The effect of a GnRH agonist on cryopreserved human ovarian grafts in severe combined immunodeficient mice

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Abstract

This prospective study compares the effect of a GnRH agonist on the number of follicles in different developmental stages in cryopreserved human ovarian grafts transplanted into gonadotropin-stimulated or not stimulated severe combined immunodeficient mice (SCID mice). Human ovarian tissue from seven patients was cryopreserved with an open-freezing system and xenotransplanted in SCID mice. The SCID mice were then treated according to different stimulation protocols. The survival of the tissue after cryopreservation was examined by LIVE/DEAD viability staining or transplanted in the neck muscle of 41 SCID mice. Development of follicles, estradiol production, vaginal cytology, and uterus weight were assessed after 15 weeks with or without gonadotropin stimulation. Viable follicles were detected in all frozen/thawed specimens using the LIVE/DEAD assay. Triptorelin, a GnRH agonist, caused a significant reduction of follicles in all developmental stages in the non-gonadotropin-stimulated animals ($P < 0.001$). In gonadotropin-stimulated animals, GnRH agonist treatment has no significant effect on primordial, primary and preantral follicle count, whereas the antral follicles were significantly fewer ($P = 0.03$). The GnRH agonist treatment is not able to prevent the primordial follicle depletion after the xenografting of ovarian tissue in SCID mice with or without gonadotropin stimulation. Furthermore, it causes an additional loss of follicles if administered during the critical neovascularization period after the transplantation.

Introduction

Aggressive chemo- and radiotherapy in young females with cancer has greatly enhanced the life expectancy of these patients, but these treatments often result in premature ovarian failure because of the massive destruction of the ovarian reserve. These young women have to face years of hormone replacement therapy as well as the prospect of definite infertility which causes an additional psychological stress (Demeestere \textbf{et al.} 2003, Siebzehnrubl 2003).

The most promising method for fertility preservation in these patients is the cryopreservation of ovarian tissue before oncological treatments, because of the large number of follicles that survive the freezing/thawing procedure (Donnez \& Bassil 1998, Aubard \textbf{et al.} 2001, Liu \textbf{et al.} 2003). The problem that arises after the cryopreservation is how to use this frozen material in order to achieve a pregnancy. The majority of follicles that survive cryopreservation are primordial (Newton \textbf{et al.} 1996, Candy \textbf{et al.} 1997, Gook \textbf{et al.} 1999, Broecke Van den \textbf{et al.} 2001).

Two pregnancies have already been achieved after cryopreservation and retransplantation of ovarian tissue (Donnez \textbf{et al.} 2004, Meirow \textbf{et al.} 2005), and cryopreservation of ovarian tissue is being offered due to its future therapeutic potential by many groups. Oktay \textbf{et al.} (1998) and Gook \textbf{et al.} (2001) observed a primordial follicle loss in xenotransplanted frozen/thawed human ovarian tissue after gonadotropin stimulation. A possible cause of the accelerated depletion of the primordial follicle pool could be a toxic effect of luteinizing hormone (LH) or follicle-stimulating hormone (FSH), as has already been suggested by Richardson \textbf{et al.} (Richardson \& Nelson 1990). Flaws \textbf{et al.} (1997) showed that chronically elevated LH levels deplete the primordial follicle pool and thus may hasten the onset of reproductive senescence. Gonadotropin-releasing hormone (GnRH)
an analogs are able to prevent LH/FSH secretion of the pituitary gland in humans and rodents (Schally et al. 1980, Recabarren et al. 1991).

The aim of the study was to examine prospectively the effect of a gonadotropin-releasing hormone agonist (GnRH-a; triptorelin) on the number of follicles in different developmental stages of cryopreserved human ovarian grafts transplanted to gonadotropin-stimulated or not stimulated severe combined immunodeficient mice (SCID mice) and to test the hypothesis that GnRH agonist treatment may be able to prevent primordial follicular depletion.

Material and Methods

Animals

Forty-one female SCID mice (C.B-17/ScICR-Hsd scid, 6 weeks of age) were obtained from Harlan-Winkelmann (Borchen, Germany). The animals were housed in a high efficiency particulate airfiltered positive pressure room. Cages (Techniplast, Milano, Italy) were filter topped and animals had free access to food (Altromin 1314, Altromin, Lage, Germany) and water under 12 h light:12 h darkness conditions. Groups of five to nine mice were housed in one cage. Upon arriving from the breeding company, mice were allowed to get acclimated for 1 week. All procedures, tests, and injections were performed under a laminar flow hood in a positive pressure room. Approval for the study was obtained from the local ethical committee on animal experiments. The animals were maintained in accordance with Animal Care and Use Committee regulations.

Patients

Seven patients between 10 and 34 (median 24) years of age were included in this study following informed consent and approval of the local university ethical committee. Patients suffered from malignant diseases (Table 1) and wanted to cryopreserve ovarian tissue for a future pregnancy before the chemotherapy. 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.

Cryopreservation protocol

The ovarian cortex was gained through an operative laparoscopy by dissecting an area of about 20×10×3 mm ovarian tissue antimesenterically. The biopsies were cut into small pieces (about 1×1×1 mm) and equilibrated in ascending equimolar concentrations of DMSO/propandiol in PBS up to a concentration of 1.5 M in six steps 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. Tissue was then placed in special cryovials (CTE, Erlangen, Germany) and loaded into an open-freezing system which provides self-seeding (CTE). The freezing protocol was as following: (a) cool at −5 °C/min to −3.8 °C; (b) cool at −1 to −5.3 °C; (c) cool at −0.2 to −6 °C; (d) unchanged for 20 min; (e) cool at −0.3 to −30 °C; (f) cool at −0.1 to −35 °C; (g) cool at −0.3 to −80 °C; (h) cool at −10 to −110 °C; (i) immersion into liquid nitrogen. After storage in liquid nitrogen for at least 1 month, probes were thawed at room temperature. Removal of cryoprotectant was done in reverse order of the freezing equilibration procedure. The thawing medium was the same as the freezing medium with the addition of 0.25 M sucrose. The tissue blocks were then cultured in an antibiotic supplemented Medicult IVF Medium (Gück, Berlin, Germany).

Transplantation procedure

Surgery was performed under narcosis with ketamine (80 mg/kg bodyweight, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and xylazine (10 mg/kg bodyweight, Rompun, Bayer, Frankfurt, Germany), irrespective of the stage of the estrus cycle. During surgery, mice were kept on a warming plate, the incision site was

Table 1 Follicular development per graft (mean ± s.d.) and uterus weight (mg, mean ± s.d.) of SCID mice after transplantation of cryopreserved human ovarian tissue from seven patients under different stimulation protocols.

<table>
<thead>
<tr>
<th>Group</th>
<th>Indication (age in years)</th>
<th>nSCID</th>
<th>HMG</th>
<th>Triptorelin</th>
<th>Primordial</th>
<th>Primary</th>
<th>Preantral</th>
<th>Antral</th>
<th>Uterus (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1</td>
<td>Hodgkin (24)</td>
<td>4</td>
<td>×</td>
<td>–</td>
<td>4.4 ± 4.8</td>
<td>4.2 ± 3.9</td>
<td>4.3 ± 2</td>
<td>4.3 ± 2.5</td>
<td>180 ± 62</td>
</tr>
<tr>
<td>B 1</td>
<td></td>
<td>2</td>
<td>×</td>
<td>–</td>
<td>19 ± 19.7</td>
<td>12.5 ± 6.3</td>
<td>3 ± 2.8</td>
<td>2.5 ± 3.5</td>
<td>148 ± 9</td>
</tr>
<tr>
<td>A 1</td>
<td>CML (10)</td>
<td>5</td>
<td>×</td>
<td>–</td>
<td>0.2 ± 0.4</td>
<td>0.8 ± 0.8</td>
<td>6 ± 3.8</td>
<td>4.4 ± 1.8</td>
<td>110 ± 64</td>
</tr>
<tr>
<td>A 1</td>
<td>Hodgkin (34)</td>
<td>3</td>
<td>×</td>
<td>–</td>
<td>3 ± 2</td>
<td>3.7 ± 2</td>
<td>4.3 ± 4.9</td>
<td>124 ± 50</td>
<td></td>
</tr>
<tr>
<td>B 1</td>
<td>Ewing sarc. (16)</td>
<td>2</td>
<td>×</td>
<td>–</td>
<td>3.5 ± 4.9</td>
<td>3.4 ± 2</td>
<td>6.5 ± 7</td>
<td>1.5 ± 2</td>
<td>119 ± 84</td>
</tr>
<tr>
<td>A 1</td>
<td>CML (16)</td>
<td>5</td>
<td>×</td>
<td>–</td>
<td>0.4 ± 0.5</td>
<td>0.6 ± 0.9</td>
<td>1 ± 1</td>
<td>164 ± 45</td>
<td></td>
</tr>
<tr>
<td>B 1</td>
<td>Hodgkin (28)</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>16.5 ± 5.8</td>
<td>7.6 ± 2.9</td>
<td>2.0 ± 1.8</td>
<td>1.3 ± 1.5</td>
<td>172 ± 28</td>
</tr>
<tr>
<td>B 2</td>
<td></td>
<td>3</td>
<td>×</td>
<td>–</td>
<td>7.0 ± 3.4</td>
<td>3.3 ± 1.5</td>
<td>0 ± 3</td>
<td>56 ± 9</td>
<td></td>
</tr>
<tr>
<td>A 2</td>
<td>Hodgkin (26)</td>
<td>2</td>
<td>×</td>
<td>–</td>
<td>1.0 ± 0.7</td>
<td>1.4 ± 1.4</td>
<td>1.5 ± 2.1</td>
<td>1.5 ± 0.7</td>
<td>92 ± 24</td>
</tr>
<tr>
<td>B 2</td>
<td></td>
<td>3</td>
<td>×</td>
<td>–</td>
<td>2.2 ± 2.6</td>
<td>2.6 ± 2.6</td>
<td>0 ± 0</td>
<td>64 ± 19</td>
<td></td>
</tr>
<tr>
<td>A 2</td>
<td>Hodgkin (26)</td>
<td>2</td>
<td>×</td>
<td>–</td>
<td>0.6 ± 1.1</td>
<td>1.6 ± 1.5</td>
<td>4.3 ± 7.5</td>
<td>1.3 ± 2</td>
<td>99 ± 31</td>
</tr>
</tbody>
</table>

*CML, chronic myeloid leukemia.
disinfected with pure alcohol and covered with a sterile towel. Both mice ovaries were removed by a small body wall incision which was sutured with absorbable thread. One frozen/thawed human ovarian tissue piece was placed in an i.m. pocket of the neck muscle.

**Study design**

All mice were ovarectomized and assigned to two different experimental groups:

**Study group A**

The effect of triptorelin on the follicular development in gonadotropin-stimulated animals.

**Study group B**

The effect of triptorelin on the follicular development in gonadotropin untreated animals.

In study group A, 23 SCID animals were treated with HMG alone (group A1, \( n = 17 \)) or with human menopausal gonadotropin (HMG) and triptorelin (group A2, \( n = 6 \)), and in study group B, 18 SCID mice were treated either with saline (group B1, \( n = 10 \)) or with only triptorelin (group B2, \( n = 8 \)).

**Gonadotropin stimulation**

Mice received daily i.p. injections of HMG (Menogon, Ferring, Kiel; 1 IU FSH/1 IU LH per animal/day) or saline, starting from day 14 after transplantation for 15 weeks. This dose was adjusted from an earlier study by Oktay et al. (1998).

**Estrus 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 estrogens to cornify the vaginal epithelium. Vaginal cells were left to dry after being smeared on a microscopic slide and then stained with methylene blue (Gunasena et al. 1997).

**Pituitary downregulation with triptorelin treatment**

Two weeks before the mice of groups A2 and B2 were ovarectomized, an i.m. injection of 8 mg GnRH agonist depot triptorelin in the form of microcapsules was administered. The injection was repeated every 4 weeks to ensure an adequate mice-pituitary negative feedback.

**LIVE/DEAD assay**

One ovarian tissue piece from each patient was examined for estimation of vitality. In order to estimate the number of follicles that survived the freezing/thawing procedure in vitro we used a fluorescence staining. 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 the simultaneous determination of live and dead cells. We used the kit with a Zeiss fluorescence microscope (IM35, Zeiss, Oberkochen, Germany) in order to examine the viability of the frozen–thawed tissue.

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeating calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em \( \sim 495/515 \) nm). Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em \( \sim 495–635 \) nm).

To perform the viability assay we used the following protocol: (a) the thawed tissue is dissected to as small as possible pieces with a scalpel; (b) 3 mg collagenase (Collagenase Type IV, Sigma-Aldrich) diluted in 3 ml Dulbecco’s PBS (D-PBS) are added to the tissue; (c) the samples are left to incubate for approximately 2 h at 37°C, while the homogenate is stirred periodically every 20 min; (d) the LIVE/DEAD reagent stock solutions are removed from the freezer and allowed to warm to room temperature; (e) the reagents are combined by adding 20 \( \mu \)l of the 2 mM EthD-1 stock solution and 5 \( \mu \)l supplied 4 mM calcein AM stock solution to 10 ml sterile, tissue culture-grade D-PBS; (f) the resulting approximately 2 \( \mu \)M calcein AM and 4 \( \mu \)M EthD-1 working solutions are added directly to the samples and left to incubate for 30 min in a dark place at room temperature; and (g) the labeled cells are viewed under the fluorescence microscope. Follicles were classified into four categories depending on the percentage of dead granulosa cells (Table 2). Only when both the oocyte and all the granulosa cells were dead, was a follicle considered as dead.

**Microscopic evaluation of number of follicles**

Seventeen weeks after ovarian transplantation, the mice were sacrificed by an anesthesia overdose. grafts were recovered and fixed in formalin. After routine paraffin embedding, the entire samples were serially sectioned (~3 \( \mu \)m), and every tenth section was stained with hematoxylin and eosin and examined microscopically as a reference section (Fig. 1).

The numbers of primordial, primary, preantral, 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 \( \approx 2 \mu m \) (Jones & Krohn 1961). Using
A section thickness of 3 μm reduces the risk of over-counting, without totally eliminating it. 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 (Myers et al. 2004, Kerr et al. 2006).

**Hormone determination and uterus weight**

After the animals were killed, serum was collected for estradiol measurement by cardiac puncture. Total serum estradiol was measured by RIA without extraction by a commercial high-sensitive assay for rat and mouse serum (Diagnostic Systems Laboratories, Sinsheim, Germany). The uterine horns were removed, trimmed, and weighed after removing the surface moisture.

**Statistical evaluation**

Statistical Product and Service Solution (SPSS) was used for data evaluation. Nominal data were expressed as mean ± s.d. and compared using t-test. A P value of 0.05 was considered statistically significant. Significance of correlation was calculated according to Pearson.

**Results**

Table 1 shows the distribution of the ovarian tissue from the seven patients to the various study groups, the follicular development, and uterus weight of every subgroup (mean ± s.d.). Table 2 shows the pre-freeze histological follicle count and the survival of follicles with the LIVE/DEAD assay after thawing. Table 3 shows the follicular development and the uterus weight of the different study groups and statistical analysis of the results.

**Table 2** Pre-freeze follicular count (per mm² of ovarian cortex) and survival of follicles assessed with LIVE/DEAD viability assay after thawing.

<table>
<thead>
<tr>
<th>Patient diagnosis</th>
<th>Age</th>
<th>Follicular count number/mm² (percentage of primordial)</th>
<th>Survival count after thawing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin 28</td>
<td>28</td>
<td>17 (94.2%)</td>
<td>Complete follicle 15 (39%)</td>
</tr>
<tr>
<td>Hodgkin 26</td>
<td>26</td>
<td>23 (95.5%)</td>
<td>Oocyte + &gt; 50% granulosa 19 (50%)</td>
</tr>
<tr>
<td>Hodgkin 24</td>
<td>24</td>
<td>21 (95.7%)</td>
<td>Oocyte + &lt; 50% granulosa 4 (11%)</td>
</tr>
<tr>
<td>CML² 10</td>
<td>10</td>
<td>36 (100%)</td>
<td>Dead 0</td>
</tr>
<tr>
<td>Hodgkin 34</td>
<td>34</td>
<td>9 (88%)</td>
<td></td>
</tr>
<tr>
<td>CML² 16</td>
<td>16</td>
<td>26 (98%)</td>
<td></td>
</tr>
<tr>
<td>Ewing 16</td>
<td>16</td>
<td>24 (97%)</td>
<td></td>
</tr>
</tbody>
</table>

*CML, chronic myeloid leukemia.*

![Figure 1](image1)

**Figure 1** SCID mouse during retrieval of the ovarian tissue graft, isolated graft with an antral follicle, and histological stainings of antral follicles (×25) after cryopreservation and grafting of human ovarian cortex.
Table 3 Summarized follicle development (mean ± S.D.) and uterus weight (mg, mean ± S.D.) in the two study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>nScid</th>
<th>HMG</th>
<th>Triptorelin</th>
<th>Primordial</th>
<th>Primary</th>
<th>Preantral</th>
<th>Antral</th>
<th>Uterus (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>17</td>
<td>X</td>
<td>–</td>
<td>1.0±2.6*</td>
<td>1.3±2.3†</td>
<td>3.5±3.0‡</td>
<td>3.3±2.6§</td>
<td>147.3±56.0¶</td>
</tr>
<tr>
<td>A2</td>
<td>6</td>
<td>X</td>
<td>X</td>
<td>0.8±0.8*</td>
<td>1.3±1.1†</td>
<td>2.6±4.7‡</td>
<td>1.1±1.4§</td>
<td>96.6±23.2¶</td>
</tr>
<tr>
<td>B1</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>14.4±9.9‡</td>
<td>7.7±4.6*</td>
<td>2.7±2.5*</td>
<td>1.6±1.8*</td>
<td>156.9±42.0¶</td>
</tr>
<tr>
<td>B2</td>
<td>8</td>
<td>–</td>
<td>X</td>
<td>3.7±3.1*</td>
<td>2.7±2.3*</td>
<td>0*</td>
<td>0*</td>
<td>58.8±12.6*</td>
</tr>
</tbody>
</table>

Statistical differences: *P=0.44; †P=0.49; ‡P=0.30; §P=0.04; ¶P=0.03; ‡P<0.001.

- Coefficient for age and pre-freeze follicular count was −0.93 (P=0.002). The correlation coefficient for age and complete follicular survival count were −0.83 (P=0.02). The correlation coefficient for pre-freeze follicular count and survival rate of complete follicles were 0.88 (P=0.007). All correlations are significant.

- The results of the fluorescence staining are also given in Table 2. Dead follicles were very rare.

**Analysis of vaginal smears**

In all mice except group B2, cornified epithelial cells were present. No regular cyclicity could be detected.

**Uterine weights**

The mean uterine weights of all study groups are shown in Table 3. We found statistical differences between groups A1 and A2 and between B1 and B2. Our results show that the uterine weight is reduced by the administration of triptorelin in stimulated (P=0.03) and non-stimulated animals (P<0.001).

**Follicular survival and development**

The results of the follicular count per patient are shown in Table 1. Table 3 shows the results of the follicular count in the various study groups. Triptorelin caused a significant reduction of follicles in all developmental stages in the non-gonadotropin-stimulated animals (group B2; P<0.001). In gonadotropin-stimulated animals GnRH agonist treatment has no significant effect on primordial, primary, and preantral follicle count, whereas the antral follicles were significantly fewer (groups A1 and A2; P=0.03).

**Estradiol**

Estradiol was measured in a highly sensitive estradiol RIA for mice. Nevertheless, the lower detection limit was 5 pg/ml. In all 41 animals, enough blood serum was collected for estradiol determination. In only six animals, estradiol concentrations of >5 pg/ml were found. In all of these animals, antral follicles were detected (only one animal had a triptorelin treatment, all animals received gonadotropin stimulation).

**Discussion**

It is estimated that by 2010, 1 in every 250 women of reproductive age will be a cancer survivor (Blatt 1999). However, this lifesaving treatment can provoke early menopause and subsequent infertility, due to the destruction of a significant proportion of ovarian follicles by chemo- and radiotherapy.

The cryopreservation of ovarian tissue before the loss of reproductive function is today a realistic option for women facing cytotoxic cancer therapy (Falcone et al. 2004, Falcone & Bedaiwy 2005). Nevertheless, this technique is still in an experimental stadium and improvements in both the cryopreservation and retransplantation methods are necessary before it becomes routine. In order to validate the maintenance of the developmental potential of primordial follicles in the stored tissue, many groups have used the xenotransplantation in SCID mice (Weissman et al. 1999, Gook et al. 2003, Maltaris et al. 2006).

The aim of the study was to examine prospectively the effect of a GnRH agonist (triptorelin) on the number of follicles in different developmental stages of cryopreserved human ovarian grafts transplanted to gonadotropin-stimulated or not stimulated SCID mice and to test the hypothesis that GnRH agonist treatment may be able to prevent primordial follicular depletion.

The pre-freezing histological follicular count showed normal age-related follicular distribution in the ovary and is in accordance with findings of other studies (Siebzehnrubl et al. 2000, Schmidt et al. 2003). 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, follicle count, and estradiol determination.

In only six animals, estradiol concentrations of >5 pg/ml were found. In all of these animals, antral follicles were present. It is known that the rodent reproductive system lacks a specific estradiol-binding globulin equivalent to the human sex hormone-binding protein (Janne et al.1999). Therefore, minute levels of estradiol are adequate for the normal reproductive function.

The cornifying of the epithelial cells of the vaginal mucosa, which is predominant in the estrus phase, demonstrated that the animals produced ovarian steroid hormones. The only group that showed no cornifying of...
the vaginal epithelial cells was the group with triptorelin treatment and no stimulation. This can be explained by the mouse pituitary downregulation in the absence of exogenous stimulation, which leads to a total estrogen production failure. Triptorelin reduced significantly the uterus weight in stimulated and not stimulated animals, which can be explained by a reduced estradiol release due to the pituitary downregulation. In the group that received no triptorelin or gonadotropin stimulation normal uterine weights were found, which also correlates with the work of Cook et al. (2001), who reported adequate circulating concentrations of FSH for follicle maturation in oophrectomized mice without exogenous FSH stimulation.

The results of the staining with the LIVE/DEAD fluorescent assay confirm that a high percentage of oocytes as well as granulosa cells survive the cryopreservation and thawing procedure. Our results are in accordance with other publications (Siebzehnrubl et al. 2000, Martinez-Madrid et al. 2004). It is known from other studies that the main reason for the follicular loss after cryopreservation and xenografting is the ischemic effect after transplantation rather than the cryopreservation (Liu et al. 2002, Maltaris et al. 2005).

The following factors influence the follicular distribution in transplantation studies: the inhomogeneous distribution of follicles in the ovarian cortex (intrapatient variation) (Schmidt et al. 2003), the age-related decline of follicles, the interpatient variation, and the size of the grafts (Broecke Van den et al. 2001). This study demonstrates that a prolonged exogenous stimulation promotes the primordial follicle maturation but causes a loss of primordial follicles in xenotransplanted frozen/thawed human ovarian tissue grafts, which is in accordance to the known literature (Oktay et al. 1998, Cook et al. 2001, Dittrich et al. 2001, Maltaris et al. 2007). This depletion of the ovarian follicular reserve could be a result of a toxic effect of FSH or LH (Richardson & Nelson 1990, Flaws et al. 1997).

In our experiments, triptorelin, a GnRH agonist, which suppresses the FSH and LH release, was not able to preserve the primordial follicle fraction. It caused a significant reduction of follicles in all developmental stages in the non-gonadotropin-stimulated animals (B1 vs B2; \( P<0.001 \)). In gonadotropin-stimulated animals, GnRH agonist treatment had no significant effect on primordial, primary, and preantral follicle count, whereas the antral follicles were significantly fewer (A1 vs A2; \( P=0.03 \)).

A possible explanation for this effect of triptorelin especially in the non-stimulated animals (B1 vs B2), could be the absence of gonadotropins in the neovascularization period directly after grafting. Dissen et al. (1994) have proven that gonadotropins play an important role in this critical period.

In the stimulated group (A1 vs A2), stimulation began 2 weeks after the ovarian transplantation, so that also in these animals a part of the follicular reserve was lost, although not statistically significant. The significant loss of the antral follicles in the downregulated and stimulated animals (A1 vs A2) can be explained by the not sufficiently compensating stimulation by exogenous gonadotropins (1 IU HMG/day per mouse) in our experiments.

We conclude that the GnRH agonist treatment with triptorelin is not able to prevent the primordial follicle depletion after the xenografting of ovarian tissue in SCID mice with or without gonadotropin stimulation, but can also cause an additional loss of follicles if administered during the critical neovascularization period after the transplantation.

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