Assessment of follicular development in cryopreserved primate ovarian tissue by xenografting: prepubertal tissues are less sensitive to the choice of cryoprotectant

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Abstract

Improvements in cancer survival rates have renewed interest in the cryopreservation of ovarian tissue for fertility preservation. We used the marmoset as a non-human primate model to assess the effect of different cryoprotectives on follicular viability of prepubertal compared to adult ovarian tissue following xenografting. Cryopreservation was performed with dimethylsulfoxide (DMSO), 1,2-propanediol (PrOH), or ethylene glycol (EG) using a slow freezing protocol. Subsequently, nude mice received eight grafts per animal from the DMSO and the PrOH groups for a 4-week grafting period. Fresh, cryopreserved–thawed, and xenografted tissues were serially sectioned and evaluated for the number and morphology of follicles. In adult tissue, the percentage of morphologically normal primordial follicles significantly decreased from $41.2 \pm 4.5\%$ (fresh) to 13.6 ± 1.8 (DMSO), 9.5 ± 1.7 (PrOH), or 6.8 ± 1.0 (EG) following cryopreservation. After xenografting, the percentage of morphologically normal primordial ($26.2 \pm 2.5\%$) and primary follicles ($28.1 \pm 5.4\%$) in the DMSO group was significantly higher than that in the PrOH group (12.2 ± 3 and $5.4 \pm 2.1\%$ respectively). Proliferating cell nuclear antigen (PCNA) staining suggests the resumption of proliferative activity in all cellular compartments. In prepubertal tissues, primordial but not primary follicles display a similar sensitivity to cryopreservation, and no significant differences between DMSO and PrOH following xenografting were observed. In conclusion, DMSO shows a superior protective effect on follicular morphology compared with PrOH and EG in cryopreserved tissues. Xenografting has confirmed better efficacy of DMSO versus PrOH in adult but not in prepubertal tissues, probably owing to a greater capacity of younger animals to compensate for cryoinjury.

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Introduction

Cryopreservation of ovarian tissue has been under investigation for many years, mainly for use in domestic animals. Improvements in the diagnosis and therapy of childhood malignancies accompanied by a significantly increased life expectancy, however, have renewed interest in the cryopreservation of human ovarian tissue. This is mainly due to the severe side effects of therapeutic regimens often including subfertility and premature ovarian failure (Meirow 2000). Among the promising strategies for fertility preservation is the laparoscopic removal and cryopreservation of ovarian cortical tissue fragments prior to gonadotoxic therapy followed by retransplantation (von Wolff *et al.* 2009). This approach facilitates the storage of a substantial number of primordial follicles without interfering with the oncological treatment, since it can be performed at any time of the menstrual cycle. To date,

ten live births have been reported after orthotopic transplantation of frozen–thawed ovarian tissue (Donnez *et al.* 2004, Meirow *et al.* 2005, Demeestere *et al.* 2007, Rosendahl *et al.* 2008, Silber *et al.* 2008, Ernst *et al.* 2010, Roux *et al.* 2010, Sanchez-Serrano *et al.* 2010), and potential long-term survival of grafted tissue has been documented (Ernst *et al.* 2010, Silber *et al.* 2010). In spite of these encouraging results, the cryopreservation and subsequent use of ovarian tissue thus far remains experimental and requires further research. There are still concerns about the safety of tissue retransplantation, as a potential reintroduction of malignant cells at least in some cancer subtypes cannot be fully excluded (Dolmans *et al.* 2010). Moreover, none of these live births originate from ovarian tissue cryopreserved at childhood or adolescence, and the developmental potential of juvenile ovarian tissue is unknown (Feigin *et al.* 2007). Due to the limited availability of donated human ovarian tissue, systematic approaches for the

comparison of different cryopreservation protocols have mainly been conducted in domestic animals. Early studies in rodents suggested that the restoration of fertility following the cryopreservation of ovarian tissue in glycerol and subsequent transplantation was possible (Parrott 1960). Since then, the preservation of reproductive potential after cryopreservation using slow-freezing methods has been demonstrated successfully in various mammalian species with differing cryoprotectants (Sztein *et al.* 1998, Candy *et al.* 2000, Salle *et al.* 2002, Bosch *et al.* 2004, Lucci *et al.* 2004, Gook *et al.* 2005).

The main purpose of ovarian cryopreservation is the storage of developmentally competent follicles. Histological evaluation may be employed to investigate the effects of cryopreservation procedures on follicular morphology: atresia is usually accompanied by nuclear pyknosis, cytoplasmic disorder, detachment of granulosa cells (GCs) from the oocyte, and changes to the basement membrane (Hulshof *et al.* 1995, Demirci *et al.* 2002). However, since not all morphologically intact follicles may convey viability, xenografting of cryopreserved ovarian tissue can demonstrate developmental potential by the resumption of follicular growth.

In this study, the common marmoset (*Callithrix jacchus*) served as a non-human primate model for the cryopreservation and subsequent xenografting of ovarian tissue fragments into immunodeficient nude mice. We analyzed the cryoinjury inflicted by different cryoprotective agents (CPAs) on prepubertal versus adult ovarian tissues. The aims of this study were to i) optimize the cryopreservation procedure for adult and prepubertal

primate ovarian cortical tissue fragments with respect to the cryoprotectant used and ii) to analyze the relationship between pre-freeze histology and follicular development following xenografting of thawed tissue.

Results

Histological analysis of adult ovarian tissues frozen with dimethylsulfoxide, 1,2-propanediol, or ethylene glycol

Typical pre- and post-cryopreservation morphology is shown in Fig. 1. In sections of freshly fixed tissue, cellular density of the follicles is high, instances of misshapen oocytes or the apparent loss of structural integrity of GCs are rather rare, and small vacuoles are found in only a few oocytes (Fig. 1A). In frozen-thawed tissue fragments (Fig. 1B–D), more oocytes have lost their spherical shape and displayed large vacuoles in the cytoplasm. GCs are more often unevenly distributed.

Cryopreservation, largely irrespective of the cryoprotectant used, challenges the morphological integrity of ovarian tissue fragments (Fig. 2): the percentage of morphologically normal primordial follicles significantly decreases from $41.2 \pm 4.5\%$ (fresh) to 13.6 ± 1.8 (dimethylsulfoxide, DMSO), 9.5 ± 1.7 (1,2-propanediol, PrOH), or 6.8 ± 1.0 (ethylene glycol, EG), respectively, following cryopreservation. DMSO displayed slightly better protective abilities compared with EG ($P=0.04$), whereas the difference between DMSO and PrOH and between PrOH and EG was not statistically significant ($P=0.83$ and 0.68 respectively). Within the class of primary follicles, the percentage of morphologically normal follicles decreased from $41.8 \pm 5.1\%$ in fresh

Figure 1 Typical morphology of freshly fixed marmoset ovarian tissue (A) and tissues fixed after freeze-thawing in DMSO (B), PrOH (C), and EG (D); unevenly distributed granulosa cells are indicated by asterisks and non-spherical oocytes are indicated by arrowheads.

Figure 2 Effects of DMSO, PrOH, and EG in adult marmoset ovarian tissue. Cryopreservation significantly decreases the percentage of morphologically normal primordial and primary follicles. Following cryopreservation with DMSO, primordial but not primary follicle morphology is significantly better compared with EG. Different letters indicate significant differences ($P < 0.05$) between treatments.

tissue to 13.3 ± 3.1 (DMSO), 8.4 ± 1.9 (PrOH), or 4.9 ± 1.5 (EG). The difference between the CPAs did not reach statistical significance ($P = 0.67$ for DMSO compared with PrOH, $P = 0.63$ for DMSO compared with EG, and $P = 0.08$ for PrOH compared with EG respectively). Morphologically intact preantral or antral follicles were not observed after cryopreservation.

Histological analysis of adult ovarian tissues frozen with DMSO or PrOH after xenografting

To assess the effects of different CPAs on the functionality of ovarian tissue fragments, thawed tissues not directly fixed for histological analysis were grafted to ovariectomized nude mice for a grafting period of 4 weeks. Due to the results of the post-freeze evaluation, six tissue fragments from each adult donor (18 fragments in total) were included in the experiment from both the DMSO and the PrOH groups, but not the EG group. For group assignment, see Table 1. Histological evaluation of all treatment groups (fresh tissues and tissues frozen with

one of the CPAs before and after grafting) comprised a total number of 16 235 primordial and primary follicles in adult marmoset tissues. The grafting procedure is commonly known to result in high follicular loss rates, and a one to three ratio of cryopreserved–thawed versus grafted tissues was chosen to achieve in each experimental group absolute follicle numbers adequate for further histological analysis (Table 1). At the end of the experiment, the graft retrieval rate was 72.9% (adult), without significant differences between treatment groups. All grafts displaying no follicles were excluded from the analysis.

The typical post-grafting morphology of adult tissue exhibits a reduced cellular density of the interstitial area compared with fresh controls. In addition to primordial and primary follicles, secondary and preantral follicles were detected in the freshly grafted control tissues and in both the DMSO (Fig. 3A) and the PrOH groups. However, due to the small overall numbers of advanced follicles in grafted tissues post-cryopreservation, the differences were not statistically analyzed. Even though the percentage of morphologically normal follicles significantly decreases after cryopreservation and grafting compared with freshly grafted controls, proliferating cell nuclear antigen (PCNA) staining of GCs, oocytes, and stromal cells suggests the initiation or resumption of proliferative activity in all xenografts (fresh and frozen, Fig. 3B).

Comparing the proportion of morphologically normal primordial and primary follicles after grafting of adult tissue with regard to the CPA used, the DMSO group displays a significantly higher percentage of primordial ($26.2 \pm 2.5\%$) and primary follicles ($28.1 \pm 5.4\%$) than the PrOH group (12.2 ± 3 and $5.4 \pm 2.1\%$, respectively, Fig. 4A). The ratio of primordial to primary follicles does not differ between the DMSO and the PrOH groups (Fig. 5). However, in comparison to freshly grafted tissues, tissues grafted after freeze–thawing show a shift towards primary follicle activation with a lower proportion of primordial follicles ($66.5 \pm 2.5\%$ (DMSO) and $71.4 \pm 4.6\%$ (PrOH) versus $86.1 \pm 2.4\%$ (freshly grafted); Fig. 5).

Table 1 Assignment of adult and prepubertal marmoset ovarian cortical tissue fragments to treatment groups.

	No. of donor animals (n)	Treatment	Overall no. of tissue fragments per donor/total	No. of grafted tissue fragments per donor/total	Total no. of analyzed follicles	
					Before grafting	After grafting
Adult	3		34/102	20/60	11 349	4886
		Fresh	10/30	8/24	2422	2011
		DMSO	8/24	6/18	2600	2296
		PrOH	8/24	6/18	1382	579
		EG	8/24	–	4945	–
Prepubertal	3		24/72	12/36	31 584	6737
		Fresh	6/18	4/12	17 729	4635
		DMSO	6/18	4/12	6706	1599
		PrOH	6/18	4/12	804	503
		EG	6/18	–	6345	–

Figure 3 Typical post-graft morphology of frozen-thawed marmoset ovarian tissue (A). Advanced follicle post-grafting (arrowhead). PCNA staining of granulosa cells (arrows), oocytes (asterisks), and interstitial cells (arrowheads) suggest proliferative activity in xenografts (B); negative control for PCNA staining (C).

Cryopreservation of prepubertal ovarian tissue with DMSO, PrOH, and EG and subsequent grafting

The effects of DMSO, PrOH, and EG on the percentage of morphologically normal primordial and primary follicles in prepubertal marmoset ovarian tissue are shown in Fig. 6: the proportion of morphologically normal primordial follicles in thawed prepubertal tissue is significantly higher after cryopreservation with DMSO

($28.2 \pm 1.6\%$) compared with PrOH ($11.7 \pm 2.2\%$) and EG groups ($14.4 \pm 1.6\%$). The difference between PrOH and EG did not reach statistical significance ($P=0.37$). Within the population of primary follicles (Fig. 6), the percentage of morphologically normal follicles is also highest in the DMSO group ($84.6 \pm 3.4\%$). However, morphologically normal follicles were not found in the PrOH group, whereas the EG group showed a rather low number of follicles with a normal morphology ($1.4 \pm 0.6\%$).

Due to the small size of prepubertal marmoset ovaries, four tissue fragments of each prepubertal donor were included into the grafting experiment (from both the DMSO and PrOH groups), yielding a total of 24 grafts. In total, 83.3% of all (prepubertal) grafts were retrieved, without statistical difference between treatment groups. For group assignment and absolute follicle numbers, also see Table 1. When grafted to immunodeficient nude mice, unlike in adult tissues, the effects of the CPAs analyzed (DMSO and PrOH) on normal morphology of primordial and primary follicles are not significantly different in prepubertal tissues (36.3 ± 2.8 (DMSO) and 28.8 ± 3.9 (PrOH)). However, the shift towards primary follicle activation in grafted tissues post-freezing when compared to freshly grafted tissues is also visible in prepubertal tissue and even more pronounced in tissues frozen with PrOH than with DMSO (Fig. 7). In freshly grafted ovarian tissue fragments, primordial follicles hold a proportion of $97.3 \pm 1.0\%$ among the pool of total primordial and primary follicles, whereas in tissues grafted after freeze-thawing this proportion is significantly reduced to $78.3 \pm 3.2\%$ in the DMSO and $61.4 \pm 5.0\%$ in the PrOH groups respectively.

Discussion

Regular ovarian function comprises a natural maturation process, during which consecutive hormonal cycles permit the development of follicles to more advanced

Figure 4 Confirmative superior effect of DMSO compared to PrOH following xenografting. The relative number of morphologically normal primordial (A) and primary follicles (B) in xenografted adult marmoset ovarian tissue is significantly higher following cryopreservation with DMSO. Different letters indicate significant differences ($P<0.05$) between treatments.

Figure 5 Higher proportion of primary follicles after xenotransplantation of frozen-thawed versus fresh grafted ovarian tissue from adult animals, irrespective of DMSO or PrOH as cryoprotectant. Different letters indicate significant differences ($P < 0.05$) between treatments.

stages, while others within the same cohort become atretic and degenerate (Gougeon 1996). Against this background of natural follicular change in the adult ovary, we investigated the effects of different cryoprotectants on non-human primate ovarian tissue from adult and juvenile donor animals. In accordance with the earlier studies for a number of different species (Gook *et al.* 1999, Paynter *et al.* 1999, Gandolfi *et al.* 2006, Maltaris *et al.* 2007, Borges *et al.* 2009), we found a pronounced decrease in the percentage of morphologically normal primordial and primary follicles following cryopreservation.

Among the cryoprotectants investigated, DMSO overall had a superior protective effect compared with PrOH and EG. In human ovarian tissue, comparisons between DMSO and PrOH proved both the cryoprotectants' ability to preserve morphological integrity (Hovatta *et al.* 1996, Van den Broecke *et al.* 2001, Maltaris *et al.* 2007) to a certain degree; however, DMSO was suggested to yield more viable follicles after freeze-thawing than PrOH (Hovatta *et al.* 1996). Other investigations found DMSO and PrOH to induce significant structural damage in human and bovine primordial follicles in particular (Gandolfi *et al.* 2006), whereas both agents preserve porcine primordial follicles well. It was also shown by one group that for PrOH, best results were achieved with a prolonged pre-freezing equilibrium period of 90 min (Gook & Edgar 1999). Possibly, better results not only for PrOH but also for EG could be obtained by prolonging equilibrium periods beyond the period used in our investigation. Also, a significant effect of post-thaw short-term tissue incubation (2–4 h) on the percentage of morphologically normal follicles has been described, suggesting a recovery of some follicles

(Borges *et al.* 2009). During this period, cells might be able to re-establish metabolic activity, cellular volume control, and cell–cell communication. A positive effect of *in vitro* culture before transplantation on viability and development of human fetal follicles in ovarian grafts has recently been demonstrated (Lan *et al.* 2010). Given that in our study tissues were either grafted or fixed as soon as possible after thawing, prolonged post-thaw incubation might improve results for all CPAs analyzed.

A significantly faster penetration of DMSO and EG versus PrOH in human ovarian tissue has been described previously (Newton *et al.* 1998). Furthermore, the higher tissue permeability coefficient of DMSO has been suggested to account for a better preservation of bi-directional interactions between germ cells and their somatic neighbors (Navarro-Costa *et al.* 2005). This communication is mediated by transzonal projections (TZPs) emanating from neighboring GCs (Anderson & Albertini 1976). DMSO maintains a higher post-thaw density of filamentous actin, which sustains TZPs (Navarro-Costa *et al.* 2005). In our study, DMSO has shown an increased protective effect compared with PrOH, but also to EG. As previously observed, differences in penetration rates have been temperature dependent (Newton *et al.* 1998); this may be due to the fact that dehydration conditions (as cooling and warming rates) have to be chosen specifically for different CPAs. Supplementing the permeating cryosolutions with components which do not penetrate the cell membrane, such as sucrose, promotes dehydration and thus improves the overall performance of the CPA solution (Kuleshova *et al.* 1999). Furthermore, in whole tissue cryopreservation as opposed to oocyte cryopreservation, optimal conditions for one cell type may not be ideal for other cellular components. One recent study on primate ovarian tissue

Figure 6 Effects of DMSO, PrOH, and EG in prepubertal marmoset ovarian tissue. Following cryopreservation with DMSO, the relative number of morphologically normal primordial and primary follicles is significantly higher compared to PrOH and EG. Different letters indicate significant differences ($P < 0.05$) between treatments.

Figure 7 Higher proportion of primary follicles after xenotransplantation of frozen–thawed versus fresh grafted ovarian tissue from prepubertal animals, significantly more pronounced following cryopreservation with PrOH. Different letters indicate significant differences ($P < 0.05$) between treatments.

cryopreservation has highlighted the cryoinjury incurred by interstitial cells, which may be of importance for long-term graft survival and the full developmental competence of retrieved oocytes (Jin *et al.* 2010). In tissue fragments, unlike in cell suspensions, the dense packing of cells creates a thermal gradient across the tissue, resulting in different cooling rates for individual cells. Particularly in primordial follicles, where pre-GCs and oocytes differ remarkably in volume, a different sensitivity to the cryopreservation procedure compared to other follicular populations can be expected. In our study, this effect would not only account for differing results between the primordial and the primary follicle compartment but may also explain differences observed between adult and prepubertal tissues, as the proportion of primordial follicles is higher in the latter.

Although morphological evaluation has proven useful to estimate the extent of injury in tissues submitted to cryopreservation, morphological integrity does not necessarily correspond to viability or developmental competence. We have, therefore, decided to assess the effects of different cryopreservation protocols by xenografting the ovarian tissue fragments to nude mice. Our results show a beneficial effect of DMSO on the percentage of morphologically normal primordial and primary follicles compared with PrOH in adult tissues. This is in accordance with the results of other groups, who found DMSO to allow for better preservation of primary follicles than PrOH in human tissue (Gandolfi *et al.* 2006), although no effects were found in the population of primordial follicles in that study. Other studies suggest that even though EG is known for its low toxicity when applied as a cryoprotectant of bovine and caprine ovarian tissue (Santos *et al.* 2006b, Celestino *et al.* 2008), DMSO preserves the ultrastructure of ovine early-staged follicles better (Santos *et al.* 2006a).

Despite a decreased percentage of morphologically normal follicles after cryopreservation compared with fresh controls, PCNA staining of interstitial cells, GCs, and oocytes suggests ongoing proliferative activity within all cellular compartments of xenografted tissues after cryopreservation.

The evaluation of tissues post-grafting as a functional test of follicular viability, however, did not show an advantage of DMSO in prepubertal tissues. In a recent paper by Abir *et al.* (2009), follicular development of xenografted frozen–thawed human fetal ovarian tissue could only be documented following cryopreservation with PrOH but not with DMSO. It is of note that contrary to our protocols, sucrose was added to the PrOH cryoprotectant solution only. The observed lack of difference between DMSO and PrOH in comparison to adult tissues in our study may be attributed to the fact that throughout life cohorts of primordial follicles are recruited to go down the differentiation pathway, leading to a steady decline of the ovarian reserve. Accordingly, ovaries of younger individuals should have a greater follicular capacity to compensate for cryoinjury and be less sensitive. Thus, the ability of the cryoprotectants to preserve morphological integrity and functionality of at least some follicles within the treated tissue fragments also seems to be dependent on the age of the tissue donor.

It has previously been shown that ovarian tissue grafting, even without cryopreservation, induces a dramatic follicular loss (Mussett & Parrott 1961, Newton *et al.* 1996, Nisolle *et al.* 2000, Shaw *et al.* 2000, Liu *et al.* 2002, Borges *et al.* 2009), irrespective of grafting site (Wang *et al.* 2002), the age of donors and recipients (Liu *et al.* 2002), and even species (Salle *et al.* 2003, Gook *et al.* 2005). This is associated with a reduced graft size and significant fibrosis in most grafts (Agca *et al.* 2002). These phenomena have been related to initial ischemia induced by the harvesting procedure and the prolonged time until neovascularization after reimplantation allows a sufficient supply of oxygen and nutrients (Baird *et al.* 1999, Laschke *et al.* 2002, Aubard 2003, Van Eyck *et al.* 2009). In this study, the superior effect of DMSO on both primordial and primary follicle morphology in adult tissue was to the full extent distinguishable only following xenografting.

Moreover, in prepubertal and adult tissues, xenografting revealed remarkable dynamics in the transfer of follicles from the primordial to the primary follicle compartment. Female fertility of most mammals is determined by the primordial follicle pool size and by the rate at which primordial follicles deplete from this reservoir to go down the maturation pathway. The local production and action of several factors are supposed to mediate cell–cell interactions required during follicular development (Skinner 2005). A disruption of GC–oocyte cross talk induced by the cryopreservation procedure has also been suggested to account for the initial follicular

loss following grafting (Navarro-Costa *et al.* 2005). Recently, it has been demonstrated that as an effect of cryopreservation, stromal cell health might be severely affected (von Wolff *et al.* 2009). Stromal cells are not only involved in producing factors regulating follicular development (Nilsson & Skinner 2003) but also provide the three-dimensional environment required for follicle maintenance. Our results show a shift towards primary follicle activation from the resting primordial follicle pool when grafting frozen-thawed tissue, whereas we do not find this effect in freshly grafted tissues. This may rather reflect a decrease in inhibitory signals on follicular activation following cryodamage of interstitial cells (Jin *et al.* 2010) than an increase in activating signals deriving from a permissive recipient milieu.

In conclusion, both the morphological appearance of follicles and any condition facilitating their recruitment in the pool of growing follicles will ultimately define the oocyte's developmental competence and long-term graft survival. The present data suggest that DMSO has a slightly advantageous effect compared to other cryoprotectives during conventional slow-freezing procedures of marmoset ovarian tissue. The whole extent of structural and functional damage inflicted on ovarian tissue by cryopreservation becomes apparent only following the grafting of thawed tissue fragments. In prepubertal tissue, significant differences between DMSO and PrOH concerning follicular morphology are abrogated following xenografting, probably owing to a greater capacity of younger individuals to compensate for cryoinjury. The propagation of these results from non-human primate tissue can assist in further developing ovarian tissue cryopreservation and grafting into a routinely successful option for fertility preservation in children and young adults.

Materials and Methods

Animals

All handling and experimental procedures concerning the animals utilized in this study were in accordance with the German animal protection law (license no. AZ 50.0835.1.0).

Ovarian tissues were taken from three healthy female common marmosets with proven fertility aged between 2 and 3 years (adult group) and three healthy females aged between 6 and 20 weeks (prepubertal group) originating from the breeding colony of our institute's animal research unit. Directly upon removal, ovaries were rinsed briefly in PBS (Gibco), transferred into fresh PBS supplemented with human serum albumin (HSA, 4 mg/ml, Irvine Scientific, Santa Ana, CA, USA), and dissected under microscopic control at room temperature (RT). Fragment sizes of 1–2 mm³ were used in all experiments. Control tissues were fixed immediately; the remaining tissues were either grafted freshly or assigned to cryopreservation with DMSO, PrOH, or EG; see also Table 1 for group assignment.

Cryopreservation

The effects of three different cryopreservation protocols were investigated as follows:

- (1) DMSO: PBS supplemented with DMSO (1.5 mol/l, Merck), sucrose (0.5 mol/l, Sigma-Aldrich), and HSA (10 mg/ml).
- (2) PrOH: PBS containing PrOH (1.5 mol/l, Sigma-Aldrich), sucrose (0.5 mol/l), and HSA (10 mg/ml).
- (3) EG: PBS containing EG (1.5 mol/l), sucrose (0.5 mol/l), and HSA (10 mg/ml).

In these solutions, fragments were gently agitated at RT for 20 min, transferred into cryostraws (Consarctic, Schoellkrippen, Germany) with 0.5 ml of cryoprotectant solution, sealed, and cryopreserved following a slow-freezing protocol (Gook *et al.* 1999) in a controlled rate programmable freezing system (Consarctic): starting at RT, the cooling rate to -7°C was $2^{\circ}\text{C}/\text{min}$. Seeding was induced manually at -7°C . Cooling then continued at $0.3^{\circ}\text{C}/\text{min}$ to -40°C followed by $-10^{\circ}\text{C}/\text{min}$ to -150°C . Cryostraws were subsequently immersed in liquid nitrogen and stored for a minimum time period of 2 weeks.

On the day of transplantation, ovarian tissue fragments were thawed following a rapid procedure: straws were warmed at RT for 40 s and then immersed in a water bath at 30°C until all ice crystals vanished. The contents of the straws were then released into cryoprotectant solution. The cryoprotectant was removed by washing the tissue fragments in PBS containing decreasing concentrations of cryoprotectant (75, 50, and 25% of the original concentration) at RT for 5 min each followed by PBS (with 10 mg/ml HSA) and either grafted as quickly as possible or fixed for histological evaluation (see below). Cooling rates, manual seeding, and rapid thawing procedures in preparation for grafting were alike in all cryoprotocols.

Surgical procedures

Totally, eight Nu/Nu nude mice (CrI:NU-Foxn1^{nu}) were obtained (Charles River Germany, Sulzfeld, Germany) and housed in the central animal facility of the university for at least 1 week prior to procedures. The environment was air filtered with a 12 h light:12 h darkness cycle at 23°C ; all animals had free access to sterilized food and water. Ovariectomies were carried out 2 weeks prior to grafting through a dorsomedian incision; surgical procedures were performed under a laminar flow hood in aseptic conditions. The animals were anesthetized with 0.15 mg/g body weight ketamine and 2% (v/v) xylazine intraperitoneally. They were kept on a warming plate; eye protection was carried out with humidified sterile swabs. On the day of transplantation, mice received eight grafts per animal. For best accessibility and to achieve a maximum number of grafts per host animal, the tissue was placed in subcutaneous pouches formed with a blunt probe in a small incision in the dorsal skin. Each graft was placed in an individual subcutaneous pouch. The grafting experiments have been carried out in multiple rounds to ensure optimal technical handling and minimize the number of animals to be killed. Grafting procedure was

scheduled to the availability of freshly donated ovarian tissue. For cryopreserved tissues, storage time was a minimum of 2 weeks and could also be longer without effect on later analysis. To avoid bias inflicted by different endogenous hormonal milieus of the recipients, cryopreserved fragments from both treatment groups along with fresh control tissues were transplanted to every mouse except in the first round of experiments, when only fresh tissue was grafted. The grafting pattern (four grafts were placed on either side of the spinal column) was retained in all animals for easy identification of the grafts at the end of the experiment. Pouches were sutured with 5/0 vicryl sutures. No animal died during surgery or was lost prior to the end of the experiment.

Graft retrieval and procession of tissue

On the day of explantation of the grafts, the mice were killed by cervical dislocation. Grafts were retrieved 4 weeks after the transplantation procedure and immediately fixed in 4% paraformaldehyde (Sigma–Aldrich). Following fixation, tissues were dehydrated in a graded ethanol series, clarified with xylene, and routinely embedded in paraffin wax. Serial sections of 5 µm thickness were cut; every fifth section was stained with hematoxylin–eosin (HE) and analyzed with a light microscope (Axiophot, Zeiss, Oberkochen, Germany) under a magnification of 400×. The size of the tissue sections was determined based on follicle sizes and previous work with marmoset and primate ovarian tissue (Gougeon 1986, Candy *et al.* 1995). Marmoset primordial follicles have a diameter of 30 µm, increasing up to 45–85 µm in primary follicles (Buse *et al.* 2008). On the opposite end, small follicles with a nucleus <19 µm are considered non-growing in primates (Gougeon & Busso 2000). Given these numbers, the aforementioned tissue section size is adequately chosen to detect the large majority of follicles while at the same time prevent double-counting of follicles. An additional oocyte staining technique has, therefore, not been employed.

Histological evaluation

All sections were analyzed by a single investigator blinded to the experimental groups. In the analyzed sections, all follicles were counted. To avoid overcounting, follicles were only counted when the nucleus of the oocyte was observed in the section. The developmental stages of the follicles were classified according to Gougeon (1996): the oocyte of a primordial follicle is surrounded by a single layer of flattened GCs or a mixture of flattened and cuboidal GCs. In a primary follicle, the single GC layer is cuboidal; a secondary follicle displays two or more layers of GCs; in a preantral follicle, small fluid-filled cavities are observed between the GCs, and in the antral follicle, an antrum is distinguishable.

Follicular morphology was evaluated based on variables such as integrity of the oocyte, the GCs, and the basement membrane. Follicles were classified as morphologically normal if they showed no pyknosis of the oocyte nucleus, no disorganized or detached GCs, or shrunken ooplasm.

Immunohistochemistry

Tissue sections were immunohistochemically stained for PCNA using an indirect method with a peroxidase-labeled secondary antibody according to the manufacturer's instructions. Briefly, sections were deparaffinized in paraclear and rehydrated in a graded series of ethanol. For antigen retrieval, sections were heated in a microwave oven in glycine/HCl buffer (50 mM, pH 3.5) for 10 min at 100 °C. Endogenous peroxidase activity was quenched by treatment with hydrogen peroxide (3% v/v for 5 min) followed by blocking of nonspecific antibody binding with 5% (v/v) normal goat serum supplemented with BSA (0.1% v/v) for 20 min at RT. All antibodies were diluted in TBS/BSA (0.1% v/v). The slides were incubated with primary rabbit-anti-PCNA antibody (1:100, Dako, Glostrup, Denmark) at RT in a humidified chamber for 1 h and rinsed in TBS (10 mM TBS, 150 mM NaCl, pH 7.6) for 3×5 min between each of the following incubations. Sections incubated in TBS/BSA without primary antibody served as negative control. All tissues were immunostained with HRP-conjugated goat-anti-rabbit IgGs (1:50; Dako) for 30 min. The label was visualized by incubation in 3,3-diaminobenzidine tetrahydrochloride in urea buffer for 5–12 min (Sigma–Aldrich). Positive staining appeared as a brown precipitate in the cells. All sections were counterstained in hematoxylin, mounted, and analyzed.

Statistical analysis

Comparisons between experimental groups were carried out using ANOVA, and in case of statistical significance, Tamahane was used for *post hoc* comparisons. Values were considered statistically significant with $P < 0.05$ and are presented as mean ± S.E.M. Data analysis was carried out utilizing SPSS for Windows (Version 15.0; SPSS Inc, Chicago, IL, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Abir R, Biron-Shental T, Orvieto R, Garor R, Krissi H & Fisch B 2009 Transplantation of frozen–thawed late-gestational-age human fetal ovaries into immunodeficient mice. *Fertility and Sterility* **92** 770–777. (doi:10.1016/j.fertnstert.2008.06.032)

- 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. *Biology of Reproduction* **67** 1493–1501. (doi:10.1095/biolreprod.102.005579)
- Anderson E & Albertini DF 1976 Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *Journal of Cell Biology* **71** 680–686. (doi:10.1083/jcb.71.2.680)
- Aubard Y 2003 Ovarian tissue xenografting. *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **108** 14–18. (doi:10.1016/S0301-2115(02)00424-4)
- 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°C . *Endocrinology* **140** 462–471. (doi:10.1210/en.140.1.462)
- Borges EN, Silva RC, Futino DO, Rocha-Junior CM, Amorim CA, Bao SN & Lucci CM 2009 Cryopreservation of swine ovarian tissue: effect of different cryoprotectants on the structural preservation of preantral follicle oocytes. *Cryobiology* **59** 195–200. (doi:10.1016/j.cryobiol.2009.07.003)
- Bosch P, Hernandez-Fonseca HJ, Miller DM, Wininger JD, Massey JB, Lamb SV & Brackett BG 2004 Development of antral follicles in cryopreserved cat ovarian tissue transplanted to immunodeficient mice. *Theriogenology* **61** 581–594. (doi:10.1016/S0093-691X(03)00244-9)
- Buse E, Zöller M & van Esch E 2008 The macaque ovary, with special reference to the cynomolgus macaque (*Macaca fascicularis*). *Toxicologic Pathology* **36** 24S–66S. (doi:10.1177/0192623308327407)
- Candy CJ, Wood MJ & Whittingham DG 1995 Follicular development in cryopreserved marmoset ovarian tissue after transplantation. *Human Reproduction* **10** 2334–2338.
- Candy CJ, Wood MJ & Whittingham DG 2000 Restoration of a normal reproductive lifespan after grafting of cryopreserved mouse ovaries. *Human Reproduction* **15** 1300–1304. (doi:10.1093/humrep/15.6.1300)
- Celestino JJ, dos Santos RR, Lopes CA, Martins FS, Matos MH, Melo MA, Bao SN, Rodrigues AP, Silva JR & de Figueiredo JR 2008 Preservation of bovine preantral follicle viability and ultra-structure after cooling and freezing of ovarian tissue. *Animal Reproduction Science* **108** 309–318. (doi:10.1016/j.anireprosci.2007.08.016)
- Demeestere I, Simon P, Emiliani S, Delbaere A & Englert Y 2007 Fertility preservation: successful transplantation of cryopreserved ovarian tissue in a young patient previously treated for Hodgkin's disease. *Oncologist* **12** 1437–1442. (doi:10.1634/theoncologist.12-12-1437)
- 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. *Fertility and Sterility* **77** 595–600. (doi:10.1016/S0015-0282(01)03205-8)
- Dolmans MM, Marinescu C, Saussoy P, Van Langendonck A, Amorim C & Donnez J 2010 Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood* **116** 2908–2914. (doi:10.1182/blood-2010-01-265751)
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B & van Langendonck A 2004 Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* **364** 1405–1410. (doi:10.1016/S0140-6736(04)17222-X)
- Ernst E, Bergholdt S, Jorgensen JS & Andersen CY 2010 The first woman to give birth to two children following transplantation of frozen/thawed ovarian tissue. *Human Reproduction* **25** 1280–1281. (doi:10.1093/humrep/deq033)
- Feigin E, Abir R, Fisch B, Kravarusic D, Steinberg R, Nitke S, Avrahami G, Ben-Haroush A & Freud E 2007 Laparoscopic ovarian tissue preservation in young patients at risk for ovarian failure as a result of chemotherapy/irradiation for primary malignancy. *Journal of Pediatric Surgery* **42** 862–864. (doi:10.1016/j.jpedsurg.2006.12.041)
- Gandolfi F, Paffoni A, Papasso Brambilla E, Bonetti S, Brevini TA & Ragni G 2006 Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. *Fertility and Sterility* **85** (Supplement 1) 1150–1156. (doi:10.1016/j.fertnstert.2005.08.062)
- Gook DA & Edgar DH 1999 Cryopreservation of the human female gamete: current and future issues. *Human Reproduction* **14** 2938–2940. (doi:10.1093/humrep/14.12.2938)
- Gook DA, Edgar DH & Stern C 1999 Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1,2-propanediol. *Human Reproduction* **14** 2061–2068. (doi:10.1093/humrep/14.8.2061)
- Gook DA, Edgar DH, Borg J, Archer J & McBain JC 2005 Diagnostic assessment of the developmental potential of human cryopreserved ovarian tissue from multiple patients using xenografting. *Human Reproduction* **20** 72–78. (doi:10.1093/humrep/deh550)
- Gougeon A 1986 Dynamics of follicular growth in the human: a model from preliminary results. *Human Reproduction* **1** 81–87.
- Gougeon A 1996 Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocrine Reviews* **17** 121–155. (doi:10.1210/edrv-17-2-121)
- Gougeon A & Busso D 2000 Morphologic and functional determinants of primordial and primary follicles in the monkey ovary. *Molecular and Cellular Endocrinology* **163** 33–42. (doi:10.1016/S0303-7207(00)00220-3)
- 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. *Human Reproduction* **11** 1268–1272.
- Hulshof SC, Figueiredo JR, Beckers JF, Bevers MM, van der Donk JA & 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. (doi:10.1016/0093-691X(95)00171-4)
- Jin S, Lei L, Shea LD, Zelinski MB, Stouffer RL & Woodruff TK 2010 Markers of growth and development in primate primordial follicles are preserved after slow cryopreservation. *Fertility and Sterility* **93** 2627–2632. (doi:10.1016/j.fertnstert.2009.11.029)
- Kuleshova LL, MacFarlane DR, Trounson AO & Shaw JM 1999 Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. *Cryobiology* **38** 119–130. (doi:10.1006/cryo.1999.2153)
- Lan C, Xiao W, Xiao-Hui D, Chun-Yan H & Hong-Ling Y 2010 Tissue culture before transplantation of frozen–thawed human fetal ovarian tissue into immunodeficient mice. *Fertility and Sterility* **93** 913–919. (doi:10.1016/j.fertnstert.2008.10.020)
- Laschke MW, Menger MD & Vollmar B 2002 Ovariectomy improves neovascularization and microcirculation of freely transplanted ovarian follicles. *Journal of Endocrinology* **172** 535–544. (doi:10.1677/joe.0.1720535)
- Liu J, Van der Elst J, Van den Broecke R & Dhont M 2002 Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. *Human Reproduction* **17** 605–611. (doi:10.1093/humrep/17.3.605)
- Lucci CM, Kacinskis MA, Lopes LH, 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. (doi:10.1016/j.theriogenology.2003.06.004)
- Maltaris T, Beckmann MW, Mueller A, Hoffmann I, Kohl J & Dittrich R 2007 Significant loss of primordial follicles after prolonged gonadotropin stimulation in xenografts of cryopreserved human ovarian tissue in severe combined immunodeficient mice. *Fertility and Sterility* **87** 195–197. (doi:10.1016/j.fertnstert.2006.05.058)
- Meirow D 2000 Reproduction post-chemotherapy in young cancer patients. *Molecular and Cellular Endocrinology* **169** 123–131. (doi:10.1016/S0303-7207(00)00365-8)
- Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, Schiff E & Dor J 2005 Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. *New England Journal of Medicine* **353** 318–321. (doi:10.1056/NEJMc055237)
- Mussett MV & Parrott DM 1961 Factors affecting the fertility of mice with orthotopic ovarian grafts. *Journal of Reproduction and Fertility* **2** 80–97. (doi:10.1530/jrf.0.0020080)
- Navarro-Costa P, Correia SC, Gouveia-Oliveira A, Negreiros F, Jorge S, Cidadao AJ, Carvalho MJ & Plancha CE 2005 Effects of mouse ovarian tissue cryopreservation on granulosa cell–oocyte interaction. *Human Reproduction* **20** 1607–1614. (doi:10.1093/humrep/deh787)
- Newton H, Aubard Y, Rutherford A, Sharma V & Gosden R 1996 Low temperature storage and grafting of human ovarian tissue. *Human Reproduction* **11** 1487–1491.

- Newton H, Fisher J, Arnold JR, Pegg DE, Faddy MJ & Gosden RG 1998 Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. *Human Reproduction* **13** 376–380. (doi:10.1093/humrep/13.2.376)
- Nilsson EE & Skinner MK 2003 Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction* **69** 1265–1272. (doi:10.1095/biolreprod.103.018671)
- Nisolle M, Casanas-Roux F, Qu J, Motta P & Donnez J 2000 Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. *Fertility and Sterility* **74** 122–129. (doi:10.1016/S0015-0282(00)00548-3)
- Parrott DM 1960 The effect of site of implantation on host reaction. *Immunology* **3** 244–253.
- 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. (doi:10.1006/cryo.1999.2170)
- Rosendahl M, Andersen CY, Ernst E, Westergaard LG, Rasmussen PE, Loft A & Andersen AN 2008 Ovarian function after removal of an entire ovary for cryopreservation of pieces of cortex prior to gonadotoxic treatment: a follow-up study. *Human Reproduction* **23** 2475–2483. (doi:10.1093/humrep/den248)
- Roux C, Amiot C, Agnani G, Aubard Y, Rohrlisch PS & Piver P 2010 Live birth after ovarian tissue autograft in a patient with sickle cell disease treated by allogeneic bone marrow transplantation. *Fertility and Sterility* **93** 2413 e2415–e2419. (doi:10.1016/j.fertnstert.2009.12.022)
- 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. *Fertility and Sterility* **77** 403–408. (doi:10.1016/S0015-0282(01)02960-0)
- Salle B, Demirci B, Franck M, Berthollet C & Lornage J 2003 Long-term follow-up of cryopreserved hemi-ovary autografts in ewes: pregnancies, births, and histologic assessment. *Fertility and Sterility* **80** 172–177. (doi:10.1016/S0015-0282(03)00554-5)
- Sanchez-Serrano M, Crespo J, Mirabet V, Cobo AC, Escriba MJ, Simon C & Pellicer A 2010 Twins born after transplantation of ovarian cortical tissue and oocyte vitrification. *Fertility and Sterility* **93** 268 e211–e263. (doi:10.1016/j.fertnstert.2009.09.046)
- Santos RR, Rodrigues AP, Costa SH, Silva JR, Matos MH, Lucci CM, Bao SN, van den Hurk R & Figueiredo JR 2006a Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. *Animal Reproduction Science* **91** 249–263. (doi:10.1016/j.anireprosci.2005.04.013)
- Santos RR, Tharasanit T, Figueiredo JR, van Haefen T & van den Hurk R 2006b Preservation of caprine preantral follicle viability after cryopreservation in sucrose and ethylene glycol. *Cell Tissue Research* **325** 523–531. (doi:10.1007/s00441-006-0193-5)
- Shaw JM, Cox SL, Trounson AO & Jenkin G 2000 Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. *Molecular and Cellular Endocrinology* **161** 103–110. (doi:10.1016/S0303-7207(99)00230-0)
- Silber SJ, DeRosa M, Pineda J, Lenahan K, Grenia D, Gorman K & Gosden RG 2008 A series of monozygotic twins discordant for ovarian failure: ovary transplantation (cortical versus microvascular) and cryopreservation. *Human Reproduction* **23** 1531–1537. (doi:10.1093/humrep/den032)
- Silber S, Kagawa N, Kuwayama M & Gosden R 2010 Duration of fertility after fresh and frozen ovary transplantation. *Fertility and Sterility* **94** 2191–2196. (doi:10.1016/j.fertnstert.2009.12.073)
- Skinner MK 2005 Regulation of primordial follicle assembly and development. *Human Reproduction Update* **11** 461–471. (doi:10.1093/humupd/dmi020)
- Sztejn J, Sweet H, Farley J & Mobraaten L 1998 Cryopreservation and orthotopic transplantation of mouse ovaries: new approach in gamete banking. *Biology of Reproduction* **58** 1071–1074. (doi:10.1095/biolreprod58.4.1071)
- Van den Broecke R, Liu J, Handyside A, Van der Elst JC, Krausz T, Dhont M, Winston RM & Hovatta O 2001 Follicular growth in fresh and cryopreserved human ovarian cortical grafts transplanted to immunodeficient mice. *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **97** 193–201. (doi:10.1016/S0301-2115(00)00507-8)
- Van Eyck AS, Jordan BF, Gallez B, Heilier JF, Van Langendonck A & Donnez J 2009 Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting. *Fertility and Sterility* **92** 374–381. (doi:10.1016/j.fertnstert.2008.05.012)
- Wang H, Mooney S, Wen Y, Behr B & Polan ML 2002 Follicle development in grafted mouse ovaries after cryopreservation and subcutaneous transplantation. *American Journal of Obstetrics and Gynecology* **187** 370–374. (doi:10.1067/mob.2002.123606)
- von Wolff M, Donnez J, Hovatta O, Keros V, Maltaris T, Montag M, Salle B, Sonmezer M & Andersen CY 2009 Cryopreservation and autotransplantation of human ovarian tissue prior to cytotoxic therapy – a technique in its infancy but already successful in fertility preservation. *European Journal of Cancer* **45** 1547–1553. (doi:10.1016/j.ejca.2009.01.029)

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