Article

Comparison of necrosis in human ovarian tissue after conventional slow freezing or vitrification and transplantation in ovariectomized SCID mice

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Abstract

This paper examines and compares necrosis in human ovarian tissue after conventional slow freezing or vitrification and ensuing xenotransplantation. Slow cryoconserved or vitrified ovarian tissue samples and fresh controls from nine patients were subcutaneously transplanted into SCID mice. The tissue samples were explanted after 6 weeks and the necrotic areas were examined by staining with Lucifer yellow SV. The size of the necrotic areas in parallel cultivated ovarian tissue samples was compared, as was necrosis in cultivated prostate tumour spheroids where the emergence of necrosis and its pathophysiological correlation have been described. Examinations showed no significant rise in the proportion of necrotic areas after slow cryoconservation/transplantation and in the controls (transplanted fresh tissue, not transplanted fresh tissue, long-term culture). The proportion of necrotic areas in the tumour spheroids was significantly higher than in the ovarian tissue. Vitrification could, after these results, be presented as an alternative to conventional slow cryoconservation.

Keywords: cryopreservation, necrosis, ovarian transplant, ovary, vitrification

Introduction

Ovarian tissue banking offers hope to cancer patients who want to safeguard their fertility against the sterilizing effects of chemotherapy and radiotherapy. In addition, a growing number of women want to preserve their fertility for childbearing later in life.

Animal studies have begun to accumulate within the past decade to show the feasibility of ovarian tissue banking and transplantation (Newton et al., 1996; Oktay et al., 1998a, 2000; Gosden, 2000; Gook et al., 2001). In addition, for preserving male germ cells to restore fertile potential, testicular biopsies that have been cryopreserved can be grafted subcutaneously to generate enough spermatozoa for intracytoplasmic sperm injection (Gosden, 2002). While many of the problems associated with slow cryoconservation or vitrification seem to have been solved for cells, there is still an enormous need for clarification in regard to the slow cryoconservation or vitrification of human ovarian tissue and its optimal use after thawing.

It is now known that both after conventional cryocConservation and after vitrification, it is most probable that vital tissue remains preserved. The optimal use of this tissue is still unclear. Autotransplantation to date has led only to temporary follicle maturation or hormone synthesis (Salle et al., 1999).

Many studies have used human xenografts in immunodeficient hypogonadotrophic or ovariectomized mice. These mice carry a mutation that renders them deficient in both T-cell and B-cell function (Bosma et al., 1983), which results in the inability to reject xenografts. It
has been shown that frozen-thawed human ovarian tissue retained >50% of its primordial follicle reserve after 18 days of grafting in severe combined immunodeficiency (SCID) mice (Newton et al., 1996). Most investigators have also confirmed the high viability of human primordial follicles after the slow freeze–rapid thaw protocol (Hovatta et al., 1996).

Most of these observations are based on programmed slow cryoconservation. At the moment, there is little experience with vitrification of human ovarian tissue (Lee et al., 2000; Isachenko et al., 2002, 2003), a procedure in which the cells or tissue are plunged directly into liquid nitrogen, saving both time and cost. Many tests on vitrification, especially on single cells, have left the future significance of vitrification unclear (Liebermann et al., 2002).

This study examines the proportion of necrotic areas in human ovarian tissue after vitrification or slow cryoconservation and subsequent transplantation in SCID mice. With every trial, a parallel necrotic area was measured in freshly transplanted ovarian tissue, in fresh ovarian tissue and ovarian tissue from long-term culture and in tumour spheroids (Wartenberg et al., 2001).

Materials and methods

Specimens

The study was approved by the Ethics Board of the University of Cologne, Germany. Informed consent was given by nine patients aged between 32 and 39 (36.6 ± 2.2) years, with a protocol approved by the Ethics Board of the University of Cologne.

Ovarian biopsies were obtained during operative laparoscopy and uniformly smooth areas of cortex fragments were recovered by antimesenteric dissection of ovarian pieces (about 4 x 4 x 2 mm). Ovarian tissue was transported at room temperature in Dulbecco phosphate-buffered saline (DPBS) with 5% fetal calf serum (FCS).

Chemicals were obtained from Sigma (Deisenhofen, Germany) and dissecting media was purchased from Gibco (Life Technologies Ltd, Berlin, Germany). DPBS was used as a basic medium for the preparation of all solutions (cryopreservation, thawing, warming).

Preparation of tissue pieces, slow cooling/thawing and vitrification/warming

Ovarian tissue pieces were cut on to strips (OTS) under a stereomicroscope with an approximate dimension of 0.5 x 1.0 x 4.0 mm using a no. 22 scalpel and tweezers. Five OTS were produced from each biopsy (totally 105 OTS) that were randomly distributed for treatment and were cryopreserved, frozen, served as controls, non-cryopreserved OTS with and without transplantation, or for long-term culture. Dissection and treatment of ovarian cortex before cryopreservation/freezing was performed in DPBS with 5% FCS at room temperature and processed within 60 min. Vitrification (group A) of OTS was performed according to a standard protocol (Isachenko et al., 2002) in three steps. Solutions for vitrification were prepared in DPBS medium supplemented with 15% FCS. The first step involved exposure of OTS to 10% glycerol + 20% ethylene glycol for 15 min. Next, the OTS were exposed to 25% glycerol + 25% ethylene glycol for 15 min. Lastly, the OTS were exposed to 25% glycerol + 25% ethylene glycol + 1% Supercool® X-100 (Cooltechnica, New York, USA) for 30–40 s with subsequent plunging into liquid nitrogen. Exposure to each vitrification solution was accompanied by shaking at 5 Hz at room temperature.

Solutions for slow freezing (group B) were supplemented with 30 mg/ml HSA (human serum albumin; IVF Science Scandinavia, Gothenburg, Sweden). Freezing was performed in three steps: 15 min exposure in DPBS with HSA, 20 min exposure in 1.5 mol/l ethylene glycol, and finally, 35 min exposure in 1.5 mol/l ethylene glycol with 0.1 mol/l sucrose. Exposure to vitrification solutions was accompanied by shaking at 5 Hz at room temperature. After placing in straws, the OTS were put into a programmable freezer (IceCube 1810CD; Neuperkersdorf, Austria) and frozen according the following protocol: from room temperature to −6°C at 2°C/min; at −6°C holding for 15 min (during the time seeding was performed); then from −6°C to −35°C cooling was performed at 0.3°C/min; at −35°C holding for 10 min; from −35°C to −130°C cooling was performed at 20°C/min, then samples were immersed into liquid nitrogen.

For slow freezing of OTS, standard insemination straws (0.5 ml; IMV, L’Aigle, France) were used. Straws were partially filled with a 1 cm column of cryoprotectant medium containing OTS (1–2 strips per straw), then sealed with a plastic plug. Straws were stored in liquid nitrogen for at least 1 month before being thawed/warmed.

Warming after slow cooling was achieved by holding the straw in the air for 6 s at room temperature, followed by immersion in a 40°C water bath and gently agitated until the ice melted. The cryoprotectant was removed immediately after thawing/warming by expelling the content of the straws into 1 ml of dilution medium. Immediately after thawing, the OTS were transferred into 1 ml of the same fresh dilution medium with subsequent step-wise dilution at room temperature by successive exposure to 0.5 mol/l, 0.25 mol/l, 0.15 mol/l sucrose solutions for 10, 10, and 10 min respectively with gentle agitation. Finally, the ovarian pieces were washed three times in DPBS supplemented with 15% FCS over 10 min for each step.

Thawing after slow freezing was achieved by holding the straw for 20 s at room temperature in air, followed by immersion in a 40°C water bath and gentle agitation until the ice melted. The cryoprotectant dilution was performed in three steps: first, 1.0 mol/l ethylene glycol with 0.2 mol/l sucrose for 10 min; second: 0.5 mol/l ethylene glycol with 0.2 mol/l sucrose for 10 min; third: 0.2 mol/l sucrose in phosphate-buffered saline (PBS)-HSA for 10 min, then strips were washed three times (10 min each time) in DPBS-HSA.
Each block, including the control, was then grafted under the skin of an ovarectomized SCID mouse (6 weeks old).

Ovarectomies were performed using the dorsolateral approach with a combination of anaesthetics (Rompun 2%; Bayer Vetal, Leverkusen, Germany) and Ketanest 50 mg/ml (Parke-Davis, Freiburg, Germany). After surgery, the animals were observed daily and showed no signs of local irritation or abnormal behaviour.

The animals were killed 6 weeks later. Grafts were recovered and stored in DPBS (three grafts were not recovered). After measurement of necrosis in the grafts (control group and cryopreserved ovarian tissue), ovarian tissue from long-term culture and also fresh ovarian tissue, the tissue pieces were fixed in Bouen solution and prepared for measurement of lethal cells.

**Culture technique for human ovarian tissue**

Ovarian tissue pieces from each patient were transferred to 24-well multiwell plates (Falcon, Heidelberg, Germany). One to three slices were placed in each well. The pieces were cultivated in Dulbecco’s modified Eagle’s medium (Gibco BRL, Life Technologies, Karlsruhe, Germany) supplemented with 15% FCS (Boehringer, Mannheim, Germany), L-glutamine (2 mmol/l) (Gibco BRL), β-mercapto-ethanol (final concentration 5 × 10⁻⁵ mol/l) (Sigma), non-essential amino acids (Gibco BRL; stock solution diluted 1:100), 100 IU per ml of penicillin, 0.1 mg per ml of streptomycin (Gibco BRL) and 300 mIU/ml human recombinant FSH (recFSH; Gonal F®, Serono, Unterschleissheim, Germany) in a humidified incubator containing 5% CO₂ in air at 37°C. The medium was changed every 48 h. After 6 weeks, the necrotic areas were measured and the tissue pieces were then fixed in Bouen solution.

**Culture technique for multicellular tumour spheroids (MTS)**

The human prostate cancer cell line DU-145 was used for this experiment. The cell line was grown in 5% CO₂/humidified air at 37°C with Ham’s F10 medium (Gibco BRL) supplemented with 10% FCS (Boehringer), 100 IU/ml penicillin and 100 μg/ml streptomycin (ICN Flow, Meckenheim, Germany), 100 μmol/l β-mercaptoethanol (Gibco) and 2 mmol/l Glutamax-1 (Gibco). Spheroids were grown from single cells. Cell monolayers were dissociated with 0.2% trypsin 0.05% EDTA, and seeded in 250 ml spinner flasks (Technomara, Fernwald, Germany) with 250 ml complete medium and agitated at 20 rpm using a cellspin stirrer system (Integra Biosciences, Fernwald, Germany). A 100 ml aliquot of the cell culture medium was changed every day.

**Measurement of necrosis**

For this study, the hydrophilic, membrane-impermeable tracer Lucifer Yellow Versus (LYVS; Sigma) with a fluorescent stimulus maximum of 450 nm (Stewart, 1981) was used. LYVS is tied by proteins in the necrotic cell areas because of its excellent affinity to SH groups. Through the comparison of measurements with confocal microscopy and the histological strips on LYVS coloured multicellular spheroids, the colouring could be characterized as a specific marker for necrotic cell areas (Gassmann et al., 1995; Wartenberg and Acker, 1996).

The OTS (after explantation, fresh tissue and those from the long-term culture) and the MTS were suspended in F-10 medium containing LYVS in a final concentration of 20 μmol/l for 40 min. The samples were washed eight times with F10 medium and stored in the same medium for 24 h in an incubator, in order to remove the absorbed colour around the endocytosis in the cells.

Necrotic areas (reversible and irreversible membrane breakdown) were evaluated by computer-assisted image analysis of LYVS stained tissue areas. Since tissues were of different size, data were given as percent fluorescent necrotic area, which was calculated from the area of stained necrosis above background level in relation to the area of unstained viable tissue. Background fluorescence was analysed in separate experiments with unstained tissues. Excitation was performed using the 488 nm line of the argon-ion confocal laser. Emission was recorded using long pass LP515-nm filter setup I.

**Measurement of lethal cells**

Imaging of lethal tissue cells was monitored using the fluorescent dye SYTOX Green Nucleic Acid Stain (Molecular Probes, Eugene, OR, USA). Short incubation with SYTOX Green stains the nucleic acids of dead cells. Fixed tissue was embedded in paraffin wax, and serially sectioned at 6 μm. The sections were deparaffinized and rehydrated. After washing, they were loaded for 5 min with 1 μmol/l SYTOX Green. After loading, the sections were washed three times in PBS. For fluorescence excitation, the 488 nm line of an argon ion confocal laser was used. Emission was recorded with a 515 nm long pass filter. The portion of pyknotic cells was counted in 160 × 160 μm² regions of interest in different areas of every sample.

**Statistical analysis**

Data are given as mean values ± SEM, with n denoting the number of experiments performed with different ovarian tissue cultures. In each experiment, at least three to five pieces of ovarian tissue from each woman were examined. Each experiment was repeated at least three times. Student’s t-test for unpaired data was applied as appropriate. A value of P < 0.05 was considered significant.

**Results**

Examination of the necrotic areas of the ovarian tissue samples after slow cryoconservation or vitrification and transplantation into SCID mice showed no significant rise in comparison with the control group. In avascular tissues, necrosis development was related to gradients in oxygen, nutrients and pH as well as accumulation of catabolic end products, which in turn are related to tissue volume. To compare necrotic areas in ovarian tissue samples with...
Figure 1. Comparison of necrotic areas in the different group of ovarian tissue pieces after cryopreservation and transplantation, in transplanted ovarian tissue without cryopreservation, in fresh tissue and tissue from long-term culture.

Figure 2. Detection of necrotic areas with Lucifer yellow SV staining. (a) Prostate tumour spheroid. (b) Human ovarian tissue after 6 weeks' culture. (c) Control (fresh tissue). (d) fresh grafted tissue. (e) Slow protocol. (f) Vitrification. Bar = 200 μm.

Figure 3. Determination of lethal cells showed no significant deviation from fresh ovarian tissue. After killing the tissue by heating to 60°C, the portion of lethal cells was 10 times higher ($P < 0.05$, data not shown).

Figure 4. Percentage of pyknotic cells. The tissue pieces were loaded with SYTOX Green. (a) Fresh ovarian tissue. (b) Pyknotic cells in human ovarian tissue after killing the cells ($* P < 0.05$). The pyknotic cells are round in form and show an increase in fluorescence. Bar = 20 μm.
necrosis in a typical multicellular avascular tissue, multicellular tumour spheroids were analysed for the occurrence of central necrosis. The share of necrotic areas in tumour spheroids was significantly higher than in ovarian tissue, and was associated with central necrosis. The development of necrosis depending on the size of the DU-145 MTS showed that up to a critical diameter of 350 μm in the MTS, no necrosis could be proven. In the centre of the tissue, necrotic cell groups then develop which can be recognized by binding of LYVS in these areas. With further growth, a large central round delimitned necrosis is formed against the surrounding tissue (Wartenberg et al., 2001). The catalyst for the development of necrosis has, to date, not been completely explained.

The apparent necrotic areas in ovarian tissue show limited size and uneven division. Figure 1 shows parts of the necrotic areas in the fresh preparation, in ovarian tissue after long-term cultivation, in the cryoconserved ovarian tissue after thawing and later transplantation, and in controls (fresh tissue after transplantation), in comparison with necrosis in the tumour spheroids. Figure 2 shows the marked necrotic areas after treatment with LYVS.

To evaluate whether during vitrification and transplantation (in culture from ovarian tissue, an increase in lethal cells can also be observed), the ovarian tissue was loaded with SYTOX Green nucleic acid stain (1 μmol/l), which binds with high affinity to the triplex structure of nucleic acids. Determination of the lethal cells in cryopreserved ovarian tissue and ovarian tissue from culture showed no significant deviation from observations in fresh ovarian tissue (Figure 3). Under the same experimental conditions, the number of lethal cells (as a percentage) was 3.08 ± 1.25% in fresh ovarian tissue, 5.2 ± 1.5% after 6 weeks of culture and 3.6 ± 1.1% after vitrification, 3.8 ± 1.6% in the slow cryopreservation group, and 2.8 ± 0.9% in freshly grafted tissue (n = 3 for each experimental condition). The lethal cells have a round shape and show increased fluorescence (Figures 4 and 5).

**Discussion**

It has been shown in different protocols (slow freezing, vitrification) that primordial follicles were intact after thawing/warming (Isachenko et al., 2003). There is now a large amount of evidence from human studies that primordial follicles retain viability in tissue when the slow freezing–thawing protocol is used. Transplantation studies have also shown that these follicles retain the capacity to develop to antral stages and even result in pregnancies in laboratory and farm animals (Parkes and Smith, 1952; Parrot, 1960; Gosden et al., 1994; Candy et al., 1995; Oktay et al., 1998b; Salle et al., 2002). Follicle presence and maturation was also shown in the transplanted ovarian tissue following both cryopreservation methods (data are not shown).

There are also studies that show follicle maturation after transplantation. It has already been shown that primordial follicles in fresh grafts can be stimulated to grow to the antral stage when FSH was administered to SCID mice (Oktay et al., 1998b). The timing of FSH stimulation may be important for optimal follicular development in cryopreserved human ovarian grafts. The effect of stimulation with FSH and the progression from the primordial to growing primary and secondary stages is most significant in the 14-week interval after grafting (Van den Broecke et al., 2002).

It is important to optimize freezing methods in order to obtain a better tissue quality (vitality, growth capacity, hormone production, and follicle differentiation) for successful transplantation. Most work in this area has been concerned mainly with follicle differentiation, oocyte morphology, development and fertility after slow cryoconservation (Candy et al., 1995; Wood et al., 1997; Gunasena et al., 1998; Campbell et al., 2000; Gook et al., 2001). In this work, the vitrification of human ovarian tissue was examined and considered as a possible alternative to conventional slow cryoconservation. Based on the size of the necrotic areas after transplantation of human ovarian tissue in ovarectomized SCID mice, tissue vitality was compared. The tests showed that the share of necrotic areas did not significantly differ after slow cryoconservation and vitrification. A slight but not significant raise in the necrotic areas and lethal cells occurred in the cultivated ovarian tissue probes over the 6 weeks.

In order to examine the pathophysiology of necrosis in ovarian tissue, necrosis in ovarian tissue was compared...
with necrosis in MTS. The measurements were performed in parallel. The cause of necrosis development is as yet not completely explained. Oxygen profiles measured with oxygen microelectrodes in small MTS, large MTS without necrosis and large MTS with central necrosis describe a large drop in the central oxygen partial pressure with increasing size of the spheroids The oxygen partial pressure on the surface of the MTS declines with the size of the spheroids (Wartenberg et al., 2001). Both the central and the peripheral oxygen partial pressure have an effect on cell proliferation and gene expression (HIF-1α and VEGF) (Wartenberg et al., 2003). It is suspected that the minimally proven necrosis in transplanted ovarian tissue, and especially in culture with a drop in the oxygen partial pressure, are related, which should be resolved by the neovascularization in the transplanted tissue. This could be possible with diffusion in smaller cultivated tissue samples. As neovascularization of the implanted ovarian tissue in the physical environment of the recipient is important for survival, tests are being carried out on the connection of the implanted tissue in the organism through the blood vessels. Unsuccessful neovascularization causes inadequate nutrient and oxygen supply to the implanted tissue and then tissue necrosis or apoptosis ensues. The insignificant amounts of lethal cells and necrotic areas in this study are signs that tissue of this size is sufficiently supplied in regard to nutrients and oxygen.

It has already been shown that human ovarian tissue shows good vitality and growth after 6 weeks’ cultivation (Rahimi et al., 2000), but because of the still existing suboptimal culture conditions, they can only be cultivated for a limited time. Cryopreservation or vitrification allows, in contrast, a longer storage time and the possibility of later use when required. It could be shown in this study that in regard to necrosis and cell vitality, cryopreservation (even though not significantly) delivers better results.

Planned subsequent examinations will concentrate on angiogenesis and the origin of endothelial cells in the transplanted ovarian tissue. Any effective method of cryopreservation must provide for the storage of cells after cooling (hypothermal anabiosis) and development after thawing/warming. Researchers have artificially classified cryopreservation of cells in liquid nitrogen in different cooling (hypothermal anabiosis) and development after thawing/warming. Researchers have artificially classified cryopreservation of cells in liquid nitrogen in different modes: ‘slow’ classical or equilibration freezing, rapid or ultrarapid or quick freezing as well as vitrification are completely equivalent methods of preserving cells in a ‘resting’ but viable state.

Based on existing publications and the reported examinations, it is expected that vitrification of human ovarian tissue will become a viable alternative to slow programmed cryopreservation. The reasons for this lie in the immense saving in cost and time. Further clinical studies on a larger patient group are necessary in order to evaluate the long-term survival of the transplants, long-term hormone synthesis and follicle maturation, the possibility and the progress of the pregnancies and the safety of autotransplants in malignant illnesses (Oktay and Karlikaya, 2000; Oktay and Buyuk, 2002; Oktay and Yih, 2002).

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