Cryopreservation of gonadal tissue and cells

H M Picton, S S Kim and **R G Gosden**

Division of Obstetrics and Gynaecology, University of Leeds, Leeds, UK

With the advent of assisted reproductive technology and an improved understanding of cryobiology, strategies have been developed which allow the long-term storage of gametes and embryos. Furthermore, in the light of the growing numbers of young adults and children who have been sterilised by successful cancer treatment, the need to protect their fertile potential has become more urgent. While semen cryopreservation is available for men, the methods for preserving oocytes are unreliable and neither method is suitable for prepubertal children. Research attention is, therefore, focusing on the low temperature banking of immature germ cells with the aim to restore natural fertility if possible or, alternatively, to use culture technology to produce ripe gametes for assisted conception. While there is no universal method for fertility conservation, gonadal tissue banking, in theory, is a practical alternative to gamete storage which can be utilised by both adults and children.

The wide-spread use of *in vitro* fertilisation technology in reproductive medicine has provided the impetus to develop strategies for the storage of surplus gametes and embryos. Consequently, the routine freezing of human embryos[1] and sperm[2] now provides the opportunity for repeated attempts at embryo transfer and pregnancy without the costs and inconvenience of repeated cycles of hormone stimulation and egg collection. In light of a better understanding of the principles of cryobiology as well as improved automation and instrumentation, we now have the potential to store oocytes as well as embryos. Furthermore, these technological advances together with long-term survival after high dose-chemotherapy and bone marrow transplantation in cancer patients have generated a surge of interest in gonadal tissue cryopreservation[3]. These methodologies will allow us to preserve the fertility of both children and adults.

Correspondence to:
Dr H M Picton, Centre for Reproduction, Growth and Development, Division of Obstetrics and Gynaecology, D Floor, Clarendon Wing (LGI), Belmont Grove, Leeds LS2 9NS, UK

Application of gonadal cryopreservation

The potential of gonadal cryobiology in the conservation of fertility is only now being fully realised. In recent years, we have seen a dramatic improvement in the management of a range of solid and haematological

malignancies and a marked increase in the chances of long-term survival[4]. Estimates now suggest that by the beginning of the new millennium at least 1:1000 adults will be survivors of childhood cancers[5]. However, the price that is paid for aggressive chemotherapy and abdominal irradiation is becoming increasingly unacceptable. The documented late effects of these treatments, including reduced pregnancy rates and a high risk of gonadal failure[6], are of particular concern because of the young median age of these patients. Furthermore, in young women, even if ovarian failure does not occur immediately, there is a substantial risk of premature menopause, particularly in those aged over 30 years[7]. Frozen banking of gonadal tissues and cells before the start of cancer treatment, therefore, represents an attractive strategy for conserving the fertility of children and young adults of both sexes.

Frozen semen banking has been available for many years to safeguard male fertility and is routinely offered to men before they undergo sterilising chemotherapy. Despite the production of often poor quality semen in this circumstance[8], the use of intracytoplasmic sperm injection (ICSI) has substantially improved the potential to use these stored cells. Furthermore, some clinical and experimental studies have indicated that the sterilising effects of cancer treatments can be reduced by hypogonadotrophism[9,10], and treatment with GnRH agonists has been advocated to protect the gonads in both sexes. However, overall, the evidence for clinical benefit is inconclusive and some experimental studies have revealed no protective effect of hypogonadotrophism on radio sensitivity[11].

The options for preserving female fertility are unreliable and complex. In theory, the only proven method for the conservation of the fertility of young women is assisted reproductive technology which enables collection and storage of mature metaphase II oocytes and, more practically, embryos. The storage of unfertilised human oocytes would avoid many of the ethical issues related to human embryo storage and facilitate egg donation programmes. Spare oocytes would be collected and stored during IVF cycles so that patients could use them at a later time or donate them to another person or for research. The opportunity to bank oocytes at low temperatures would be welcomed by young women undergoing sterilising chemotherapy as well by as others who wish to safeguard fertility from an early menopause or from the deleterious effects of ageing[12]. There is little doubt that the freeze storage of human oocytes would be a valuable technique in clinically-assisted reproduction, in animal husbandry and in the conservation of endangered species.

Numerous attempts have been made to freeze-thaw human oocytes, but success rates have been disappointing and can be attributed, in part, to the biological properties of this cell type. Ovulated oocytes are blocked at the metaphase of the second meiotic division with 23 bivalent chromosomes bound to the microtubules of the meiotic spindle. In this

state, the oocytes are extremely sensitive to both temperature and toxic shocks. Cryoprotectant exposure and/or ice crystal formation during the freeze-thaw process can lead to depolarisation of the spindle microtubules, disruption of chromatid separation at the moment of fertilisation, and the potential induction of aneuploidy after the extrusion of the second polar body. Furthermore, zona hardening can occur as a result of the premature release of cortical granules from the ooplasm[13] or lesions can be induced in the zona and plasma membrane by large ice crystals[14]. Finally, parthenogenetic activation can be induced by physical conditions such as freezing[15]. It is not surprising, therefore, that the first attempts to cryopreserve metaphase II oocytes resulted in poor cryosurvival with few live births[16]. However, since the introduction of different cryoprotective agents and ICSI, as a means of bypassing the zona pellucida, fertilisation and pregnancy rates of cryopreserved oocytes have improved[17,18]. However, the protocols that have been used in oocyte cryopreservation are still not optimal for this cell and authors have argued both for[19,20] and against[17,21] the maintenance of the cumulus oophorus to optimise oocyte survival rate. Despite this, cryopreservation of mature human oocytes has resulted in the production of healthy offspring with normal karyotypes suggesting that the process of freezing may select the most resistant oocytes which are best able to survive different stresses.

An alternative strategy to the banking of mature oocytes is to cryopreserve mature oocytes before they resume nuclear maturation and progress to metaphase II. At this stage of prophase I – the so-called germinal vesicle (GV) stage – the chromosomes are not aligned along the spindle, and there should, therefore, be a reduced risk of cytogenetic errors at subsequent divisions. However, cytoskeleton damage may still occur and the risk of zona hardening may be increased as the oocytes must undergo an extended period of culture to enable nuclear maturation to progress before fertilisation can occur *in vitro*. Using cryopreserved GV oocytes, high survival rates and birth rates have been achieved in mice[22]. However immature human oocytes have low post-thaw survival (37%[23], and 43%[24]) and *in vitro* maturation rates (20%[23] and 27%[24]). Furthermore, very few pregnancies have been achieved after the *in vitro* maturation of fresh oocytes[25–27] and none after cryopreservation. At present, and until the efficiency of *in vitro* maturation improves, cryopreservation of GV oocytes offers little advantage over freeze-storage of metaphase II oocytes.

In contrast to cryopreservation of mature oocytes, freeze storage of embryos is routinely used in assisted reproductive technology and has occasionally proved successful before chemotherapy[28]. While this approach is relatively straight-forward, it hosts a number of inherent disadvantages for the conservation of fertility of cancer patients. IVF treatment and embryo storage is only suitable for patients of reproductive

age and treatment is both costly and lengthy – in many cases taking 4–6 weeks[29]. What is more, ovarian hyperstimulation, which is required if several oocytes are to be collected, may be contra-indicated in the case of steroid related cancers, and where there is no male partner, donor sperm is required. More importantly, this strategy is clearly not an option for prepubertal girls.

A radical, and perhaps more acceptable, approach which can be used by both young girls as well as adults is the cryopreservation of ovarian tissue. Ovarian tissue banking has the added potential of restoring normal ovarian function and natural fertility. Gonadal cryopreservation is of particular relevance to young women and girls who are diagnosed with cancer when they are too young to have either started or completed their families. In addition, high-dose chemotherapy is now being used for an increasing number of non-malignant conditions, such as autoimmune diseases and thalassaemias, for which tissue storage is also potentially attractive. Furthermore, fertility conservation by ovarian tissue cryopreservation may be a viable option for women with a familial history of premature ovarian failure as the methodology offers the potential to restore natural fertility by autografting the thawed tissue at either an orthotopic or heterotopic site.

The technique of ovarian cryopreservation involves freezing immature primordial follicles *in situ* in slices of ovarian cortex. In comparison with mature oocytes, primordial follicles are smaller and more tolerant to freezing and thawing as they lack a zona pellucida and cortical granules, are relatively metabolically quiescent and undifferentiated and have more time to repair sublethal damage to organelles and other structures during their prolonged growth phase. Above all, primordial follicles are the most abundant stage of follicular development present at every age, and hundreds of small follicles can be obtained using a simple biopsy procedure, or thousands if an oophorectomy is performed. Primordial follicles from humans[30–33] and other species[34,35] have successfully survived cryopreservation to liquid nitrogen temperatures. Paradoxically, while primordial follicles may be a more effective subject for tissue banking than mature oocytes, animal studies have demonstrated that it is precisely this stage of follicle development which is most susceptible to the effects of ionising radiation and alkylating agents[36,37].

Cryopreservation and storage of testicular tissue slices may also be effective, and could provide an opportunity to preserve germ cells for prepubertal boys. Storage of spermatogonial stem cells or intact seminiferous tubules may provide the possibility of restoring spermatogenesis *in vivo* either by transferring stem cells back to the sterilised testes or by grafting tubules heterotopically for spermatogenesis *in vivo* and recovery of spermatozoa for an ICSI procedure[38,39]. In the longer term, it may even be possible to produce enough spermatozoa for ICSI from cultured

testicular tissue years after frozen banking testicular biopsies from children. In contrast to the successful cryopreservation of small pieces of gonadal tissue, at the present time, the freeze storage of whole gonads has only been achieved with the ovaries of small animals[40] or fetuses[41] and, therefore, remains only a remote possibility for use in humans.

Cryobiology of gonadal cells

When cells are cooled to temperatures between –5°C and –15°C, ice crystal formation is induced in the extracellular medium. As the temperature decreases further, the amount of ice increases, the solutes concentrate in the extracellular medium creating an osmotic gradient, and there is net water movement from the cytoplasm to the extracellular medium causing cellular shrinkage and dehydration. Cells undergoing cryopreservation are, therefore, liable to damage both from the formation of intracellular ice and from the build-up of salts in the cells as they dehydrate.

Protection from damage during freezing and thawing can be achieved through the use of a cryoprotective agent (CPA). The agents penetrate the cell membranes and are thought to stabilise intracellular proteins, reduce the temperature at which cells undergo lethal intracellular ice formation and moderate the impact of concentrated intra- and extracellular electrolytes[42]. Historically, glycerol, by virtue of its relative non-toxicity, has been extensively used as the CPA for sperm banking in animals and humans. However, its use for tissue preservation has been limited by its poor penetration qualities[30]. Consequently, in recent years, its use has been superseded by a number of new generation CPAs, namely, dimethylsulphoxide (DMSO), 1,2-propanediol (PROH) and ethylene glycol[43]. These cryoprotectants have high water-solubility, rapid penetrability and produce less osmotic damage at the high concentrations normally used in cryopreservation protocols; however, their effects on cellular viability are still largely unknown. For example, CPA exposure in mature oocytes has been implicated in alterations in the cytoskeleton[44], microtubular structure, and spindle organisation[45]. Such effects may disrupt the normal organisation and traffic of molecules and organelles, impair embryonic development and increase the incidence of aneuploidy and other chromosomal anomalies. More recently, CPA exposure has been shown to promote the passive influx of Ca^{2+} across the plasma membrane[46], possibly by stimulating the release of Ca^{2+} from storage sites in the mitochondria and endoplasmic reticulum. Such increases in intracellular Ca^{2+} can lead to parthenogenetic activation of mature oocytes and are known to activate intracellular phospholipases, proteases, ATPases and endonucleases, resulting in altered plasma membrane integrity, denaturation of

cystolic proteins, and chromosomal fragmentation – all of which can lead to irreversible cell injury and apoptosis.

In addition to the potential cytotoxic effects of CPA exposure, cryopreservation solutions are hyperosmotic and CPAs penetrate cells more slowly than water. Video microscopy of human oocytes has revealed that CPA exposure causes a 40% shrinkage of cellular volume as water is withdrawn down the osmotic gradient, followed by a return to normal volume as the CPA penetrates the cell[47]. When the oocyte is returned to the isotonic solution after thawing, there is an inward osmotic flux with the possibility of excessive swelling to 140–150% of the physiological volume (Fig. 1). These volumetric changes may contribute to the mechanism for the induction of cytogenetic damage during cryopreservation of mature oocytes and, in extreme cases, can cause cellular lysis.

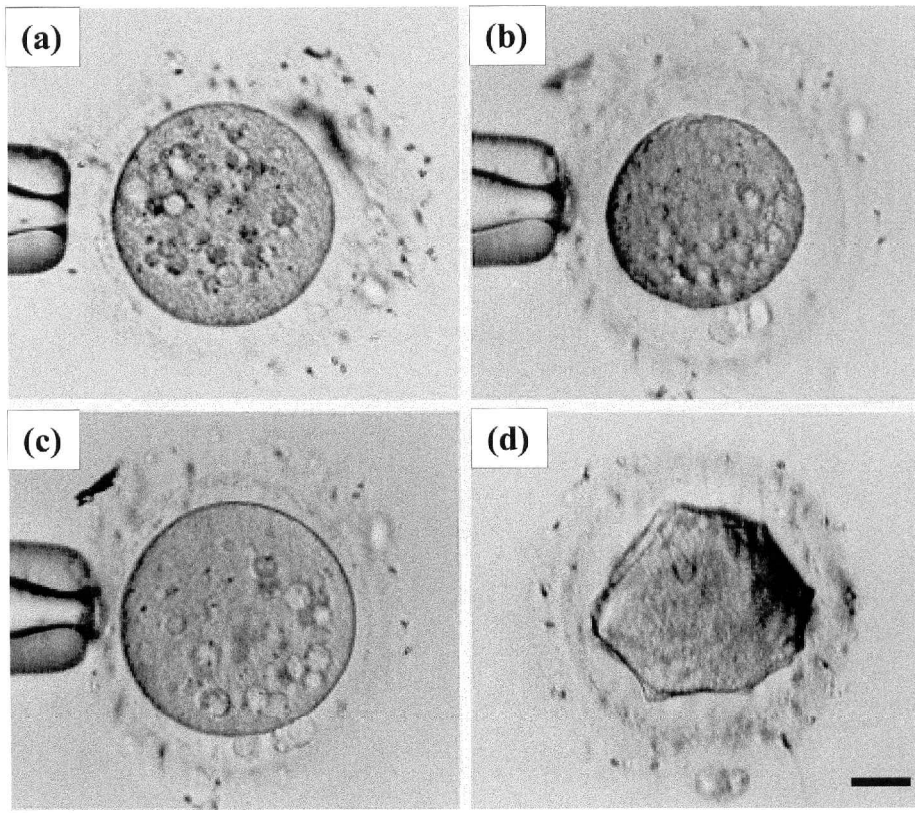

Fig. 1 The morphological appearance of mature human oocytes during exposure to isotonic and non-isotonic solutions at room temperature. (a) A morphologically normal oocyte in isotonic Leibovitz medium; (b) a shrunken oocyte after 30 s of exposure to 1.5 M DMSO; (c) a swollen oocyte after sequential exposure to 1.5 M DMSO, 1.5 M DMSO supplemented with 0.1 M sucrose and re-introduced to isotonic Leibovitz medium; (d) an oocyte exposed to 1.5 M DMSO at room temperature without prior incubation in 1 mM EDTA l-1. Scale bar = 28 μm. Reproduced from Newton *et al*[47] with permission of the *Journal of Reproduction and Fertility*.

Unlike cryopreservation of isolated cells, freeze-storage of tissue presents new problems because of the complexity of tissue architecture and protocols must strike a compromise between the optimal conditions for each different cell type. For example, the cooling and warming rates together with dehydration conditions which result in optimal survival of one cell type may not be ideal for other cellular components of the same piece of tissue. In addition, problems can arise when ice forms extracellularly, because it can cleave tissues into fragments. Furthermore, rapid solute penetration of highly compacted tissue is vital to ensure high final concentrations of cryoprotectant at temperatures which will minimise cytotoxicity[48]. These requirements necessitate optimisation of freeze/thaw protocols for each cell type, as post-thaw survival of reproductive cells and tissues are profoundly affected by both the type of cryoprotectant used[30] and the equilibration times required for cryoprotectant uptake and removal[48].

Despite the apparent difficulties compared with single cells, the storage of gonadal, and particularly ovarian, tissue has proved surprisingly successful. The suitability of ovarian tissue for freezing is enhanced by the developmental plasticity of the tissue as the ovary is capable of functioning even when its complement of follicles has been severely reduced such as naturally occurs during ageing, after partial ablation or after injury. Furthermore, the cortical distribution of primordial follicles permits the preparation of thin strips of tissue which provide maximal surface area for rapid CPA penetration, as evidenced by NMR spectroscopy[49].

Overall, the consensus of opinion suggests that, to minimise freeze-thaw injury, cooling rates should be fast enough to minimise exposure of cells to high intracellular concentrations of electrolyte and slow enough to avoid intracellular ice formation. Furthermore, these processes should be carried out in the presence of low concentrations of non-permeable osmolytes, such as sucrose and mannitol, to act as osmotic buffers against swelling during the addition and removal of CPAs[48]. In support of this recommendation, significantly more pregnancies have been achieved in mice after orthotopic ovarian transplantation when fast (86%), rather than slow (25%), thaw protocols were used[50]. Successful cryopreservation of human ovarian tissue with high post-thaw follicle survival rates of 84% and 74% have been achieved using the slow freeze, rapid rewarming protocol after immersion of ≤1 mm thick slices of ovarian cortex for 30 min at 4°C in freezing solution containing either 1.5M EG or DMSO with 0.1 M sucrose[30,51]. The tissue can then be stored at liquid nitrogen temperatures (–196°C) for as long as required. Morphological assessments of cryopreserved human ovarian cortex at the light microscope level[30], and examination of the fine structural integrity of tissue by electron microscopy[52] have confirmed that, in the hands of experienced cryobiologists, cellular damage in the tissue is minimal (Figs 2 & 3). However,

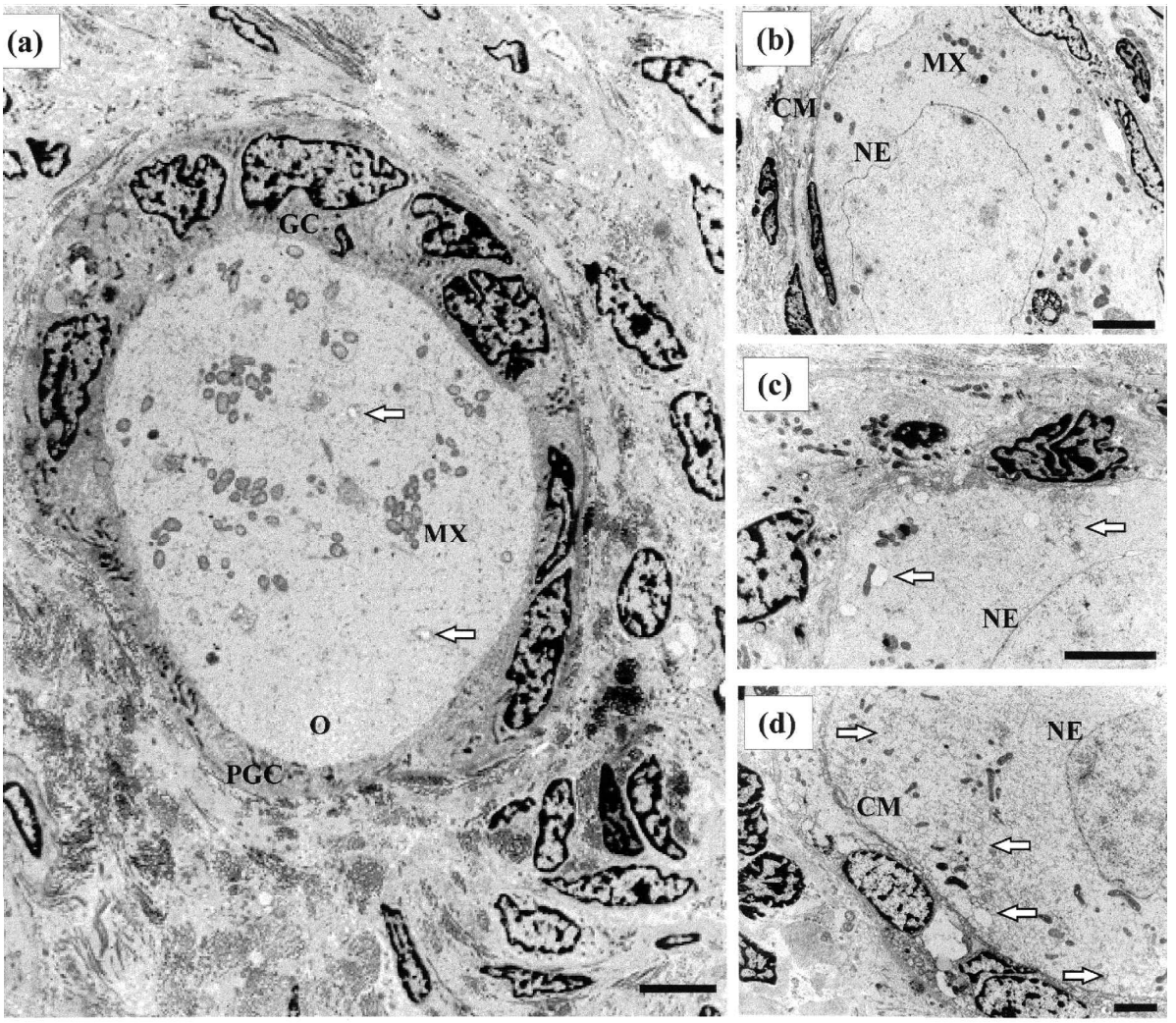

Fig. 2 Transmission electron micrographs of primordial oocytes from fresh and cryopreserved tissue (c,d). The cell membrane (CM), nuclear envelope (NE), and mitochondria (MX) are marked, vacuolation is indicated by the arrows. Tissue before freezing (**a**,**b**), showing a primordial oocyte (O) recruited into the growing pool. The oocyte is enclosed by pregranulosa cells (PGC) and granulosa cells (GC). (**c**) Morphologically normal tissue after cryopreservation showing limited vacuolation, an intact nuclear envelope and normal mitochondria. (**d**) Tissue damaged during cryopreservation showing intact cell and nuclear membranes but extensive vacuolation throughout the cytoplasm and in the mitochondria. Scale bar = 5 μm.

the choice of an inappropriate CPA together with poor laboratory practice may lead to increased cellular damage, and compromise tissue viability on thawing.

An alternative to the slow freezing protocols detailed above is rapid freezing. Here, increased concentrations of CPA dehydrate the cells before the ultra-rapid cooling rates allow the solutions to form glass (vitrify)

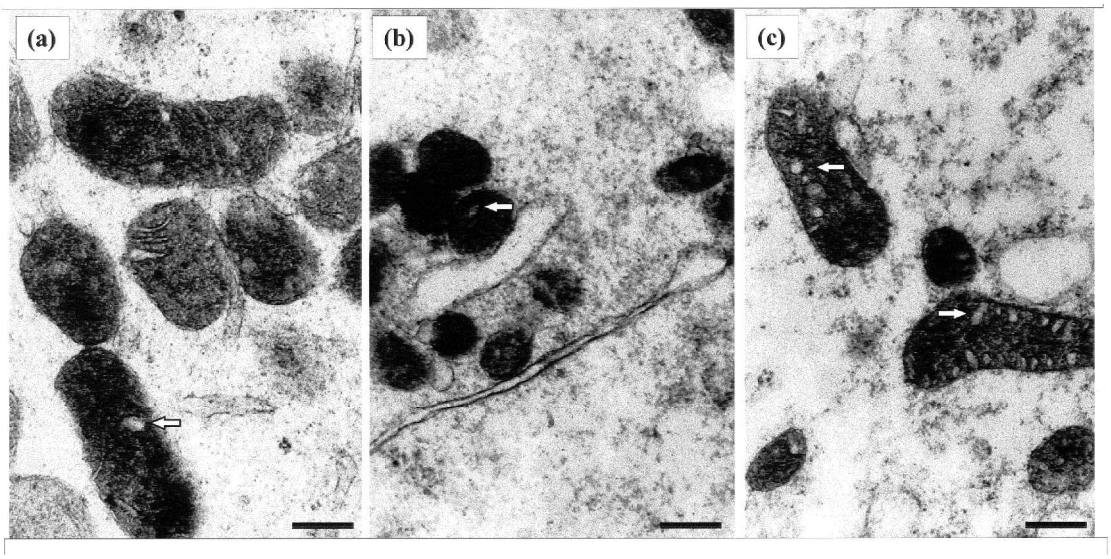

Fig. 3 Transmission electron micrographs of mitochondria in human ovarian cortex before and after cryopreservation. Healthy tissue before freezing (a) and tissue after cryopreservation with low (b) or high (c) levels of vacuolation (indicated by the arrows). Scale bar = 0.5 μm.

rather than ice crystals (freeze) when they are plunged into liquid nitrogen[53]. Increasing both cryoprotectant concentration and the rate of cooling by using metal grids[54] and minimal volumes has enabled the successful vitrification of oocytes and embryos from a number of species[55,56]. However, ultrarapid freezing regimens have proved less successful with human oocytes[57] and both the solute concentrations and cooling rates need to be optimised to prevent irreversible chromosome damage occurring.

Developmental potential of cryopreserved ovarian tissue

Although the histological evaluations detailed above indicate that human follicles can survive freezing, these morphological evaluations give no indication of the viability or long-term developmental potential of the stored tissue. Post-thaw, short-term maintenance of functional integrity of follicles from cryopreserved human ovarian cortex has been indicated by viability staining[31], and follicular structure has been maintained in tissue culture[58]. Furthermore, encouraging results have been obtained using transplantation technology in experimental studies using both animals and humans. The *in vivo* developmental potential of primordial follicles from a number of species has been demonstrated by autografting or xenografting of both fresh and cryopreserved tissue

under the kidney capsules of immunologically tolerant mice with severe combined immune deficiency[30,34,59]. For example, high rates of follicular survival have been obtained in the human xenograft model[30] and follicle survival was further improved by the use of exogenous antioxidants, such as vitamin E, to reduce ischaemia[60]. Furthermore, follicle growth was initiated up to early antral stages (10–12 layers of granulosa cells) by recombinant human FSH[59]. Experiments in sheep, whose ovaries more closely resemble the human ovary in size and follicle and fibre composition, have confirmed the practicability of restoring natural fertility by ovarian banking and autografting[51,61,62].

While autografts and, controversially, xenografts are already providing a practical basis for the restoration of natural fertility after ovarian tissue banking, the important proviso remains that the tissue does not harbour malignant cells that could reseed the old disease after grafting. Long-range hopes for the safe usage of cryopreserved gonadal tissues must, therefore, rest on the complete growth and development of follicles *in vitro* as a desirable method of generating oocytes and avoiding any risk of transmitting residual disease in autografts[63]. However, follicle culture technology is still in its infancy and the lack of detailed knowledge about the early stages of human follicle growth and survival is compounded by the large size of the Graafian follicle and a growth period of several months[64]. At present, our ability to preserve cells at low temperatures is ahead of methods to realise their fertile potential, and the techniques must, therefore, be regarded as experimental in both males and females. Furthermore, the human oocyte is clearly a difficult cell to cryopreserve being large with only a short fertile life-span and little capacity to repair damage unlike cleaving embryos which tolerate the loss of one or more blastomeres. Future advances in the technology to conserve fertility must, therefore, rely on an improved understanding of the cryobiology of gonadal cells, further optimisation of freezing protocols for each cell type, a new generation cell culture techniques and the development of new skills in reproductive surgery.

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