e 3**). Contudo, manutenção da viabilidade celular após criopreservação não consiste em garantia de desenvolvimento folicular, uma vez que foi observado um significativo declínio da qualidade de folículos pré-antrais criopreservados após cultivo in vitro por 24 h.

Provavelmente por causa do efeito tóxico dos crioprotetores, os folículos não são capazes de manter sua viabilidade nos mesmos níveis observados in vivo (controle). Um dos efeitos danosos causados pelos crioprotetores é chamado estresse osmótico, causado pela alta osmolaridade dos agentes crioprotetores. No **Capítulo 4** foi demonstrado que altas concentrações de crioprotetores, por exemplo EG 4.0 M (5242 mOsm), não são responsáveis pelo dano osmótico. Por outro lado, quando folículos pré-antrais são expostos a uma solução iônica de NaCl 9% (2800 mOsm), a maioria das células (92%) perderam sua viabilidade (**Capítulo 4**), mostrando que íons são a causa desse tipo de dano celular e não os crioprotetores (solutos não iônicos).

O objetivo da criopreservação de folículos pré-antrais consiste na preservação, por um período indeterminado e sem perda de viabilidade, seguida do completo desenvolvimento folicular após descongelação/aquecimento. Apesar do sucesso obtido com o completo desenvolvimento in vitro de folículos pré-antrais murinos, incluindo o desenvolvimento de folículos congelados-descongelados seguido de fecundação, desenvolviemtno embrionário e nascimento de animais saudáveis, similar sucesso é difícil de ser atingido em grandes mamíferos in vitro.

Por causa da ausência de um protocolo para o cultivo in vitro de folículos pré-antrais de grandes mamíferos e a importância da obtenção do completo desenvolvimento folicular após a criopreservação, o transplante do tecido ovariano aparece como um método plausível. No **Capítulo 5**, o sucesso da criopresevação foi demonstrado pela completo desenvolvimento folicular (primordial até estágio pré-ovulatório) após congelação-descongelação de fragmentos de tecido ovariano caprino e seu re-implante em doadoras ovariectomizadas. Além disso, fragmentos do córtex ovariano sofreram uma remodulação em uma nova estrutura ovariana, da qual o suprimento sanguineo e a função endócrina (estradiol e progesterona) foram restaurados.

Imediatamente após descongelação, folículos pré-antrais parecem viáveis e capazes de se desenvolverem até estágios pré-ovulatórios. O próximo passo antes da aplicação clínica consite em avaliar alguns pontos relacionados à criopreservação e transplante. Por exemplo, ausência de danos no DNA e alta taxa de sobrevivência após criopreservação não significa que o resfriamento não afeta o DNA através da ativação de genes relacionados a doenças e mal formações. Logo, posteriores experimentos precisam ser realizados em adequados modelos animais, tais como caprinos, até que os protocolos possam ser satisfatoriamente repetidos e o nascimento de indivíduos sadios seja garantido. Finalmente, a criopreservação de tecido ovariano não deve promover o adiamento da maternidade em mulheres saudáveis, e sim a utilização de tal técnica para salvaguardar a maternidade de mulheres submetidas a tratamentos quimio- e radioterápicos. Além disso, em um futuro próximo, esta técnica será importante para a preservação de raças e espécies animais em via de extinção.

Acknowledgements

Curriculum Vitae

Publications

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Curriculum Vitae

Regiane Rodrigues dos Santos was born on the 31st of August 1976 in Juazeiro do Norte, Ceará, Brazil. She studied Veterinary Medicine at the State University of Ceará, Brazil, and received her DVM degree in 2000. During her graduation, 1998 to 2000, she started her scientific training by a fellowship program at the Laboratory of Manipulation of Oocytes Enclosed in Preantral Follicles, at State University of Ceará. In 2001 she started her Master studies and in 2002 obtained her Master of Science degree in Animal Health and Reproduction, under supervision of Dr. José Ricardo de Figueiredo, at the State University of Ceará. During her Master's study, she was involved in the organization of local scientific symposia and was member of the Board of the Cearense Society of Animal Science. The research project of her Master's study focused on short-term preservation of ovine and caprine goat early-staged follicles. In March 2003 she started her PhD studies at the Faculty of Veterinary Medicine, State University of Ceará, with a project focused on the development of cryopreservation procedures for isolated and enclosed in ovarian tissue ovine early-staged follicles. Her PhD studies were partially, 2004-2005, performed at Utrecht University, where together with the experiments in ovine ovarian tissue, she started the development of conventional and vitrification procedures for caprine ovarian follicles. In October 2005 she obtained her first PhD degree in Animal Reproduction, under supervision of Dr. José Ricardo de Figueiredo, at the State University of Ceará. During her PhD studies she was co-supervisor of a master student and was member of the Board of the Brazilian Society of Embryo Technology (2003-2004). From 2005 to July 2006, she has been working in a PRODOC project financed by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and has been supervising three undergraduate students. From September 2006 onwards she has been collaborating with Dr. Ana Paula R Rodrigues, in a project for the comparative study of cryoprotectant perfusion in domestic small ruminant ovaries, by co-supervising two master students in Brazil. In addition, during this period, she has been working at the Department of Farm Animal Health – Utrecht University, where she performed her second PhD project under the supervision of Prof. Dr. Ben Colenbrander, Dr. Bernard A J Roelen and Dr. Rob van den Hurk, resulting in this thesis.

Published

Santos RR, Tharasanit T, Van Haeften T, Figueiredo JR, Silva JR, Van den Hurk R (2007) Vitrification of goat preantral follicles enclosed in ovarian tissue using conventional and solid-surface vitrification (SSV) methods. Cell Tissue Res 327:167-176

Santos RR, Van den Hurk R, Rodrigues AP, Costa SH, Martins FS, Matos MH, Celestino JJ, Figueiredo JR (2007) Effect of cryopreservation on viability, activation and growth of in situ and isolated ovine early-stage follicles. Anim Reprod Sci 99:53-64

Santos RR, Tharasanit T, Figueiredo JR, Van Haeften T, Van den Hurk R (2006) Preservation of caprine preantral follicle viability after cryopreservation in sucrose and ethylene glycol. Cell Tissue Res 325:523-531

Santos RR, Rodrigues AP, Costa SH, Silva JR, Matos MH, Lucci CM, Báo SN, Van den Hurk R, Figueiredo JR (2006) Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. Anim Reprod Sci 91:249-263

Silva JR, Tharasanit T, Taverne MA, Van der Weijden GC, *Santos RR*, Figueiredo JR, Van den Hurk R (2006) The activin-follistatin system and in vitro early follicle development in goats. J Endocrinol 189:113-125

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

Rodrigues AP, Costa SH, *Santos RR*, Amorim CA, Lucci CM, Báo SN, Nunes JF, Rondina D, Figueiredo JR (2006) In vitro culture of cryopreserved caprine ovarian tissue pieces and isolated follicles. Cell Preserv Tech 4:290-298

Rodrigues AP, Amorim CA, Costa SH, *Santos RR*, Lucci CM, Nunes JF, Figueiredo JR (2005) Cryopreservation and short-term culture of isolated caprine primordial follicles. Small Rumin Res 56:103-111

Rodrigues AP, Amorim CA, Costa SH, Matos MH, *Santos RR*, Lucci CM, Ohashi OM, Figueiredo JR (2004) Cryopreservation of caprine ovarian tissue using dimethyl sulfoxide and propanediol. Anim Reprod Sci 84:211-227

Rodrigues AP, Amorim CA, Costa SH, Matos MH, *Santos RR*, Lucci CM, Báo SN, Ohashi OM, Figueiredo JR (2004) Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology 61:1009-1024

Silva JR, Van den Hurk , Matos MH, *Santos RR*, Pessoa C, Moraes MO, Figueiredo JR (2004) Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. Theriogenology 61:1691-1704

Matos MH, Andrade ER, Lucci CM, Báo SN, Silva JR, *Santos RR*, Ferreira MA, Costa SH, Celestino JJ, Figueiredo JR (2004) Morphological and ultrastructural analysis of sheep primordial follicles preserved in 0.9% saline solution and TCM 199. Theriogenology 62:65-80

Silva JR, Ferreira MA, Costa SH, *Santos RR*, Carvalho FC, Rodrigues AP, Lucci CM, Báo SN, Figueiredo JR (2002) Degeneration rate of preantral follicles in the ovaries of goats. Small Rumin Res 43:203-209

Silva JR, Lucci CM, Carvalho FC, Costa SH, *Santos RR*, Figueiredo JR (2000) Effect of coconut water and Braun-Collins solutions at different temperatures and incubation times on the morphology of goat preantral follicles preserved in situ. Theriogenology 54:809-822

In press

Celestino JJ, *Santos RR*, Lopes CA, Martins FS, Matos MH, Melo MA, Báo SN, Rodrigues AP, Silva JR, Figueiredo JR (2007) Preservation of bovine preantral follicle viability and ultra-structure after cooling and freezing of ovarian tissue. Anim Reprod Sci [epub ahead of print]

Submitted

Santos RR, Van Haeften T, Roelen BA, Knijn HM, Colenbrander B, Gadella BM, Van den Hurk R (2007) Osmotic tolerance and freezability of isolated caprine early-staged follicles.

In preparation

Santos RR, Knijn HM, Vos PLAM, Oei C, Van Loon T, Colenbrander B, Gadella BM, Van den Hurk R, Roelen BA. Ovarian re-modulation, endocrine

function restoration, and complete follicular development from frozen-thawed, auto-transplanted caprine ovarian cortex.

Santos RR, Tharasanit T, Van Haeften T, Rodrigues AP, Van den Hurk R, Figueiredo JR. Viability of early-staged follicles recovered from frozen-thawed or vitrified-warmed ovine ovarian tissue.

Bruno JB, *Santos RR*, Celestino JJ, Melo MA, Figueiredo JR, Rondina D, Rodrigues AP. Food restriction interferes on the freezability and further in vitro development of caprine early-staged follicles.

Conference proceedings

Santos RR, Van Haeften T, Roelen BA, Knijn HM, Colenbrander B, Gadella BM, Van den Hurk R (2007) Osmotic tolerance and freezability of isolated caprine early-staged follicles. Proceedings International Conference on Farm Animal Reproduction, Limburg, The Netherlands, 1:28

Lima-Verde IB, Bruno JB, Martins FS, Matos MH, Maia Jr JE, Silva-Filho FJ, Silveira E, *Santos RR*, Silva JR, Figueiredo JR, Rodrigues AP (2006), In vitro culture of preantral follicles using antioxidant substances (α-tocoferol and ternatin). Proceedings XX Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Araxá, MG. Acta Scientiae Veterinariae, 34:575-575

Melo ME, Braga MM, Celestino JJ, Rodrigues AP, Figueiredo JR, *Santos RR* (2006) Vitrificação de folículos pré-antrais ovinos incluso no tecido ovariano. Proceedings 58ª Reunião Anual da Sociedade Brasileira pro Progresso da Ciência, Florianópolis, SC, Brasil, 58:60

Melo ME, Van den Hurk R, Celestino JJ, Lopes CA, Rodrigues AP, Figueiredo JR, *Santos RR* (2006). Protective effect of granulosa cells on the viability of cryopreserved ovine preantral follicles. Proceedings X Congresso da Sociedade Brasileira de Reprodução Assistida, Rio de Janeiro, RJ, Brasil, 10:103

Santos RR, Van den Hurk R, Tharasanit T, Van Haeften T, Figueiredo JR (2005) Determination of the viability of cryopreserved goat preantral follicles. Proceedings ESHRE meeting in 'Mammalian Oogenesis and Folliculogenesis', Paris, France, 1:93

Celestino JJ, *Santos RR*, Matos MH, Martins FS, Costa SH, Silva JR, Rodrigues AP, Figueiredo JR (2005) Cryopreservation of bovine preantral follicles.

Proceedings Proceedings XIX Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Angra dos Reis, RJ, Brasil, Acta Scientiae Veterinariae, 33:341

Santos RR, Rodrigues AP, Costa SH, Silv JR, Martins FS, Matos MH, Celestino JJ, Silva GA, Figueiredo JR (2004) Cryopreservation and in vitro culture of sheep preantral follicles. Proceedings XVIII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Barra Bonita, SP, Brasil, Acta Scientiae Veterinariae, 32:163

Celestino JJ, *Santos RR*, Matos MH, Costa SH, Silva JR, Martins FS, Silva GA, Figueiredo JR (2004) Effect of 0.9% saline solution and TCM 199 at different temperatures and incubation times on the morphology of bovine preantral follicles. Proceedings XVIII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Barra Bonita, SP, Brasil, Acta Scientiae Veterinariae, 32:135

Martins FS, *Santos RR*, Celestino JJ, Matos MH, Silva GA, Ferreira FV, Figueiredo JR (2004) Development of goat preantral follicles in coconut water solution. Proceedings XVIII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Barra Bonita, SP, Brasil, Acta Scientiae Veterinariae, 32:136

Rodrigues AP, Costa SH, *Santos RR*, Lucci CM, Nunes JF, Ohashi OM, Rondina D, Figueiredo JR (2004) Cryopreservation and short-term culture of isolated caprine primordial follicles. Proceedings XVIII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Barra Bonita, SP, Brasil, Acta Scientiae Veterinariae, 32:91

Santos RR, Rodrigues AP, costa SH, Matos MH, Silva JR, Celestino JJ, Câmara AC, Martins FS, Figueiredo JR (2003) Toxicity test and cryopreservation of ovine preantral follicles in situ. Proceedings XVII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Beberibe, CE, Brasil, Acta Scientiae Veterinariae, 31:555

Matos MH, Silva JR, Lucci CM, Báo SN, *Santos RR*, Costa SH, Celestino JJ, Figueiredo JR (2003) Morphological and ultrastructural analysis of sheep primordial follicles preserved in 0.9% saline solution and TCM 199. Proceedings XVII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Beberibe, CE, Brasil, Acta Scientiae Veterinariae, 31:469

Rodrigues AP, Amorim CA, Costa SH, Matos MH, *Santos RR*, Lucci CM, Báo SN, Ohashi OM, Figueiredo JR (2003) Cryopreservation of caprine ovarian tissue using different cryoprotectants. Proceedings XVII Reunião Anual da

Sociedade Brasileira de Tecnologia de Embriões, Beberibe, CE, Brasil, Acta Scientiae Veterinariae, 31:547

Silva JR, Van den Hurk R, Matos MH, *Santos RR*, Ferreira MA, Martins FS, Figueiredo JR (2003) Influence of FSH and EGF on the activation of goat primordial follicles after in vitro culture of ovarian cortical tissue. Proceedings XVII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Beberibe, CE, Brasil, Acta Scientiae Veterinariae, 31:581

Santos RR, Rodrigues AP, Costa SH, Matos MH, Celestino JJ, Figueirêdo EL, Figueiredo JR (2002) Efeito tóxico de diferentes crioprotetores sobre folículos pré-antrais ovinos isolados. Proceedings I Congresso Norte-Nordeste de Reprodução Animal, Recife, PE, Brasil, Revista Brasileira de Reprodução Animal, 5:80-82

Santos RR, Silva JR, Matos MH, Rodrigues AP, Ferreira MA, Damasceno CB, Rassy FB, Figueiredo JR (2002) Atresia rate of preantral follicles in adult pregnant or non-pregnant sheep ovaries. Proceedings 28th Annual Conference of the International Embryo Transfer Society, Foz do Iguaçu, RS, Brasil, Theriogenology, 57:623

Brasil AF, Rodrigues AP, *Santos RR*, Costa SH, Matos MH, Machado VP, Ferreira MA, Silva JR (2002) Degeneration of goat primordial follicles enclosed in fragments of ovarian tissue preserved in TCM 199 and phosphate buffer saline at different temperatures and incubation times. Proceedings 28th Annual Conference of the International Embryo Transfer Society, Foz do Iguaçu, RS, Brasil, Theriogenology, 57:622

Silva JR, Costa SH, Andrade ER, *Santos RR*, Matos MH, Figueiredo JR (2002) Ativação e crescimento in vitro de folículos primordiais caprinos. Proceedings I Congresso Norte-Nordeste de Reprodução Animal, Revista Brasileira de Reprodução Animal, suplemento 5, Recife, PE, Brasil, 89-91

Silva JR, *Santos RR*, Machado VP, Matos MH, Costa SH, Rassy FB, Figueiredo JR (2002) Incidence of atresia in primordial follicles from fetal, pregnant and non-pregnant adult goat ovaries. Proceedings 28th Annual Conference of the International Embryo Transfer Society, Foz do Iguaçu, RS, Brasil, Theriogenology, 57:626

Santos RR, Silva JR, Matos MH, Machado VP, Brasil AF, Rodrigues AP, Figueiredo JR (2000) Conservação de folículos pré-antrais caprinos in situ. Proceedings II Simpósio Cearense de Ciência Animal, Fortaleza, CE, Brasil, Revista Ciência Animal, 10:142

Costa SH, *Santos RR*, Ferreira MA, Machado VP, Brasil AF, Rodrigues AP, Figueiredo JR (2000) Preservation of goat preantral follicles in small fragments of ovarian tissue in saline solution of coconut water. Proceedings II Simpósio cearese de Ciência Animal, Fortaleza, CE, Brasil, Revista Ciência Animal, 10:108-110

Ferreira MA, Brasil AF, Costa SH, *Santos RR*, Rodrigues AP, Figueiredo JR (2000) Effect of the addition of indole-3-acetic acid to TCM 199 for preservation of goat isolated preantral follicles. Proceedings II Simpósio Cearense de Ciência Animal, Fortaleza, CE, Brasil, Revista Ciência Animal, 10:113-114

Machado VP, Rodrigues AP, Brasil AF, *Santos RR*, Matos MH, Figueiredo JR (2000) Comparação da eficiência dos métodos mecânico e enzimático de isolamento de folículos ovarianos pré-antrais de fetos caprinos. Proceedings II Simpósio Cearense de Ciência Animal, Fortaleza, CE, Brasil, Revista Ciência Animal, 10:124-125

Silva JR, Báo SN, Andrade ER, Ferreira MA, Costa SH, *Santos RR*, Carvalho FC, Figueiredo JR (2000) Efeitos das soluções à base de água de coco e Braun-Collins sobre as características morfológicas e ultra-estruturais de folículos pré-antrais caprinos conservados in situ. Proceedings XV Reunião da Sociedade Brasileira de Tecnologia de Embriões, Porto alegre, RS, Brasil, Arq Fac Vet UFRGS, 28:333

Silva JR, Ferreira MA, Costa SH, *Santos RR*, Carvalho FC, Rodrigues AP, Figueiredo JR (2000) Taxa de degeneração de folículos pré-antrais em ovários de cabra sem raça definida. Proceedings XV Reunião da Sociedade Brasileira de Transferência de Embriões, Porto Alegre, RS, Brasil, Arq Fac Vet UFRGS, 28:332

"Eu sou de uma terra que o povo padece
Mas não esmorece e procura vencer.
Da terra querida, que a linda cabocla
De riso na boca zomba no sofrer
Não nego meu sangue, não nego meu nome.
Olho para a fome , pergunto: que há ?
Eu sou brasileiro, filho do Nordeste,
Sou cabra da Peste, sou do Ceará."

Patativa do Assaré