Abstract. The number of follicles were compared in different developmental stages after the cryopreservation of human ovarian tissue by open freezing system followed by xenotransplantation into severe combined immunodeficient (SCID)-mice under stimulation, with and without ovariectomy. Ovarian tissue, cryopreserved for fertility preservation was partly examined by LIVE/DEAD viability staining or was transplanted in the neck muscle of 32 SCID-mice. The development of follicles, estradiol production, vaginal cytology and uterus weight was assessed after 15 weeks under gonadotropin stimulation, with or without ovariectomy. Viable follicles were detected in all frozen/thawed specimens using the LIVE/DEAD assay. Ovariectomy caused a significant improvement of survival of follicles in the preantral and antral stages in the gonadotropin-stimulated animals (p<0.001), whereas there was no significant effect on the primordial and primary follicle counts. In the non-ovariectomised group, only isolated primordial and primary follicles could be detected. The total follicle amount was significantly higher in the ovariectomised group (n=17, 9.2 ± 7.8, mean±SD) than in the non-ovariectomised group (n=15, 0.3 ± 1.0). This study demonstrates that ovariectomy of stimulated recipient SCID-mice is essential for the development of follicles after xenotransplantation of cryopreserved human ovarian grafts.

Progress in the treatment of oncological diseases has resulted in the last years to a great improvement of the survival prognosis, especially in children and juvenile cancer patients (1). In the USA alone, more than 20,000 children and young people of reproductive age are exposed every year to known mutagens in the form of chemo- and/or radiotherapy for cancer. In the developed or Westernised countries, women utilise better methods of contraception and delay childbearing for social or financial reasons; as a result an increasing number of women are anxious to preserve their fertility, when their early-stage cancers are discovered. A rising number of patients with non-malignant auto-immune diseases, such as rheumatoid arthritis or systemic lupus and haematological diseases are treated successfully with chemotherapy or radiation (2, 3). One of the serious side-effects of cytotoxic therapies (chemo and/or radio therapy) is the threatening partial or complete destruction of the gonadal function (3). Quality-of-life is increasingly important to long-term survivors of cancer and one of the major quality-of-life issues is the ability to produce and raise normal children. In a recent survey, 72% of women with a breast cancer diagnosis have been reported to discuss fertility concerns with their doctors; 51% felt their concerns were addressed adequately (4).

One of the most promising methods for fertility preservation is the cryopreservation of ovarian tissue before oncological treatments because of the large number of follicles that survive the frozen/thawing procedure (5, 6). The problem that arises after the cryopreservation is how to use this frozen material in order to achieve a pregnancy. The vast majority of follicles that survive cryopreservation are primordial (7-10).

There are three ways to achieve the development of these follicles to maturity. The first method is autografting, either...
Animals.

Materials and Methods

Cryopreservation protocol. The ovarian cortex was obtained through an operative laparoscopy by dissecting an area of about 20x10x3 mm ovarian tissue antimesenterically. The biopsies were cut into small pieces (approximately 1x1x1 mm) and were equilibrated in ascending aequimolar concentrations of dimethyl sulfoxide (DMSO)/propandiol up to a concentration of 1.5 M in stages of 0.25 M. The tissue pieces remained in each concentration at 37°C for 7 min and at the last concentration of 1.5 M for 30 min. The tissue was then placed in special cryovials (CTE, Erlangen, Germany) and was loaded into an open freezing system which provides self seeding (CTE). The freezing protocol was as follows: a) cool at -5°C/min to -3.8°C; b) cool at -1°C to -5.3°C; c) cool at -0.2°C to -6°C; d) unchanged for 20 min; e) cool at -0.3°C to -30°C; f) cool at -0.1°C to -35°C; g) cool at -0.3°C to -80°C; h) cool at -10°C to -110°C; and i) immersion in liquid nitrogen. After storage in liquid nitrogen for at least 1 month, the probes were thawed at room temperature. Removal of the cryoprotectant was done in reverse order of the freezing equilibration procedure. The tissue blocks were then cultured in an antibiotic supplemented Medicult IVF-Medium (Gück, Berlin, Germany).

Transplantation procedure. Surgery was performed under narcosis with ketamin (80 mg/kg bodyweight, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and xylazin (10 mg/kg bodyweight, Rompun, Bayer, Frankfurt, Germany), irrespective of the stage of the oestrus cycle. During surgery, mice were kept on a warming plate, the incision site was disinfected with pure alcohol and covered with a one way sterile towel. Both ovaries were removed by a small body wall incision which was sutured with absorbable thread. Xenografting of ovarian cortex was performed within 2 h after removal from the patient to minimize ischemic damage. The ovarian tissue pieces were placed in an intramuscular pocket of the neck muscle.

Gonadotropin stimulation. Mice received daily i.p. injections of human menopausal gonadotropin (hMG, Menogen, Ferring, Kiel; 1 IU FSH / 1 IU LH per animal/day) or saline, starting from day 14 after transplantation for 15 weeks.

Patients. Six patients between 10 and 34 (median 20.5) years of age were included in this study, following informed consent and approval of the local university ethical committee. All patients suffered from malignant diseases (Table I) and wanted to preserve ovarian tissue for a future pregnancy. A maximum of 5% of frozen tissue from each patient was used for our experiments. Prior to cryopreservation, a histological examination of the ovarian cortex was performed in order to secure a sufficient amount of primordial follicles. All patients had age-related normal follicular distribution.

Table I. Follicular development (mean±SD) of SCID-mice (ovariectomized or not) after transplantation of cryopreserved human ovarian tissue from 6 patients under stimulation.

<table>
<thead>
<tr>
<th>Stgr</th>
<th>Indication (age)</th>
<th>nSCID</th>
<th>Ovariec.</th>
<th>hMG</th>
<th>Primordial</th>
<th>Primary</th>
<th>Pre-antral</th>
<th>Antral</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hodgkin (24)</td>
<td>4</td>
<td>x</td>
<td>x</td>
<td>4±4.8</td>
<td>4.2±3.9</td>
<td>4.3±2</td>
<td>4.3±2.5</td>
</tr>
<tr>
<td>1</td>
<td>CML (10)</td>
<td>5</td>
<td>x</td>
<td>x</td>
<td>0.2±0.4</td>
<td>0.8±0.8</td>
<td>6±3.8</td>
<td>4.4±1.8</td>
</tr>
<tr>
<td>1</td>
<td>Hodgkin (34)</td>
<td>3</td>
<td>x</td>
<td>x</td>
<td>0</td>
<td>0</td>
<td>3.7±2</td>
<td>4.3±4.9</td>
</tr>
<tr>
<td>1</td>
<td>Ewing sarc. (16)</td>
<td>5</td>
<td>x</td>
<td>x</td>
<td>0</td>
<td>0.4±0.5</td>
<td>0.6±0.9</td>
<td>1±1</td>
</tr>
<tr>
<td>2</td>
<td>Hodgkin (17)</td>
<td>9</td>
<td>-</td>
<td>x</td>
<td>0.1±0.3</td>
<td>0.3±1.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Ovar. Ca. (29)</td>
<td>6</td>
<td>-</td>
<td>x</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Stgr = study groups; ovariec = ovariectomized; hMG = human menopausal gonadotropin.
Oestrus cycle stage determination. Vaginal smears were taken once a week from all mice starting at day 10 after transplantation using sterile pipettes, in order to examine if follicular tissue survived the transplantation and could produce enough oestrogens to cornify the vaginal epithelium. Vaginal cells were left to dry after being smeared on a microscopic slide and were then stained with methylene blue. The epithelium cells were classified into one of the following categories: a) pro-estrous, b) estrous, c) metestrous, d) diestrous (21).

LIVE/DEAD assay. One ovarian tissue piece from each patient was examined for vitality estimation. A fluorescence stain was used to estimate the number of follicles that had survived the freezing/thawing procedure in vitro. The LIVE/DEAD viability/cytotoxicity assay kit (L-3224, Molecular Probes ®, Leiden, The Netherlands) provides a two-color fluorescence cell viability assay that is based on simultaneous determination of alive and dead cells. The method has been described elsewhere (17). In brief, the tissue was digested in PBS-medium supplemented with 1 mg/ml collagenase (Collagenase Type IV, Sigma-Aldrich®, Steinheim, Germany) and incubated at 37°C for 2 h. The samples were stained according to the assay instructions and were subsequently examined under a Zeiss fluorescence microscope (IM 35®, Zeiss, Oberkochen, Germany). The follicles were classified into 4 categories depending on the percentage of dead granulosa cells (Table II), and were considered dead, only when both the oocyte and all the granulosa cells were dead.

Microscopic evaluation of the number of follicles. The grafts were recovered by a skin and neck muscle incision and were fixed in formalin. After routine paraffin embedding, the samples were serially sectioned (3 μm) and every tenth section was stained with haematoxylin and eosin and examined microscopically as a reference section. The numbers of primordial, primary, pre-antral and antral follicles that had survived the transplantation procedure (intact and with ooplasm) were examined. The diameter of the nucleolus of primordial follicles was estimated to be ≈ 2 μm (17). Using a section thickness of 3 μm reduces the risk of overcounting, without totally eliminating it. The follicles were classified as follows: primordial follicles with one layer of flattened granulosa cells surrounding the oocyte; primary follicles with one layer of cuboid granulosa cells; preantral follicles with two or more layers of granulosa cells, but no antrum; and antral follicles with an antral cavity.
Statistical evaluation. SPSS was used for data evaluation. Nominal data were expressed as mean±SD and compared using the Student’s t-test. A p-value of 0.05 was considered statistically significant.

Results

The distribution of the ovarian tissue from the 6 patients in the two study groups, the follicular development and uterus weight (mean±SD) are presented in Table I. The pre-freeze histological follicle count and the survival of follicles with the LIVE/DEAD assay after thawing are provided in Table II. The follicular development and the uterus weights in the study groups, as well as the statistical analysis of the results are presented in Table III.

Analysis of vaginal smears. Both study groups showed changes in vaginal cytology characteristics of oestrus cycles at days 10-14 after surgery. These changes were evident through the presence of cornified epithelial cells.

Pre-freeze follicular histological count and follicular viability after thawing assessed with the LIVE/DEAD assay. All patients showed age-related normal follicular counts (Table II), and were within 2 orders of standard deviation except the pre-pubertal patient. The primordial follicle was the most predominant follicle type.

The results of the fluorescence staining are also given in Table II. Dead follicles were very rare.

Follicular survival and development. The results of the follicular count per patient are shown in Table I. Table III shows the results of the follicular count in the various study groups. Ovariectomy caused significant development of follicles in the pre-antral and antral stages in the gonadotropin-stimulated animals (p<0.001), whereas there was no significant effect on the primordial and primary follicle counts (Figure I). In the non-ovariectomised group, only isolated primordial and primary follicles could be detected. The total follicle amount was significantly higher in the ovariectomised group (n=17, 9.2±7.8, mean±SD) than in the non-ovariectomised group (n=15, 0.3±1.0).

Discussion

It is estimated that by 2010, one in every 250 women of reproductive age worldwide will be a cancer survivor (22). However, the lifesaving treatments administered can provoke early menopause and subsequent infertility, due to the destruction of a significant proportion of ovarian follicles by chemo- and radiotherapy. Methods to preserve fertility in these young patients are chemoprotection with GnRH-analogs (23) before the planned chemotherapy, ovarian transplantation before radiation and the cryopreservation of cells or ovarian tissue.

In April 2004, the first pregnancy after autotransplantation of cryopreserved ovarian tissue was announced by a team of Belgian scientists (11). Many groups are cryopreserving ovarian tissue for future clinical use (1).

The aim of the study was to examine the effect of ovariectomy on the number of follicles in different developmental stages of cryopreserved human ovarian grafts transplanted to gonadotropin-stimulated SCID-mice.

A slow freezing protocol was used with cryoprotective agents and an open freezing system was already successfully applied for the cleavage stage embryos (24). Many study groups have been using slow freezing equilibrium protocols for the ovarian tissue cryobanking (summarized in 25).

The pre-freezing histological follicular count showed normal age-related follicular distribution in the ovary and was in accordance with findings of other studies (26, 27). After thawing, we performed a LIVE/DEAD assay, xenotransplanted the tissue in SCID-mice and tested the viability of the transplanted ovarian tissue by vaginal cytology, uterus weight and follicle count.

The cornifying of the epithelial cells of the vaginal mucosa demonstrated that both groups produced ovarian steroid hormones.

The results of the staining with the LIVE/DEAD florescent assay confirm that a high percentage of oocytes, as well as granulosa cells survived the cryopreservation and thawing procedure. Our results are in accordance with those of other publications (27, 28). It is known from other studies that the main reason for the follicular loss after cryopreservation and xenografting is the ischaemic effect after transplantation rather than the cryopreservation (29).

The following factors influenced the follicular distribution in transplantation studies: the inhomogeneous distribution of follicles in the ovarian cortex (intra-patient variation) (26), the age-related decline of follicles, the inter-patient variation and the size of the grafts (7).

Most researchers use human gonadotropins to maximise follicular development in the xenografted ovarian tissue (30, 31). We also stimulated the SCID mice with exogenous gonadotropins in order to promote the development of primordial follicles.

Our results indicate that ovariectomy improved the survival of follicles in all developmental stages in the xenotransplanted grafts in the gonadotropin-stimulated SCID mice. Since the stimulation was initiated two weeks after the ovarian transplantation, we assumed that high gonadotropin levels are necessary in the neovascularisation period directly after grafting, which is in accordance with the work of Dissen et al. (32).
Since the serum gonadotropins regulate the female reproductive system and are essential for the cyclic growth of follicles after the onset of antrum formation (33), it has been assumed that one effect mediated by these hormones could be the local activation of angiogenic factors in developing follicles and corpora lutea (34, 35). Bex and Goldman (36) were able to show that in bilaterally ovariectomised hamsters, both serum LH and FSH concentrations were significantly elevated when compared with unmanipulated animals.

Laschke et al. demonstrated by direct in vivo observation that in ovariectomised hamsters, ovarian graft (although not cryopreserved) vascularisation was not only accelerated but also markedly enhanced (37).

In conclusion, the xenotransplantation of small pieces of frozen/thawed human ovarian tissue in SCID mice provides a practical method to assess the development potential of stored ovarian tissue. The observation of even a few growing follicles is sufficient for this purpose, since almost only primordial follicles survive the cryopreservation. Ovariectiony of the mice improves the success of the xenotransplantation procedure as a diagnostic method.

Acknowledgements

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References


