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Cryopreservation of oocytes from pre-antral follicles

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Cryobiology is a very important tool in reproductive biology. Research in this area focuses on the possibility of restoring fertility in women with reproductive problems or after cancer treatments. Another goal is to establish a genetic resource bank for endangered or commercially important animal species. Cryopreservation of oocytes from pre-antral follicles has been studied during the past decade. Procedures can be divided between the cryopreservation of either ovarian tissue or isolated follicles. Most studies describe a slow freezing/rapid thawing protocol to cryopreserve ovarian fragments. Histology shows that the follicles maintain their morphological integrity, and transplantation of ovarian tissue demonstrates that the follicles can restart their growth and eventually ovulate. Some research groups have obtained offspring using this procedure in mice and sheep. With regard to the cryopreservation of isolated follicles, the few studies reported in this area used the same freezing protocol, and some of them described follicular growth using in-vitro culture. The best result was obtained in mice, with animal birth after follicular cryopreservation and culture. However, additional studies are necessary for a better understanding of the events during follicular cryopreservation and to establish a standard protocol for ovarian transplantation or follicle culture.

Key words: cryoprotectant/freezing/isolated follicles/oocyte/ovarian tissue

Introduction

During the 1940s, reproductive biology took a very important step when Polge and colleagues (Polge *et al*., 1949) published their study describing, for the first time, the use of glycerol (GLY) to preserve spermatozoa. Since then, several research groups have attempted to develop procedures to cryopreserve not only spermatozoa, but also embryos and female gametes in different developmental stages. During the 1950s, GLY was used to freeze pre-antral follicles enclosed in ovarian tissue fragments (Parkes and Smith, 1953; Green *et al*., 1956; Deanesly, 1957), but unfortunately the results were inferior to those obtained with frozen spermatozoa.

In the 1970s, new materials were used as cryoprotective agents (CPAs), including dimethyl sulphoxide (DMSO), ethylene glycol (EG) and propylene glycol (PROH). In humans, these were tested for the cryopreservation of platelets (Raymond *et al*., 1975; Odink and Sprokholt, 1976), lymphocytes (Simon *et al*., 1977; Pandolfi *et al*., 1979), monocytes (Shah *et al*., 1984), embryos (Lassalle *et al*., 1985; Mandelbaum *et al*., 1987; Siebzehnrübl *et al*., 1989) and oocytes (Al-Hasani *et al*., 1987; Siebzehnrübl *et al*., 1989). However, regardless of the successful results described in the cryopreservation of different cell types with these CPAs, pre-antral follicle freezing was not reinvestigated until two decades later.

The cryopreservation of embryos and female gametes proved to be a very important tool for re-establishing fertility in humans and safeguarding genetic material from animals. In the case of embryo cryopreservation in humans, the pregnancy rate using cryopreserved embryos is about 15–25%, and the probability of pregnancy increases with repeated transfers (Wood *et al*., 1997). However, this procedure is still far from satisfactory for preserving a woman's reproductive potential (Revel and Laufer, 2002). In addition, the in-vitro production of embryos requires IVF, which is a costly, stressful and relatively inefficient procedure (Rutherford and Gosden, 1999). Embryo cryopreservation is suitable for women who have partners or accept donor

sperm, but not for young girls; moreover, it can also lead to legal and ethical complications (cases of divorce, death, unmarried couples and disposing of unwanted embryos) (Wood *et al*., 1997; Shaw *et al*., 2000a; Kim *et al*., 2001a). In cases involving women before anti-cancer treatment, embryo cryopreservation can be risky as it requires hormonal stimulation, which is not suitable for estrogen-dependent malignancies such as breast cancer (Wood *et al*., 1997; Revel and Laufer, 2002). It is not indicated for patients with polycystic ovarian syndrome who might be sensitive to hormonal stimulation (Trounson *et al*., 1994). Moreover, the protocols for obtaining the embryos are time-consuming as oocyte retrieval for IVF requires a few weeks of treatment, and a harvest of viable embryos is uncertain (Radford *et al*., 2001). This can lead to a delay in anti-cancer treatment which, in some cases, is not recommended by oncologists and haematologists (Revel and Laufer, 2002).

Improvements in the cryopreservation of mature human oocytes and their subsequent fertilization have been achieved in recent years, with the establishment of ICSI as a fertilization method. One group (Porcu *et al*., 2000) obtained 19 pregnancies with this technique, showing that it is a safe and efficient procedure to achieve pregnancies in women. Despite extensive research being conducted during the past 15 years, progress in this field remains limited, and the current live birth rate is less than one in every 100 oocytes (Wood *et al*., 1997; Rutherford and Gosden, 1999). Although mature oocytes have been cryopreserved using all disposable protocols (slow or rapid freezing and vitrification), the fertilization and in-vitro development rates achieved are much lower than those obtained with fresh oocytes. In addition, similar to embryo cryopreservation, this technique is risky in patients with estradiol-sensitive malignancies, ovarian epithelial tumours or severe endometriosis (Wood *et al*., 1997) as oocyte retrieval for cryopreservation requires hormonal stimulation. This method is also not suitable for young girls.

Another option is to preserve oocytes at an earlier developmental stage (immature oocytes from tertiary or pre-antral follicles). The collection of oocytes from tertiary follicles differs from the harvesting of mature oocytes in that it does not require ovarian hormone stimulation. One group (Arav *et al*., 1996) reported that, in cattle, the membrane of immature oocytes is more sensitive to cryoinjury than is that of mature oocytes, despite the former having a lesser sensitivity to freezing and cryoinjury. Furthermore, the number of oocytes that can be collected from tertiary follicles is still low.

The cryopreservation of immature oocytes from pre-antral follicles is an experimental alternative to restore fertility (Moomjy and Rosenwaks, 1998), and the technique has also been shown to preserve a higher number of oocytes from the ovary. Immature oocytes have several features which make them less sensitive to cryoinjury (Gosden *et al*., 1994; Shaw *et al*., 2000b). Among the classes of pre-antral follicles, the primordial follicles have been most commonly used in these studies due to their special characteristics: (i) they represent 90% of the entire follicular population in the ovary; (ii) they have a relatively inactive metabolic rate; (iii) their small size (~20 μm diameter); (iv) a low number of granulosa cells around the small oocyte; and (v) an absence of the zona pellucida and peripheral cortical granules (Oktay *et al*., 2000a; Shaw *et al*., 2000a,b).

In humans, the main application of this technique is fertility restoration in women at risk of premature menopause, which can have several causes: recurrent or severe ovarian diseases such as cysts, benign tumours and endometriomas; ovary removal to treat endometriosis or genital cancer (Wood *et al*., 1997); and chemotherapy or radiotherapy to treat cancer or other systemic diseases (Oktay and Buyuk, 2002). This technique might be a good option for women with certain types of chromosomal anomalies, such as Turner's syndrome (Wood *et al*., 1997; Aubard *et al*., 2000), which can also cause premature ovarian failure. It has been reported (Hreinsson *et al*., 2002) that follicles may be found in ovaries from adolescent girls with Turner's syndrome. This finding is very important as it indicates that infertility in these patients could be avoided, rescuing ovarian fragments for cryopreservation procedures (Abir *et al*., 2001a). This technique could also be used in healthy women who choose to delay childbearing until later in life. It would also facilitate oocyte donation as the procedure for harvesting the ovarian fragment is very simple and does not require hormonal stimulation, which causes side effects to the donor (Van den Hurk *et al*., 2000). Hypothetically, cryopreservation of ovarian tissue followed by transplantation could delay natural menopause, avoiding the innumerable and uncomfortable side effects of this process in women and the need for exogenous hormonal replacement, which is not suitable in women who are sensitive to ovarian stimulation.

Ovarian tissue can be easily collected by laparoscopy without delaying radio/chemotherapy treatment (Schnorr, 2001). The procedure is simple and rapid, and does not require any further time or hormone treatment (Wood *et al*., 1997). In addition, none of the ethical or legal problems related to embryo or mature oocyte cryopreservation applies to pre-antral follicle freezing, as it can be performed in unmarried women as well as in young girls.

In animals, the cryopreservation of pre-antral follicles would provide a source of oocytes that could be used to propagate genetic material from commercially valuable animals or species, thereby increasing exploitation of female genetic material and propagation of valuable animal stocks (Van der Hurk *et al*., 2000). This technique could also be used to increase livestock production and benefits, help to avoid the sexual transmission of diseases, and increase the volume of the animal trade. It could also reduce the generation interval in valuable animals as no waiting period would be required between generations (Betteridge *et al*., 1989). The technique might also be used in wildlife research and conservation, through the formation of genome resource banking, which is important in preserving genetic diversity, facilitating the movement of genetic material, reducing genetic problems, and increasing efficiency in captive breeding (Wildt, 2000). Finally, the cryopreservation of pre-antral follicles would provide a great number of fertilizable oocytes for protocols such as IVF, embryo transfer, cloning and transgenesis (Van den Hurk *et al*., 2000), and this in turn would permit an intensified study of these biotechniques.

Cryopreservation of pre-antral follicles

Pre-antral follicles have been cryopreserved using two different techniques: (i) cryopreservation of follicles enclosed in ovarian tissue; or (ii) after isolation of pre-antral follicles from the ovarian

cortex. Most reported studies refer to ovarian tissue cryopreservation (Hovatta *et al*., 1996; Newton *et al*., 1996; Gunasena *et al*., 1997a,b; Baird *et al*., 1999), and only a few reports have been made of the cryopreservation of isolated follicles (Cortvrindt *et al*., 1996; Jewgenow *et al*., 1998; Amorim *et al*., 2003a,b).

Cryopreservation of ovarian tissue

Ovarian tissue can be cryopreserved as either small fragments (Nisolle *et al*., 2000; Oktay *et al*., 2000b; Radford *et al*., 2001), halves (Daniel *et al*., 1983; Sztein *et al*., 1998; Salle *et al*., 1999; Demirci *et al*., 2001) or as whole ovary (Deanesly, 1957; Sugimoto *et al*., 1996; 2000; Aubard *et al*., 1998). Usually, ovaries from humans and large animals, such as cows and sheep, are frozen in small pieces as they have a dense fibrous stroma (Newton *et al*., 1999). Cutting the ovary into pieces of ~0.2 mm^3 allows the CPAs to permeate the tissue (Shaw and Trounson, 2002). Mice have sponge-like ovaries which are rich in pores and, consequently, are best frozen entire (Harp *et al*., 1994; Candy *et al*., 1997) as this facilitates CPA permeation.

The cryopreservation of ovarian tissue has been reported in women (Hovatta *et al*., 1996; Newton *et al*., 1996; 1998; Oktay *et al*., 1997; Qu *et al*., 2000a,b), mice (Harp *et al*., 1994; Cox *et al*., 1996; Shaw *et al*., 1996; Candy *et al*., 1997; Gunasena *et al*., 1997a,b), sheep (Gosden *et al*., 1994; Salle *et al*., 1998; 1999; 2002; Baird *et al*., 1999; Demirci *et al*., 2001, 2002), rats (Sugimoto *et al*., 1996; 2000; Aubard *et al*., 1998), marmosets (Candy *et al*., 1995), cows (Paynter *et al*., 1999), elephants (Gunasena *et al*., 1998), wombats (Wolvekamp *et al*., 2001), tammar wallabies (Mattiske *et al*., 2002) and rabbits (Daniel *et al*., 1983). These studies are summarized in Tables I to VI. Promising results have been obtained in these studied species. In humans, follicle growth was observed after either autotransplantation (in the same individual) (Radford *et al*., 2001) or xenotransplantation (between members of different species) to mice (Gook *et al*., 2001; Van den Broecke *et al*., 2001a,b; Kim *et al*., 2002), and hormonal secretion was also observed (Callejo *et al*., 2001; Radford *et al*., 2001). The experiments in mice resulted in live offspring after cryopreservation and transplantation of the ovarian fragments (Parrott, 1960; Cox *et al*., 1996; Gunasena *et al*., 1997a,b; Sztein *et al*., 1998). However, others (Liu *et al*., 2001) demonstrated a different method for obtaining offspring. After transplantation, they isolated follicles from ovarian pieces and submitted them to in-vitro maturation and fertilization and transferred the embryos to recipient animals. In sheep, birth was first obtained in the early 1990s (Gosden *et al*., 1994) and more recently by another group (Salle *et al*., 2002). Other important results were also reported with this species. One group (Baird *et al*., 1999) showed that it is possible to preserve cyclical ovarian function after xenotransplantation of cryopreserved ovarian fragments. Follicular growth until the antral stage (Salle *et al*., 1998, 1999; Newton *et al*., 1999) and ovulation (Aubard *et al*., 1999) were also reported in sheep. In rats, the restoration of ovarian cyclicity and follicular growth in previously cryopreserved autografts was also demonstrated (Sugimoto *et al*., 2000). Follicular growth after freezing, thawing and xeno- or autotransplantation of ovarian tissue was also observed in rabbits (Daniel *et al*., 1983), elephants (Gunasena *et al*., 1998), wombats (Wolvekamp *et al*., 2001) and marmosets (Candy *et al*., 1995).

To cryopreserve ovarian tissue, slow freezing/rapid thawing methods (Oktay *et al*., 1997; Aubard *et al*., 1998) have been commonly used, while only a few studies tested rapid freezing (Zhang *et al*., 1995; Gook *et al*., 1999; 2000) or vitrification (Sugimoto *et al*., 1996, 2000). Such slow freezing procedures are usually adaptations of protocols previously developed for embryos and mature oocytes. However, it is important to point out that the cryopreservation of isolated cells or structures, such as embryos and mature oocytes, is different from that of ovarian tissue fragments, as they are more complex structures, with a compact stroma tissue and different cell types. In addition, follicles are multicellular and heterogeneous (Gosden *et al*., 2002) and different in size and structure from mature oocytes or embryos. Therefore, despite the successful results obtained in some studies, better results could most likely be achieved if protocols specially designed for follicles were to be developed.

To cryopreserve pre-antral follicles, ovaries or ovarian fragments are exposed to CPA at room temperature or below (until 0°C) and loaded into specialized cooling machines (Hovatta *et al*., 1996; Oktay *et al*., 1997; Newton *et al*., 1998; Salle *et al*., 1998). The temperature is then reduced to –7°C (some studies describe different temperatures, but always around –7°C) at 2°C/min and seeding is accomplished. After this, the samples are cooled to –40°C (or close to it) at 0.3°C/min. Some papers also describe another freezing step before the follicles are plunged into liquid nitrogen, during which the temperature falls to about –140°C at a rate of 10°C/min.

With regard to the CPA used in the freezing procedure, most studies describe the use of DMSO at concentrations of 1.0 and 1.5 mol/l (Harp *et al*., 1994; Cox *et al*., 1996; Newton *et al*., 1998; Callejo *et al*., 2001; Radford *et al*., 2001; Van den Broecke *et al*., 2001b). However, some researchers have used PROH (Newton *et al*., 1996; Candy *et al*., 1997; Demirci *et al*., 2001; Van den Broecke *et al*., 2001a), EG (Newton *et al*., 1996; Candy *et al*., 1997; Aubard *et al*., 1998) and GLY (Newton *et al*., 1996; Candy *et al*., 1997), as well as combinations of some of these CPAs with sucrose (Shaw *et al*., 1996; Newton *et al*., 1998; Gook *et al*., 2000; Oktay *et al*., 2000b).

In humans, one group (Newton *et al*., 1996) used the four penetrating CPAs mentioned above, and reported that freezing using DMSO or EG resulted in survival of 84 and 74% of the follicles respectively, while survival decreased to 44 and 10% when PROH or GLY respectively were used. Others (Oktay *et al*., 1997) also reported a high survival rate of the follicles cryopreserved with EG, which was similar to that of controls. In mice, the effect of the four CPAs on the survival of follicles was compared (Candy *et al*., 1997); these authors reported that 81 to 94% of the follicular population survived when frozen in DMSO, EG or PROH, and only 4 to 28% were preserved when GLY was used. Others (Newton and Illingworth, 2001) demonstrated that the percentage of live follicles after cryopreservation with DMSO, PROH or GLY followed by in-vitro culture was 43, 24 and 0% respectively. It was also found that GLY has a detrimental effect on oocyte survival, as ~80% of the oocytes in ovarian tissue were destroyed after CPA exposure and cryopreservation (Parrott, 1960). In rats, one group (Aubard *et al*., 1998) compared DMSO with EG and reported similar results between these CPAs, whilst others (Parkes and Smith, 1953; Green *et al*., 1956) reported that in using GLY to cryopreserve the ovarian

Table I. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of human ovarian tissue during the past decade

Ovarian tissue	CPA	After thawing	Main results	Reference
0.5–1 mm^3	4.2 mol/l DMSO + 0.35 mol/l S	In-vitro culture and histology or follicular isolation and viability test	After isolation, 75% survived cryopreservation. After culture, maturation rate did not differ from that of controls	Zhang *et al.* (1995)
1 mm^3	1.5 mol/l DMSO, EG, PROH or GLY	XT to mouse	74% (DMSO), 84% (EG), 44% (PROH) and 10% (GLY) of follicles survived	Newton *et al.* (1996)
0.3×2 mm	1.5 mol/l DMSO or 1.5 mol/l PROH + 0.1 mol/l S	Histology	No difference among treatments and control	Hovatta *et al.* (1996)
2×2×1 mm	1.5 mol/l EG	Follicular isolation	Number of isolated follicles after cryopreservation was similar to control	Oktay *et al.* (1997)
2 mm^3	1.5 mol/l DMSO alone or with 0.05, 0.1 or 0.25 mol/l S	Analysis with LDH[a]	No difference among treatments	Newton *et al.* (1998)
0.3×2 mm	1.5 mol/l PROH + 0.1 M	Follicular isolation and IVC	No difference between fresh and frozen follicles	Abir *et al.* (1999)
~ 4×3×1 mm	1.5 mol/l PROH + 0.1 mol/l S	Histology and TEM	Best results obtained with exposition in PROH + sucrose for 90 min	Gook *et al.* (1999)

[a]Lactate dehydrogenase assay.
DMSO = dimethyl sulphoxide; EG = ethylene glycol; GLY = glycerol; IVC = in-vitro culture; PROH = propylene glycol; S = sucrose; TEM = transmission electron microscopy; XT = xenotransplantation.

tissue, more than 90% of pre-antral follicles did not survive the cryopreservation procedure and only a small number of primordial follicles survived and achieved maturity. In a study in which GLY toxicity was assessed it was found that, after cryopreservation, almost all remaining oocytes were eliminated in the ovarian tissue (Deanesly, 1957). Finally, in sheep, comparison of DMSO with PROH as CPA showed the former agent to produce a higher survival rate (Demirci *et al.*, 2001).

Cryopreservation of human ovarian tissue using a penetrating CPA combined with sucrose was first studied in the mid-1990s (Hovatta *et al.*, 1996). These authors showed that when PROH was used with sucrose, the percentage of follicular atresia was similar to that in control (non-frozen) tissue. The addition of non-penetrating CPAs such as sucrose, threalose and mannitol resulted in protection of the cells during freezing and thawing procedures (Széll and Shelton, 1987). These sugars act as an osmotic buffer, maintaining isotonic equilibrium in the medium. They also prevent cellular swelling during the addition and removal of penetrating CPAs (Gordon, 1994; Newton *et al.*, 1998). Sucrose also prevents membrane damage caused by extreme dehydration, and lowers the transition temperature of phospholipids (Awad, 2000). Other authors also tested the addition of different sucrose concentrations to DMSO (Shaw *et al.*, 1996; Newton *et al.*, 1998; Oktay *et al.*, 2000b; Clearly *et al.*, 2001; Kim *et al.*, 2001b, 2002; Newton and Illingworth, 2001; Wolvekamp *et al.*, 2001) or PROH (Shaw *et al.*, 1996; Gook *et al.*, 1999; 2000; 2001; Van den Broecke *et al.*, 2001a).

After cryopreservation, ovarian tissue can be used for either follicle isolation (Zhang *et al.*, 1995; Oktay *et al.*, 1997; Abir *et al.*, 1999; 2001b; Newton *et al.*, 1999; Newton and Illingworth, 2001) or in-vitro culture (Sugimoto *et al.*, 1996; Paynter *et al.*, 1999). After culture, follicles can be isolated and oocytes can be collected for further in-vitro maturation, fertilization and embryo transfer. However, most researchers preferred to transplant fragments (Cox *et al.*, 1996; Salle *et al.*, 1998; Aubard *et al.*, 1999; Oktay *et al.*, 2000b; Shaw *et al.*, 2000a; Sugimoto *et al.*, 2000; Van den Broecke *et al.*, 2001a) as this would ideally re-establish ovarian hormone synthesis and propitiate oocyte growth and maturation (Nugent *et al.*, 1997). Ovarian tissue has been xenotransplanted (Nisolle *et al.*, 2000; Kim *et al.*, 2001b; Van den Broecke *et al.*, 2001a), allotransplanted (between members of the same species) (Harp *et al.*, 1994; Cox *et al.*, 1996; Gunasena *et al.*, 1997a) or autotransplanted (Aubard *et al.*, 1998; Sugimoto *et al.*, 2000; Callejo *et al.*, 2001; Radford *et al.*, 2001). The ovarian tissue has been transplanted in their original anatomical location (orthotopic transplantation) (Gosden *et al.*, 1994; Aubard *et al.*, 1998; Sztein *et al.*, 1998; Radford *et al.*, 2001) or to a different position (heterotopic transplantation), such as under the skin (Aubard *et al.*, 1999; Nisolle *et al.*, 2000; Van den Broecke *et al.*, 2001a; Kim *et al.*, 2002; Wang *et al.*, 2002) and under the kidney capsule (Gosden *et al.*, 1994; Oktay *et al.*, 2000b; Wolvekamp *et al.*, 2001).

Despite the promising results, cryopreservation of ovarian tissue and its further transplantation also has certain disadvantages. First, the quality and/or quantity of the follicle population

Table II. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of human ovarian tissue during this decade

Ovarian tissue	CPA	After thawing	Main results	Reference
1 mm^3	1.5 mol/l DMSO + 0.1 mol/l S	XT to mouse	Follicular growth	Oktay *et al*. (2000b)
1 mm^3 or 1×1×4–6 mm	10% DMSO	Histology	Follicular number and morphology not affected by cryopreservation	Qu *et al*. (2000a)
1×1×1 mm	10% DMSO	IHC	Immunoreactivites for TGF-α, EGF, and EGF receptor did not decrease	Qu *et al*. (2000b)
1×1×1 mm	10% DMSO	XT to mouse	The number of follicles did not differ from control	Nisolle *et al*. (2000)
1×3×4 mm	1.5 mol/l PROH with 0.1 or 0.2 mol/l S	Histology	One-step dehydration (90 min) and slow cooling obtained the higher follicular survival	Gook *et al*. (2000)
0.3×2 mm	1.5 mol/l PROH + 0.1 M	Follicular isolation and IVC	No difference between fresh and frozen follicles	Abir *et al*. (2001)
1.0×0.5 mm	1.5 mol/l DMSO	AT	Estradiol and antral follicle detection	Radford *et al*. (2001)
Small piece	1.5 mol/l DMSO, 1.5 mol/l PROH + 0.2 mol/l S or 1.5 PROH	XT to mouse	Follicular growth (antral stage)	Van den Broecke *et al*. (2001a)
2×1×1 mm	1.5 mol/l DMSO	XT to mouse	Follicular growth (until secondary follicle)	Van den Broecke *et al*. (2001b)
2–3 mm^3	1.5 mol/l DMSO	AT	Hormone secretion can be re-established	Callejo *et al*. (2001)
Strips ⩽8 mm	1.5 mol/l DMSO + 0.1 mol/l S	XT to mouse	None of the animals grafted with ovarian tissue from lymphoma patients developed disease	Kim *et al*. (2001b)
~ 4×2×0.5 mm	1.5 mol/l PROH + 0.1 mol/l S	XT to mouse	Follicular growth (antral stage)	Gook *et al*. (2001)
1 mm×1 mm^2 or 1 cm^2	1.5 mol/l DMSO + 0.1 S	Histology	The number of follicles varied to the chemotherapy dosage and patient age	Poirot *et al*. (2002)
5×5×1 mm	1.5 mol/l DMSO + 0.1 S	XT to mouse	Follicular maturation	Kim *et al*. (2002)

AT = autotransplantation; EGF = epidermal growth factor; IHC = Immunohistochemistry; IVC = in-vitro culture; TGF-α = transforming growth factor-α; S = sucrose; XT = xenotransplantation.

cannot easily be assessed in the ovarian tissue (Shaw *et al*., 2000a), which can represent a major problem when it is not possible to dispose of a significant amount of tissue. Second, the few studies of ovarian tissue transplantation were carried out because of the danger of malignancy transmission. One group (Shaw *et al*., 1996) demonstrated lymphoma transmission in ovarian grafts, while others (Meirow *et al*., 1998) showed that metastasis is possible in ovaries from patients with breast cancer, illustrating the risk of transmitting diseases or infections present in cryopreserved tissue. However, another group (Kim *et al*., 2001b) showed that ovarian fragments from women with Hodgkin's lymphoma did not transmit the disease when grafted to mice. The transplant procedure to preserve fertility in cancer patients has also been reviewed (Oktay, 2001; Oktay and Buyuk, 2002). These authors described the indications for ovarian transplantation as well as the risk of ovarian involvement in several types of cancer, reporting that some types may not be indicated for transplantation at the present time. Third, follicular death resulted from damage in the cellular membrane caused by lipid peroxidation during ischaemia–reperfusion injury (Nugent *et al*., 1998). One group (Baird *et al*., 1999) observed a massive loss of follicle population after transplantation when compared with cryopreservation; these authors reported that, after freezing, the follicular population decreased by 7%, and when the cryopreserved fragments were transplanted, the population decreased by 65%. Some authors (Newton *et al*., 1996; Candy *et al*., 1997; Gunasena *et al*., 1997b) also reported such loss during the ischaemic phase after transplantation, showing the risk of rapid exhaustion of follicle population in the grafts (Cortvrindt *et al*., 1996). Finally, it is important to point out that the distribution of pre-antral follicles in human ovaries seems to be irregular (Hovatta *et al*., 1996) and this may represent a risk when ovarian tissue is cut into fragments of 1 to 2 mm^3.

Cryopreservation of isolated follicles

Research into the cryopreservation of isolated follicles is conducted less frequently than are studies of the freezing of ovarian tissue, perhaps for two main reasons: (i) the preference for transplantation, which can represent a possibility of restoring normal reproductive life for women, by re-establishing not only fertility but also the production of ovarian hormones; and (ii) the poor results obtained with some follicular isolation procedures.

Table III. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of ovarian tissue from mouse

Ovarian tissue	CPA	After thawing	Main results	References
¼ ovary	15% GLY	Transplantation	Offspring	Parrott (1960)
Ovary	1.4 mol/l DMSO	Transplantation	Restoration of estrous cyclicity	Harp *et al.* (1994)
Fetal ovary	1.5 mol/l DMSO	Transplantation	Offspring	Cox *et al.* (1996)
1 mm^3	1.5 mol/l DMSO, PROH, EG or GLY + 0.1 mol/l sucrose and 5% PVP	Transplantation	Transmission of lymphoma to receptors	Shaw *et al.* (1996)
Ovary	1.5 mol/l DMSO, PROH, EG or GLY	Transplantation	DMSO and PROH showed results similar to control	Candy *et al.* (1997)
Ovary	1.4 mol/l DMSO	Transplantation	Offspring	Gunasena *et al.* (1997a)
Ovary	1.4 mol/l DMSO	Transplantation	Offspring	Gunasena *et al.* (1997b)
1×1×1.5 mm	1.4 mol/l DMSO	Transplantation	Offspring	Gunasena *et al.* (1998)
Half ovary	1.5 mol/l DMSO	Transplantation	Offspring	Sztein *et al.* (1998)
Fetal ovary	1.5 mol/l DMSO	Transplantation	Offspring	Shaw *et al.* (2000)
Ovarian pieces	1.5 mol/l DMSO + 0.1 mol/l sucrose	Transplantation	No significant difference among cooling treatments	Clearly *et al.* (2001)
Ovary	1.5mol/l DMSO	Transplantation, isolation, IVM, IVF and ET	Offspring	Liu *et al.* (2001)
Ovarian fragments	1.5 mol/l DMSO, PROH or GLY added with 0.1 mol/l S or 20% DMSO and PROH + 0.03 mol/l S	Follicle isolation and IVC	Follicle maturation after 8 days of culture	Newton and Illingworth (2001)
Ovary	1.5 DMSO	Transplantation	hMG injection increased the number of follicles in cryopreserved grafts	Wang *et al.* (2002)

ET = embryo transfer; hMG = human menopausal gonadotrophin; IVC = in-vitro culture; IVM = in-vitro maturation; PVP = polyvinyl pyrrolidone; S = sucrose.

Table IV. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of ovarian tissue from sheep

Ovarian tissue	CPA	After thawing	Main results	Reference
Slices <50 mg	1.5 mol/l DMSO	Transplantation	Birth	Gosden *et al.* (1994)
3×3 mm	1.4 mol/l DMSO	XT to mouse	Viability of ovarian fragments	Gunasena *et al.* (1997a)
Ovarian slices	10% DMSO	AT	Follicular growth (antral stage)	Salle *et al.* (1998)
~ 5×5×1 mm	1.5 mol/l DMSO	XT to mouse and AT	Preservation of cyclical ovarian function	Baird *et al.*, 1999
5×5 mm	1.5 mol/l DMSO + 0.1 mol/l sucrose	Follicular isolation and IVC	Follicular growth (antral stage)	Newton *et al.* (1999)
1 cm^2	10% DMSO	AT	Follicular growth and ovulation	Aubard *et al.* (1999)
Half ovary	10% DMSO	AT	Follicular growth (antral stage) and P secretion	Salle *et al.* (1999)
Half ovary	1.0, 1.5 or 2.0 mol/l DMSO or PROH	Histology	Higher follicular survival with 2 mol/l DMSO	Demirci *et al.* (2001)
Half ovary	1.5 mol/l or 2.0 mol/l DMSO	Histology	Percentage of normal follicles was similar in the treatments	Demirci *et al.* (2002)
Half ovary	2.0 mol/l DMSO	AT	Offspring	Salle *et al.* (2002)

AT = autotransplantation; DMSO = dimethyl sulphoxide; IVC = in-vitro culture; P = progesterone; PROH = propylene glycol; XT = xenotransplantation.

Moreover, it is possible that isolated follicles cannot develop without surrounding stroma or theca cells. In addition, in both humans and farm animals, it is not yet possible to describe a standard protocol for the in-vitro culture of pre-antral follicles with the aim of obtaining a large number of mature oocytes. However, the isolation of pre-antral follicles from ovarian tissue followed by cryopreservation with the aim of in-vitro culture and use of mature oocytes in IVF programmes represents a potential possibility for avoiding the problems related to cryopreservation and the transplantation of ovarian tissue.

It has been hypothesized that, by using in-vitro culture of follicles, it may be possible to obtain more mature oocytes than by using transplantation procedures, as this would avoid follicle loss caused by the ischaemia period and/or atresia process that characterizes normal follicular growth (Gosden *et al.*, 2002). Others (Cortvrindt *et al.*, 1996) showed that the in-vitro culture of isolated follicles was an alternative to transplantation, affirming that this procedure could be more economically favourable. The cryopreservation of isolated follicles offers other advantages when compared with cryo-

Table V. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of ovarian tissue from rat

Ovarian tissue	CPA	After thawing	Main results	Reference
Fragments <1 mm^3	15% or 25% GLY	Transplantation	Significant reduction of number of follicles	Parkes and Smith (1953)
1/12 ovary	15% GLY	AT	Significant reduction of number of follicles	Green *et al*. (1956)
Ovary	15% GLY	Transplantation	Significant reduction of number of follicles	Deanesly (1957)
Neonatal ovary	SV	IVC	Number of viable follicles inferior to control	Sugimoto *et al*. (1996)
Ovary	1.5 mol/l DMSO or EG	AT	Number of follicles similar between CPAs	Aubard *et al*. (1998)
Infantile ovary	SV	AT	Estrous cyclicity and follicular growth with presence of CL	Sugimoto *et al*. (2000)

AT = autotransplantation; CL = corpora lutea; DMSO = dimethyl sulphoxide; EG = ethylene glycol; GLY = glycerol; IVC = in-vitro culture; SV = 20.5% DMSO, 15.5% acetamine, 10% PROH and 6% polyethylene glycol.

Table VI. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of ovarian tissue from different animal species

Species	Ovarian tissue	CPA	After thawing	Main results	Reference
Rabbit	Half ovary	1.4 mol/l GLY	AT	Follicular growth	Daniel *et al*. (1983)
Marmoset	Ovarian fragments	1.5 mol/l DMSO	XT to mouse	Follicular growth to antral stage	Candy *et al*. (1995)
Elephant	1–2 cm^3	1.4 DMSO	XT to mouse	Follicular growth (antral stage)	Gunasena *et al*. (1998)
Cow	1×2 mm^3	1.5 mol/l DMSO	IVC	Number of normal follicles in cryopreserved fragments similar to control	Paynter *et al*. (1999)
Wombat	1 mm^3	1.5 mol/l DMSO + 0.1 S	XT to rat	Follicular growth	Wolvekamp *et al*. (2001)
Tammar wallaby	Ovary	1.5 mol/l DMSO	XT to mouse	Follicular growth	Mattiske *et al*. (2002)

AT = autotransplantation; DMSO = dimethyl sulphoxide; GLY = glycerol; IVC = in-vitro culture; S = sucrose; XT = xenotransplantation.

preservation of ovarian tissue: (i) qualitative and quantitative evaluation of the follicle before cryopreservation; (ii) thawing of a known and limited number of follicles; and (iii) subsequent in-vitro culture and maturation avoids possible disease transmission. In addition, this procedure—when combined with in-vitro culture—would offer a great opportunity for studies regarding folliculogenesis. A large number of follicles could be stored and used to investigate the mechanisms involved in their growth and development.

Only a few studies have reported experiments in isolated follicle cryopreservation (Carroll *et al*., 1990; 1991; Carroll and Gosden, 1993; Cortvrindt *et al*., 1996; Jewgenow *et al*., 1998; Amorim *et al*., 2003a,b; dela Peña *et al*., 2002). With one exception (dela Peña *et al*., 2002), all of these described a slow freezing method similar to the one described for the cryopreservation of ovarian tissue. The same group (dela Peña *et al*., 2002) tested a vitrification procedure that cryopreserved isolated follicles from mice; the results of this experiment showed that such a method allowed the survival of murine follicles.

The first study describing follicular cryopreservation used murine isolated follicles (Carroll *et al*., 1990). These authors obtained live offspring after isolation of primary follicles and cryopreservation followed by in-vitro culture, maturation and fertilization and embryo transfer. One year later, this group (Carroll *et al*., 1991) used the same freezing protocol and reported that after in-vitro culture of primary follicles in collagen gels, it was possible to observe follicular growth and acquisition of meiotic competence of oocytes. Using a different approach, others (Carroll and Gosden, 1993) obtained animal births; these authors isolated primordial follicles and enclosed them in fibrin clots before transplanting into the host animals. Successful results in mice were also obtained in a study which reported embryo growth using isolated secondary follicles that were previously cryopreserved and cultured *in vitro* (Cortvrindt *et al*., 1996). Others (Jewgenow *et al*., 1998) compared two CPAs (DMSO and PROH) for freezing follicles from cats, and observed that 19% of follicles survived the cryopreservation and culture. In another study in sheep (Amorim *et al*., 2003a,b), different DMSO and EG concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mol/l) were compared, and it was observed that the percentage of live follicles frozen with concentrations of 1.0 and 1.5 mol/l did not differ from those of controls (Table VII).

Methods for evaluating the efficiency of cryopreservation

In ovarian tissue cryopreservation, histology has been the most often used method for evaluating follicles after thawing (Hovatta *et al*., 1996; Gook *et al*., 2000; Demirci *et al*., 2001; 2002) or after procedures following cryopreservation, such as transplantation and in-vitro culture (Cox *et al*., 1996; Newton *et al*., 1996; Sztein

Table VII. Ovarian tissue size, cryoprotectant agent (CPA), procedure after thawing and main results in cryopreservation of isolated follicles in mouse, cat and sheep

Species	Isolation procedure	CPA	After thawing	Main results	Reference
Mouse	Collagenase + DNase	1.5 mol/l DMSO	IVC/IVF/ET	Offspring	Carroll *et al*. (1990)
Mouse	Collagenase + DNase	1.5 mol/l DMSO	IVC	Follicular growth and acquisition of meiotic competence of oocytes	Carroll *et al*. (1991)
Mouse	Collagenase	1.5 mol/l DMSO	24 h IVC → transplantation	Offspring	Carroll and Gosden (1993)
Mouse	25-gauge needles	1.5 mol/l DMSO	IVC/IVF	Embryo stage	Cortvrindt *et al*. (1996)
Cat	Cell-dissociation sieves	1.5 mol/l DMSO or PROH	IVC	19% of the follicles cryopreserved with DMSO or PROH survived in culture	Jewgenow *et al*. (1998)
Mouse	Needle	6 mol/l EG + 0.3 mol/l raffinose	IVC/IVF/ET	Offspring	dela Peña *et al*. (2002)
Sheep	Tissue chopper	0.5, 1.0, 1.5, 2.0 or 2.5 mol/l EG	Supravital stain (trypan blue)	1.0, 1.5 and 2.0 mol/l EG had a number of live follicles similar to control	Amorim *et al*. (2003a)
Sheep	Tissue chopper	0.5, 1.0, 1.5, 2.0 or 2.5 mol/l DMSO	Supravital stain (trypan blue)	1.0 and 1.5 mol/l DMSO had a number of live follicles similar to control	Amorim *et al*. (2003b)

DMSO = dimethyl sulphoxide; EG = ethylene glycol; ET = embryo transfer; GLY = glycerol; IVC = in-vitro culture; PROH = propylene glycol.

et al., 1998; Shaw *et al*., 2000a). With this analysis, it is possible to observe if follicles maintain their normal morphology after freezing and thawing. However, histology does not show freezing damage that could occur in cellular organelles, nor subtle changes in membranes. Therefore, histology can be a good adjunct procedure that provides a rapid and simple answer before more specific analysis can be carried out. Nevertheless, one group (Hovatta *et al*., 1996) incubated ovarian fragments before preparing them for histology, while others (Gosden, 2000) suggested that after thawing, this incubation period is necessary to detect changes in organelles caused by the cryopreservation procedure before preparing the specimens for histological procedures. However, most studies (Abir *et al*., 1999; 2001b; Gook *et al*., 1999; 2000; Demirci *et al*., 2001; 2002) did not incubate the ovarian tissue before histological evaluation. Another method used to evaluate follicle morphology is that of transmission electron microscopy (Oktay *et al*., 1997; Gook *et al*., 1999; Nisolle *et al*., 2000; Abir *et al*., 2001b); this permits a deeper examination of the cells, identifying even slight damage in the follicle ultrastructure caused by cryopreservation.

Despite the importance of evaluating follicular morphology after freezing and thawing procedures, this analysis provides no certainty as to cellular viability (Picton *et al*., 2000). Indications can be provided by viability staining, as morphological integrity does not guarantee follicular survival after thawing. After cryopreservation of ovarian tissue, follicles can be isolated and analysed using viability stains (Zhang *et al*., 1995; Oktay *et al*., 1997), or frozen isolated follicles can be evaluated soon after thawing (Amorim *et al*., 2003a,b), before starting in-vitro culture of the follicles. Viability stains such as trypan blue (Carroll and Gosden, 1993; Jewgenow *et al*., 1998; Amorim *et al*., 2003a,b), propidium iodine (Oktay *et al*., 1997), carboxyfluorescein diacetate succinimidyl ester (CMSE) (Oktay *et al*., 1997; Newton *et al*., 1999), Hoechst 33258 (Jewgenow *et al*., 1998), a commercial kit called Live/Dead® Viability/Cytotoxicity Kit (Siebzehnrübl *et al*., 2000), Calceim AM and ethidium homodimer-1 (Schotanus *et al*., 1997; Cortvrindt and Smitz, 2001) or Calceim AM and rhodamine 123 (Schotanus *et al*., 1997) can be used to show the short-term maintenance of functional integrity of follicles after thawing (Picton *et al*., 2000).

The best alternative to evaluating the follicles after thawing is to observe their capacity to initiate or restart growth by means of transplantation into hosts such as immunodeficient mice. These procedures reported successful results in humans (Oktay *et al*., 2000b; Callejo *et al*., 2001; Van den Broecke *et al*., 2001a), mice (Cox *et al*., 1996; Shaw *et al*., 1996; Clearly *et al*., 2001), sheep (Salle *et al*., 1998; 1999; 2002), rats (Aubard *et al*., 1998; Sugimoto *et al*., 2000), marmosets (Candy *et al*., 1995), wombats (Wolvekamp *et al*., 2001), elephants (Gunasena *et al*., 1998) and rabbits (Daniel *et al*., 1983). Hormonal assays can be conducted after implantation of ovarian tissue with the aim of following graft re-establishment in the oophorectomized hosts (Kim *et al*., 2002). Using this procedure, one group (Baird *et al*., 1999) followed the grafts for 22 weeks, another group (Gosden *et al*., 1994) for approximately 9 months, and another (Salle *et al*., 1999) for one year, showing that the grafts remained functional during these periods. In xeno- or allotransplantation to mice, it is also possible to follow ovarian cyclicity by using vaginal cytology (Candy *et al*., 1997; Gunasena *et al*., 1997b; Sugimoto *et al*., 2000).

Another method for the evaluation of follicular quality is by in-vitro culture of isolated thawed follicles and ovarian tissue. Some authors cultured ovarian fragments from rats (Sugimoto *et al*.,

1996) or cows (Paynter *et al*., 1999), or isolated follicles from the ovarian fragments in humans (Newton *et al*., 1999; Abir *et al*., 1999; 2001a) or mice (Newton and Illingworth, 2001). The main advantages of in-vitro culture are the possibility of following follicular growth and eliminating the risk of transmission of malignant cells via grafts in humans.

Additional evaluation methods can be performed after the cryopreservation procedure, such as DNA analysis (Newton *et al*., 1999; Demirci *et al*., 2002) and immunohistochemical reactions (Nisolle *et al*., 2000; Kim *et al*., 2001b), such as 5-bromo-2'-deoxyuridine (BrdU) (Cortvrindt and Smitz, 1998) and proliferating cell nuclear antigen (PCNA) immunoperoxidase (Oktay *et al*., 2000b).

Final considerations and perspectives

Cryopreservation of immature oocytes from pre-antral follicles has been shown as a considerable alternative for preserving fertility in women and protecting genetic material from rare animal breeds or species. Successful results obtained during the past 12 years have shown that such oocytes can restart their growth (humans, Oktay *et al*., 2000b; Gook *et al*., 2001; Van den Broecke *et al*., 2001a,b; mice, Newton and Illingworth, 2001; sheep, Salle *et al*., 1998; 1999; Aubard *et al*., 1999; Newton *et al*., 1999; rabbits, Daniel *et al*., 1983; marmosets, Candy *et al*., 1995; elephants, Gunasena *et al*., 1998; wombats, Wolvekamp *et al*., 2001; tammar wallabies, Mattiske *et al*., 2002) and eventually ovulate (sheep, Aubard *et al*., 1999). However, a better understanding of follicular behaviour during freezing and thawing procedures as well as CPA exposition would improve the results. Fundamental cryobiological properties, such as the permeability coefficient of CPAs and hydraulic conductivity should be determined in follicular cells (granulosa cells and oocytes) and, based on these findings, cryopreservation protocols could be more accurately designed for pre-antral follicles.

Follicular growth after thawing proved possible in transplanted ovarian fragments as well as in-vitro culture. With regard to transplantation, further studies are necessary to evaluate the risk of disease transmission, the survival period of ovarian tissue, and to estimate the number of follicles necessary to produce a pregnancy. The discovery of methods or substances, such as vitamin E (Nugent *et al*., 1998), superoxide dismutase (Parks *et al*., 1982) or allopurinol (Lindell *et al*., 1991) that protect the tissue from oxidative damage caused during the ischaemic period might also help to improve results in transplantation procedures. With regard to in-vitro culture, it is necessary to develop systems to maintain follicular growth and support oocyte maturation. However, this is a highly complex process which requires a complete knowledge of folliculogenesis and most likely a dynamic and variable system in the different stages of follicular development (Depalo *et al*., 2002). Therefore, the first step to develop such culture systems is to investigate the biology of oocyte growth and granulosa cell multiplication and differentiation and then try to imitate, *in vitro*, the conditions normally found in the ovary.

The possibility of re-establishing fertility potential in women and of safeguarding animal biodiversity is an enormous recompense for those researchers investigating follicle cryopreservation. We believe that in future, with the understanding of follicular cryobiology and the consequent development of optimum cryopreservation protocols for pre-antral follicles, this procedure will form part of the routine in medical centres and research institutes.

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