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Viability of human ovarian tissue confirmed 5 years after freezing with spontaneous ice-formation by autografting and chorio-allantoic membrane culture ☆

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ABSTRACT

To achieve optimal and uniform outcomes, slow cooling protocols for human ovarian tissues generally initiate ice formation at high sub-zero temperatures (−6 to −9 °C). The aim of the study was to investigate the function of ovarian tissue that had unintentionally self seeded at −20 °C during the freezing step, by examining its development following chicken embryonic chorioallantoic membrane (CAM) grafting and after transplantation back to the patient. Ovarian tissue was frozen in 6% (v/v) dimethyl sulfoxide, 6% (v/v) ethylene glycol and 0.15 M sucrose which had self-seeded at −20 °C. Five years after cryopreservation, 8 pieces were thawed and transplanted back to the patient. Two small (1 × 2 × 1 mm) pieces of this thawed tissue were cultured in a CAM-system for 5 days to assess the tissue viability. The autografted ovarian tissue re-established spontaneous menstrual bleeding within five months and raised serum 17-β Estradiol from 19 to 330 pg/ml. Ultrasound revealed a dominant follicle at the site of the transplanted tissue in the follicular phase after the menstrual bleed. Analysis of the CAM cultured tissue established that 88% of the primordial follicles are degenerated and there was limited in growth of blood vessels. In conclusion, in spite of the damage caused by the cryopreservation with spontaneous ice-formation the viability could be confirmed by CAM culture and the restoration of ovarian function after auto-transplantation.

Introduction

Cancer is a major public health problem in the world. Currently, in the United States alone one in three women will develop cancer in her lifetime [38]. Cancer is also one of the major death causes: in the USA alone a total of 1,638,910 new cancer cases and 577,190 deaths from cancer are projected to occur in 2012 [39].

Childhood cancers (from birth to age 14 years) are rare, representing less than 1% of all new cancer diagnoses, but they are the second leading cause of death in children, exceeded only by accidents. It is estimated that there are 58,510 survivors of childhood cancer living in the United States, and an additional 12,060 children will be diagnosed in 2012 [38].

However, due to the increasing of effectiveness of anti-cancer therapy, in USA alone the overall 5-year relative survival rate for childhood cancer has improved markedly over the past three decades, from 58.1% for cases diagnosed from 1975 to 1977 to 82.5% for diagnoses during 2001–2007, due to new and improved treatments [38].

Cancers in patients of reproductive ages are treated with a combination of therapies (surgery, radiation, and chemotherapy) chosen based on the type and stage of cancer [38]. The problem of post-cancer infertility is playing an increasingly significant role because chemotherapy can be gonadotoxic and lead to the functional death of ovaries [28,29,43,44].

Cryopreservation of ovarian tissue before cancer therapy with re-implantation after convalescence is one potential key solution of this problem [5,9,43,44].

Several cases of restored ovarian function after implantation of cryopreserved ovarian cortex in patients with premature ovarian failure after cancer treatment have been published since 1998 and now live birth after thawing and transplantation is reality [1,4,6–8,10,11,28–31,33–37,40].

Investigations show that for optimal cryopreservation of human ovarian tissue, slow cooling protocols should include a step of initiated ice formation (manual or automated seeding) and not rely on spontaneous ("self") seeding [12].

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Human ovarian tissue can be cultured on the embryonic chorio-allantoic membrane (CAM) of hen's eggs [13,27].

The CAM system is an intermediate stage between in vitro culture and animal experiments, and it could be considered as an interface between in vitro and in vivo models (including xeno-transplantation). Importantly, it does not raise ethical or legal questions, nor does it violate animal protection laws.

The aim of the study was to investigate the health of thawed ovarian tissue that was frozen with spontaneous ice formation by examining its development following CAM grafting and after transplantation back to the patient.

Materials and methods

The protocol of investigations was approved by the Ethics Board of Cologne University.

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Tissue collection and dissection

Patient S. was born in 1983, developed a Ewing-Sarcoma and obtained low dose chemotherapy in 1996 (aged 13). In 2007 (aged 24) this Patient S. was scheduled to receive a high-dose chemotherapy because lung-metastases were diagnosed.

In January 2007, before starting of chemotherapy, this Patient S. underwent laparoscopic removal and cryopreservation of ovarian tissue in the Cologne University Maternal Hospital.

Approximately 25% of the ovarian tissue from both ovaries was removed and cryopreserved.

Informed written consent for performing investigations was obtained before collection of ovaries. According to this protocol 10% of ovarian tissue was used for patient-oriented research. For transportation of ovarian tissue from surgical section, preparation of ovarian strips, freezing and thawing one medium, referred to below as 'basal medium', was used: Leibovitz L-15 with 5% Serum Substitute Supplement (Irvine Scientific, Santa Ana, CA, USA).

Following the surgical operation (04.01.2007) the fresh ovarian tissue fragments were transported to the laboratory within 20 min with the temperature maintained at 32–34 °C. Using tweezers and scalpel no. 22, ovarian fragments were dissected and divided into 20 pieces derived from both ovaries (4–5 × 2–3 × 0.8–1 mm) (length × width × depth) and frozen as described below.

Freezing and thawing

Cooling regime of the protocol for cryopreservation of ovarian tissue was based on an embryo cryopreservation protocol [42].

The 20 pieces were each equilibrated in one step in 20 ml of freezing basal medium supplemented with 6% (v/v) dimethyl sulfoxide, 6% (v/v) ethylene glycol and 0.15 M sucrose for 20 min. at room temperature. After equilibration the pieces were transferred to ten 2 ml cryovials (Nunc, Roskilde, Denmark) (2 pieces in each vial) filled with 1.8 ml of fresh cryopreservation medium at room temperature and then placed in an IceCube 14S freezer (SyLab, Neupurkersdorf, Austria), with the freezing chamber previously stabilized at −6 °C for 10 min.

The cryopreservation programme was as follows: (i) the starting temperature was −6 °C; (ii) samples were cooled from −6 °C to −34 °C at a rate of −0.3 °C/min; (iii) at −34 °C cryo-vials were plunged into liquid nitrogen. The freezing protocol for cryopreservation of this ovarian tissue included an autoseeding step at −6 °C. However, the agitation to trigger the ice formation was not performed correctly and the cooling ramp commenced without ice formation. One temperature probe monitored the chamber temperature and a second probe was inserted in a dummy cryo-vial without ovarian tissue (Fig. 2).

The procedure of thawing was achieved by holding the cryo-vials for 30 s at room temperature followed by immersion in a 100 °C (boiling) water bath for 65 s.

The exposure time of the cryo-vials to the boiling water was visually controlled by monitoring the amount of ice left in the vial. As soon as the ice at the center of the vial had shrunk to 1–2 mm, the cryo-vial was removed from the boiling water, at which point the temperature of the medium was between +4 and +10 °C. Within 5–10 s after thawing, the all pieces from the cryo-vials were expelled into 10 ml thawing solution ("basal medium"containing 0.5 M sucrose) in one single 100 ml specimen container (Sarstedt, Nuembrecht, Germany). The container was placed on a shaker and continuously agitated with 200 osc/min for 15 min at room temperature.

The procedure of stepwise dilution of cryoprotectants was realised by drop-wise addition of 1.6 ml/min basal medium for a further 30 min at room temperature (see Fig. 1 in Isachenko et al. [14]). The final sucrose concentration was 0.083 M, equivalent to almost isotonic conditions. Finally, the pieces were washed thrice each in basal medium for 10 min, and used for transplantation and CAM-culture.

CAM-culture

Obtaining of permission from the Ethics Board for use of CAM-Xenotransplantation methodology is not presupposed.

Fertilized, newly laid, White Leghorn chicken eggs, were purchased from a local hatchery and incubated at 37 °C in air with 60% relative humidity. These were prepared for implantation on day 4 of incubation. Standard microbiology assessment was performed to exclude subclinical infections. Preparation of the chorio-allantoic membranes was performed essentially as previously described [2,13,18]. Each egg was swabbed with warm 70% ethanol, and a hole then drilled through the pointed pole of the shell. On day 5 the hole was widened into a 1.5–2.0 cm window in the shell. This window was covered with tape until day 10 when forceps were used to remove the outer, peridermal, layer of the CAM and to place a 0.5 mm high, 5 mm inner diameter, 6 mm outer diameter, silicone ring on the intact, inner, vascularized CAM basal layer. An ovarian piece was placed within this ring (Fig. 1) and the shell window then covered again. The two eggs were then incubated for a further 5 days at 37 °C.

Histology of follicles

For histological investigation, one fresh piece collected in 2007, and about half of the CAM cultured tissue was fixed in Bouin's solution, embedded in paraffin wax, serially sectioned at 4 μm, stained with hematoxylin/eosin, and analyzed under a microscope (×6400, Olympus Co., Tokyo, Japan). Every fifth section was analyzed. The number of viable and degenerated follicles was counted.

Ovarian tissue pieces were sectioned, coded and scored blind. To avoid counting of the same follicles, only the section with a visible oocyte nucleus was counted.

The morphology of the primordial, primary and secondary follicles was evaluated using criteria described by Paynter et al. [32]. The primordial follicles had an oocyte surrounded by a single layer of flattened follicular cells while the primary and secondary follicles had an oocyte surrounded by one to two layers of spherical granulosa cells. The quality of the follicles was graded on a scale from one to three. A grade 1 follicle was spherical in shape and contained a spherical oocyte surrounded by evenly distributed granulosa cells. The oocyte had to have homogenous cytoplasm and slightly granulated nucleus, with condensed chromatin