Outlook

Methods for cryopreservation of human ovarian tissue

Outi Hovatta is Professor in Obstetrics and Gynaecology in the Karolinska Institute in Stockholm, Sweden, since 1998. Earlier, from 1970, she worked as a clinician and reproductive scientist in the field of infertility, particularly assisted reproduction. She has ties in Helsinki and London.

Dr Outi Hovatta

Outi Hovatta
Karolinska Institutet, Department of Clinical Science, Karolinska University Hospital Huddinge, SE 141 86 Stockholm, Sweden
Correspondence: Outi.Hovatta@klinvet.ki.se

Abstract

Human ovarian tissue can be successfully cryopreserved, with good survival and function after thawing. Experimental animal studies regarding ovarian tissue cryopreservation resulting in live-born offspring preceded the present freezing systems in humans. On the basis of current knowledge, the standard method for human ovarian cryopreservation is slow programmed freezing, using human serum albumin-containing medium, and propanediol, dimethylsulphoxide (DMSO) or ethylene glycol as a cryoprotectant, combined with sucrose. Vitrification is still at the experimental stage. Whole organ cryopreservation is an interesting experimental option. Transplantation of the frozen–thawed tissue is a feasible method to utilize the tissue in infertility treatment. Ovarian function has been restored in humans. Because one healthy child has already been born from cryopreserved tissue, tissue cryopreservation should perhaps be offered to all young girls and women who can be predicted to undergo premature ovarian failure due to cancer treatment or genetic causes. Maturation of follicles *in vitro* from frozen–thawed tissue is another option that is still under development.

Keywords: *cryopreservation, culture, follicle, ovary transplantation*

Introduction

It is believed that cryopreservation of human ovarian tissue would benefit women who can be predicted to undergo premature ovarian failure due to chemotherapy or radiotherapy, or due to genetic causes or certain other diseases. For prepubertal girls, it is the only option to store oocytes. It is, however, a good option for post-pubertal young women because the vast majority of the oocytes are located in the ovarian cortical tissue (Gosden, 2002; Hovatta, 2003).

Cryobiologically, ovarian cortical tissue is challenging because it contains many different cell types, such as stromal cells, and follicles formed by the oocytes, granulosa cells and theca cells. In addition, the blood vessels and nerves are important for function after transplantation. In human ovary, tight fibrous stroma surrounds primordial and primary follicles with a single layer of granulosa cells around an oocyte with a diameter of about 35 μm (Hovatta *et al*., 1996). Proper penetration of the cryoprotectant through the stroma and granulosa cells to the oocytes is necessary, at the same time avoiding possible toxicity of the cryoprotectant. Ice crystal formation has to be minimized by choosing optimal freezing and thawing rates. The choice of a cryoprotectant with optimal permeation with lowest toxicity and least ice crystal formation is specific for each cell and tissue type (Fuller and Paynter, 2004). In the ovary, it is a compromise between the stroma and the follicles.

Primordial ovarian follicles, which are formed by the oocyte surrounded by a single layer of flat granulosa cells, comprise a majority, 70–90% of all follicles in human ovary (Gougeon, 1986; Lass *et al*., 1997). They are located in the ovarian cortical tissue within 1 mm from the surface. These follicles are the main target of cryopreservation programmes. Primary follicles, in which the cuboidal granulosa cells have initiated their growth, are the next largest group, up to 20–30%. Primary follicles also survive freezing and thawing well.

About 6% of the follicles in the cortical tissue are secondary follicles, with the granulosa cells forming at least two layers around the oocytes. It has been supposed that these follicles do not tolerate freezing and thawing as well as those at earlier stages. Early secondary follicles can occasionally be seen in thawed tissue. Antral follicles are only rarely encountered in the cortical tissue, and the oocytes at growing stages, and probably do not survive cryopreservation (Gosden, 2002).

Cryopreservation of ovarian tissue is not new. Slices of ovarian tissue from mice, rats and hamsters have been cryopreserved and autografted subcutaneously (Deanesly 1954; Green *et al*. 1956; Parkes 1958). Frozen–thawed ovarian tissue has been transplanted to mouse irradiated ovary (Parrot, 1960), and live offspring were born. The cryoprotectant used at that time was glycerol, which permeates the tissue slowly. This may have influenced the results of those early pioneering studies. Gosden *et al*. (1994) used dimethylsulphoxide as a cryoprotectant for sheep ovarian tissue by slow programmed freezing. Transplantation of frozen–thawed tissue slices resulted in cyclic activity and pregnancies, with one live lamb born. Since then, several successful studies regarding freezing of ovarian tissue from several animal species, such as sheep (Almodin *et al*. 2004; Baird *et al*. 2004; Cecconi *et al*. 2004), goat (Rodrigues *et al*., 2004), zebu bovine (Lucci *et al*., 2004) and cats (Bosch *et al*., 2004), have been performed.

Cryopreservation of human ovarian tissue

Human ovarian tissue has been cryopreserved since 1996 (Hovatta *et al*., 1996; Newton *et al*., 1996). Hovatta *et al*. (1996) cryopreserved human ovarian cortical tissue taken by biopsy during gynaecological laparoscopies or laparotomies as donations for research, using propanediol (PROH)–sucrose and dimethylsuphoxide (DMSO) as cryoprotectants. The morphology of the tissue appeared good, with no light microscopic changes seen in the follicles. The densities of the follicles and the proportions of follicles at different developmental stages were similar in the frozen–thawed tissue to those in tissue taken to histology before cryopreservation. These frozen–thawed follicles were also cultured for up to 2 weeks in organ culture as cortical slices, and they survived and grew as well as the non-frozen follicles (Hovatta *et al*., 1997). They remained functional after transplantation to immunodeficient mice subcutaneously or under the kidney capsule (Van den Broecke *et al*., 2001).

In an initial study (Hovatta *et al*., 1996), the tissue was transferred from the operating theatre to the laboratory in buffered culture medium to minimize apoptosis. The cortical tissue was cut into pieces or slices, about 1 mm in thickness and placed into an incubator at 37°C for 30 min. The pieces were then transferred to cryovials containing freezing medium. The starting temperature in the programme with PROH–sucrose was +18°C, and when DMSO was used, it was 0°C. Slow programmed freezing was used in a programmable freezing apparatus (Planer Kryo 10). Single step dehydration was used, with 20% serum in phosphate-buffered saline (PBS) as the freezing medium. Rapid thawing was used, as in most studies on cryopreservation of human ovarian tissue (**Table 1**).

Newton *et al*. (1996) studied, in addition to PROH and DMSO, glycerol and ethylene glycol-containing freezing protocols. The frozen–thawed tissue was viable after transplantation under the kidney capsule of immuno-incompetent mice. Ethylene glycol gave the best survival result in their study, and glycerol was significantly worse than the others. Newton *et al*. (1998) also tested the penetration times of these cryoprotectants to tissue, with very good results for ethylene glycol. Ethylene glycol has been used clinically, and fertilizable oocytes have been obtained

Table 1. Cryopreservation programmes for human ovarian tissue with propanediol (PROH)–sucrose and dimethylsulphoxide (DMSO) (Hovatta *et al*., 1996).

	PROH–sucrose	*DMSO*
Concentration of cryoprotectant	PROH 1.5 mol/l, sucrose	0.1 mol/l DMSO 1.5 mol/l
Starting temperature	+18°C	0°C
Dehydration time	15 min	15 min
Initial cooling rate	2°C/min	2°C/min
Seeding temperature	–8°C	–7°C
Keeping at that temperature	10 min	10 min
Followed by cooling	0.3°C/min to –30°C	0.3°C/min to –40°C
Continued by	50°C/min to –150°C	10°C/min to –150°C
Transferred to	Liquid nitrogen	Liquid nitrogen
Thawing	PROH–sucrose	DMSO
	RT 30 s	RT 2 min
	Water bath 30°C until ice thawed	Water bath 30°C until ice thawed
	Medium with 1.5 mol/l PROH	Culture medium
	+ 0.2 mol/l sucrose + 20% serum at RT, 5 min	Washed in medium 3 times
	0.5 mol/l PROH, 0.2 mol/l sucrose 10 min	
	0.2 mol/l sucrose 10 min	
	Medium RT 10 min, then to incubator	

RT = room temperature.

from the tissue after transplantation (Schmidt *et al*. 2004).

Gook *et al*. (1999) used PROH–sucrose as a cryoprotectant for cryopreservation of human ovarian tissue, and showed that slow-programmed freezing was significantly better than rapid freezing. A long time in cryoprotectant medium before freezing, 90 min, gave the best electron microscopic morphology for both stroma cells and follicles, even though the tissue contained vacuoles. A shorter time in the freezing medium (15 min) (Hovatta *et al*., 1996, 1997) may protect tissue from vacuole formation. Chemically defined media are regarded as better in clinical practice because of being quality controlled. Serum-containing freezing medium has been compared with the same freezing medium in which serum has been replaced by human serum albumin (Hreinsson *et al*., 2003a), and tissue survival was similar in both media. The cryopreservation programmes described are summarized in **Table 2**.

Table 2. Methods used for human ovarian tissue freezing.

Article	*Technique used*	*Cryoprotectant*	*Time in medium before freezing (min)*	*Starting temperature ºC*	*Seeding temperature ºC*	*Freezing rate ºC/min*	*Function showed after thawing*	*Morphology after thawing*
Hovatta *et al*., 1996	Slow freezing	PROH–sucrose	15	18	–8	2 to –8, 0.3 to –30, 50 to –150	Organ culture, nude mice	Normal light microscopy
		DMSO	15	0	–7	2 to –7, 0.3 to –50, 10 to –150	Organ culture, nude mice	Normal light microscopy
Newton *et al*., 1996	Slow freezing	Glycerol					SCID mice	Poorest follicle survival and morphology
		PROH					SCID mice	Fairly good follicle survival and morphology
		DMSO					SCID mice	Fairly good follicle survival and morphology
		Ethylene glycol					SCID mice	Best follicle survival and morphology
Gook *et al*., 1999	Slow freezing	PROH	15, 30, 60, 90	18	–8	2 to –8, 0.3 to –30, 50 to –150		EM and light microscopy, some vacuoles in oocytes
	Rapid freezing	PROH	15, 30, 60, 90	18		Immediate		Clearly poorer morphology
Hreinsson *et al*., 2003	Slow freezing	PROH–sucrose	15	18	–8	2 to –8, 0.3 to –30, 50 to –150	Culture, live/dead assay	Electron and light microscopy, normal oocytes and granulosa cells, stroma not as good
Schmidt *et al*., 2004	Slow freezing	Ethylene glycol–sucrose	30	4	–9	2 to –9, 0.3 to —40, 10 to –140	Trans-plantation Fertilizable oocytes	Normal light microscopic morphology
Ramihi *et al*., 2004	Slow freezing	Ethylene glycol	75, 3 steps	RT	–6	2 to –6, 0.3 to –35, 20 to –130	Culture, SCID mice	Necrotic areas in histology
	Vitrification	Glycerol, DMSO, ethylene glycol	30, 3 steps	RT		Immediate	Culture, SCID mice	Necrotic areas in histology

An ultra-rapid freezing technique with a high concentration of DMSO has been used for fetal human ovarian tissue, with central necroses after thawing, but with a part of the stroma and follicles looking normal. After this type of freezing, the tissue was vital in organ culture (Zhang *et al*., 1995). Slow programmed freezing using PROH or DMSO has given good survival results also for human fetal ovarian tissue (Abir *et al*., 2003).

Vitrification is a freezing method in which high concentrations of cryoprotectants induce formation of amorphous freezing instead of crystal formation. This freezing procedure has to be fast to achieve optimal results. A combination of cryoprotectants is normally used to avoid the suspected toxicity of each individual cryoprotectant. Human blastocysts and oocytes (Lieberman *et al*., 2003) have been successfully frozen by vitrification, but the dense human ovarian tissue is much more demanding to vitrify.

Vitrification of ovarian tissue has been successfully used in mice, which have a much looser structure of the ovary than humans (Salehnia, 2002; Tokieda *et al*., 2002; Migishima *et al*., 2003). Vitrification of human ovarian tissue has also been studied (Isachenko *et al*., 2003; Ramihi *et al*., 2004) by using combinations of 40% ethylene glycol with 0.35 mol/l sucrose and egg yolk, 18% Ficoll and 0.35 mol/l sucrose, or 20% DMSO as cryoprotectants. Vitrification was carried out by plunging the tissue into liquid nitrogen. Necrotic areas were seen in the thawed tissue, but the authors point out that vitrification is an alternative for slow freezing. More research is needed to show the value of this interesting new method in cryopreservation of human ovarian tissue.

Human ovarian tissue has contained viable follicles also after transfer from one city to another in Denmark (Schmidt *et al*., 2003) with a transport time of 3 h. This allows the preservation of fertility in operations that are carried out in hospitals with no freezing facility.

To avoid ischaemic injury after re-transplantation of the tissue, experimental systems have been developed to freeze whole ovaries or hemi-ovaries and carry out vascular anastomosis after thawing (Bedaiwy and Falcone, 2003; Salle *et al*., 2003; Yin *et al*., 2003). In rat, all eight freshly transplanted ovaries resumed function, and one pregnancy was achieved after transplantation of seven frozen–thawed ovaries (Wang *et al*., 2002; Yin *et al*., 2003). In sheep, cryopreservation of hemi- or whole ovary and microvascular anastomosis has resulted in functioning ovaries (Salle *et al*., 2003; Revel *et al*., 2004). Studies in humans are ongoing (Martinez-Madrid *et al*., 2004).

Utilisation of the frozen tissue

Transplantation

The viability of human ovarian tissue after thawing has been proven first in xeno-transplantation to immuno-incompetent mice either under kidney capsule (Newton *et al*., 1996; Van de Broecke *et al*., 2001) or subcutaneously (Van der Broecke *et al*., 2001), and it has been shown that mature follicles and oocytes can be obtained after transplantation under kidney capsule (Oktay *et al*., 1998; Gook *et al*., 2003).

Transplantation of frozen–thawed tissue back to the same woman has been successful. Ovarian function with growing follicles was proved first after subcutaneous transplantation (Oktay and Karlikaya, 2000) and then also after transplantation to the ovary (Radford, 2003). Mature oocytes were obtained after stimulation, and embryo transfers were carried out after subcutaneous transplantation (Oktay *et al*., 2004) or after transplantation to the ovary (Schmidt *et al*., 2004).

The first infant, a healthy girl, was born from cryopreserved and transplanted ovarian tissue in 2004 (Donnez *et al*., 2004). The tissue was transplanted under the pelvic peritoneum close to the ovarian vessels. After several years without any ovarian function, the woman's menstrual cycles resumed after the transplantation, and she became spontaneously pregnant. This demonstrated that cryopreservation of ovarian tissue is a viable option in humans.

An important risk factor in transplantation is the possible transmission of malignant cells back to the patient (Shaw and Trounson, 1977). The risk is particularly high in haematological and ovarian malignancies. A large proportion of follicles are also lost during the initial ischaemia after transplantation (Aubard *et al*., 1999). Relatively good numbers of follicles in the tissue are probably needed for good ovarian function after transplantation.

Maturation of follicles *in vitro*

By maturing the follicles *in vitro*, the risk of malignancy could be avoided. Live offspring from primordial follicles have been obtained in mice (O'Brien *et al*., 2003). In humans, the procedure is more difficult because of the longer time required for the maturation. The dense human ovarian stroma and the relatively low numbers of follicles in adult human ovarian tissue make the cultures more challenging (Hovatta *et al*., 1997; Lass *et al*., 1997). Isolation of the ovarian follicles from the tissue is harmful for their survival and growth (Abir *et al*., 1999; Hovatta *et al*., 1999). Some success has been achieved using non-isolated follicles. It is now possible regularly to obtain secondary and occasionally antral follicles within slices or cubes about 1 mm in thickness, of ovarian cortical tissue cultured on extracellular matrix (Hovatta *et al*., 1997, 1999; Hreinsson *et al*., 2002; Scott *et al*., 2004a). Adding FSH, insulin, growth differentiation factor 9 and cyclic guanosine monophosphate improves the development and survival of the follicles in culture (Wright *et al*., 1999; Louhio *et al*., 2000; Hreinsson *et al*., 2002; Scott *et al*., 2004b). These cultures are being further improved, and it is hoped that in the future it will be possible to isolate cumulus–oocyte complexes from these follicles for further maturation of the oocytes *in vitro*, which is already a clinical treatment (Mikkelsen *et al*., 1999; Suikkari *et al*., 2000; Hreinsson *et al*., 2003b)

Conclusion

Cryopreservation of human ovarian tissue is a feasible method for preserving fertility potential of young women who can be predicted to undergo premature ovarian failure due to chemotherapy or genetic causes. The first infant after transplantation of frozen–thawed tissue (Donnez *et al*., 2004) has already been born. The standard freezing method for the

time being is programmed slow freezing using DMSO, ethylene glycol or PROH as a cryoprotectant.

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