

A Novel Ovarian Xenografting Model to Characterize the Impact of Chemotherapy Agents on Human Primordial Follicle Reserve

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Abstract

Many chemotherapeutic agents, especially of the alkylating family, alter fertility in premenopausal females. However, it is not practically possible to quantify and characterize the impact of cancer drugs on ovarian reserve in a clinical setting. Thus, our specific aim was to develop a xenograft model to characterize the *in vivo* impact of chemotherapy agents on human ovary. Ovarian pieces from 24 weeks old abortuses were xenografted s.c. to severe combined immunodeficient mice ($n = 52$). Animals received either a single dose of 200 mg/kg of cyclophosphamide or the vehicle. Grafts were recovered from the control and treated mice 12 to 72 h after the cyclophosphamide injection and serially sectioned for primordial follicle counts. Apoptosis was assessed with terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay. Platelet/endothelial cell adhesion molecule 1 (PECAM-1) staining, as well as intra-vital fluorescein-conjugated lectin and Evans blue labeling were done to assess microvasculature by confocal microscopy. Although there was 12% reduction in primordial follicle density by 12 h following treatment ($P > 0.05$), the follicle loss increased significantly at 24 h (53%, $P < 0.01$) and peaked at 48 h (93%, $P < 0.0001$). TUNEL staining peaked at 12 h, earlier than the diminishment in follicle numbers, and decreased thereafter. Xenograft vascularization pattern was similar to non-xenografted tissue, indicating appropriate *in vivo* drug delivery. The impact of cyclophosphamide on primordial follicle reserve in our human ovarian xenograft model is consistent with the clinical gonadotoxicity of this drug. Human ovarian xenografting is a promising model to characterize the gonadotoxic effects of current and emerging cancer drugs without a need for lengthy clinical studies. [Cancer Res 2007;67(21):10159–62]

Introduction

Although the progress in multiagent chemotherapy improved the cure rates of both adult and childhood cancers, long-term consequences of these treatments on the quality of life and especially fertility are now being recognized. The impact of cancer treatments on fertility is greater than generally perceived. The probability of developing cancer under the age of 40 is 2%, and this probability increases to 11% between the ages 40 and 50 in females (1). Based on the types of cancers seen in these young age groups,

approximately half of these females may receive a cancer treatment that would impact their future fertility. Thus, cancer treatment is probably the most common known cause of premature ovarian failure in the general population.

Some chemotherapeutic agents are more commonly associated with gonadal damage. These include alkylating agents such as cyclophosphamide, chlorambucil, melphalan, busulfan, nitrogen mustard, and procarbazine (2). Although animal studies have shown the direct impact of these agents on primordial follicle (PF) reserve (3, 4), there is no real-time quantitative evidence of chemotherapy in human ovary. Moreover, as new agents are introduced to adjuvant setting, their long-term impact on human ovary is extremely difficult to determine from short-term studies. If the comparative ovarian toxicity of various chemotherapy agents is known, those patients who wish to preserve their fertility can be offered fertility-friendly regimens. A bioassay that can obviate lengthy clinical trials and one that can quantify the ovarian impact of chemotherapeutics would offer enormous benefit in drug development and choice. We did this study to address the latter need.

The severe combined immunodeficient (SCID) mouse has a T and B cell deficiency, allowing xenografting without tissue rejection. Using immunodeficient mice, xenografting models have been successfully used in the cancer field (5). We previously showed that human PFs go through normal stages of maturation and respond to hormone treatment in tissue xenografted to SCID mice (6). Based on that earlier model, we aimed to develop a human ovarian xenograft model to quantify and characterize the relative impact of cancer drugs *in vivo*. At 24 weeks of gestational age, all follicles in the fetal ovary are at primordial stage, providing an opportunity to study the impact of chemotherapeutic agents on ovarian reserve.

Materials and Methods

Reagents. Platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) goat polyclonal and Mouse Vasa Homologue (MVH) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnologies Inc.. Rabbit polyclonal phospho-histone H₃ antibody was obtained from Upstate USA Inc. DMEM-F12 was from Life Technologies (Invitrogen Co.). Fluorescein-labeled GSL I-isolectin B₄ was obtained from Vector Laboratories. Evans blue dye was from Sigma.

Animals. Female SCID mice 5 to 8 weeks old were obtained from Jackson Laboratories. A total of 52 animals were used (five to six animals for each time point). The animals were maintained on a 12-h/12-h light/dark cycle with food and water available ad libitum. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University.

Human ovarian tissue. Three pairs of 24-week-old human fetal ovaries were used in the study. The ovaries were obtained during therapeutic abortions and were provided by the Human Fetal Tissue Repository at the Jacobi Medical Center of Albert Einstein University School of Medicine. The study was approved by the Institutional Review Boards of Weill Medical

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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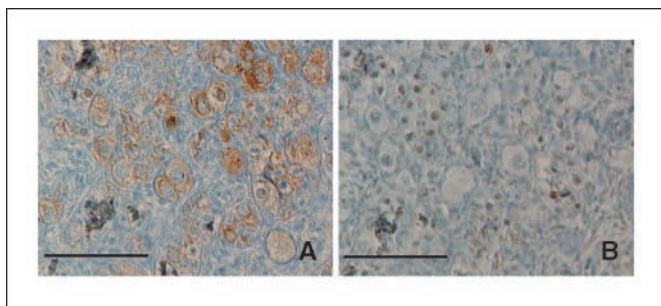


Figure 1. Germ cell mitosis is minimal in 24-wk-old human fetal ovaries. Adjacent sections of fetal ovary stained immunohistochemically with MVH (A) and with mitosis marker phospho-histone H₃ (B) show only a few cells staining positive for both markers, confirming that germ cell mitosis is nearly completed at this gestational stage. Bars, 100 μ m.

College of Cornell University and Albert Einstein University School of Medicine.

Xenografting and chemotherapy administration. Fetal ovaries were first minced into 2 \times 2-mm pieces in HEPES-buffered DMEM-F12 culture media and maintained at 4°C until grafting. The animals were anesthetized with ketamine-xylazine cocktail. Small subcutaneous pouches were created bilaterally on the dorsum of the animals. Fetal ovarian pieces were transplanted into the pockets followed by injection of 200 μ L of Matrigel. Two weeks after the transplantation, cyclophosphamide was administered at a single dose of 200 mg/kg i.p. The dose was chosen based on previous work (7). Grafts were recovered at 12, 24, 48, and 72 h postinjection. Control animals received saline injection only.

Matrigel plug assay–intravital fluorescein dye injection–confocal imaging of graft vasculature. The assay was done as described previously (8). Matrigel is a solubilized basement membrane preparation and its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin (9). To visualize revascularization in the graft and surrounding Matrigel, mice were injected with either 0.1 mL FITC–lectin (100 μ g/mL) or 0.1 mL Evans blue (1% w/v) in PBS via the tail vein 30 min before sacrifice to visualize vascular endothelium. Entire grafts were

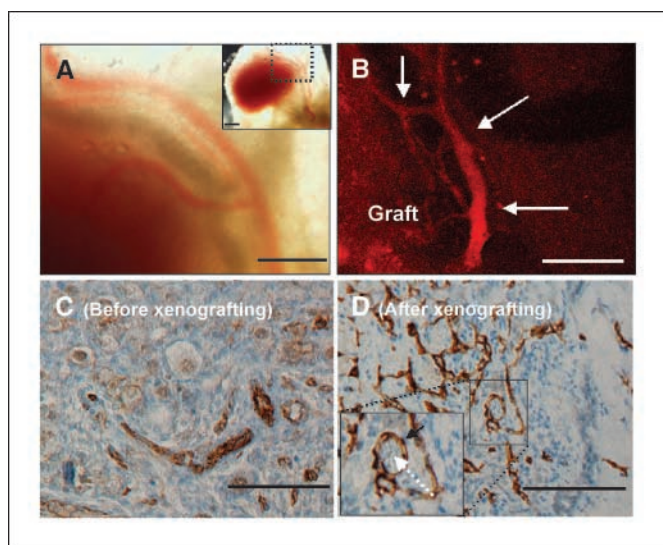


Figure 2. Assessment of graft revascularization. Gross revascularization of the grafts as shown by transillumination. Bar, 300 μ m (A). Microvasculature of the grafts after labeling with Evans blue. Bar, 100 μ m (B). Similar pattern of immunohistochemical staining with vascularization marker CD31 in non-grafted (C) and grafted fetal ovaries (D). Note the presence of RBC (white dotted arrow) in PECAM-1 positive vascular structure (black solid arrow) showing functional competency and circulation in these vessels. Bars, 100 μ m.

scanned at 0.5- μ m thickness using Leica confocal inverted microscope, and vascular tree was visualized after excitation under appropriate channels.

Histomorphometric assessment and immunohistochemistry. Paraffin-embedded sections were serially sectioned at 7- μ m thickness to determine PF density. PF density was determined from serial sections as the mean of follicle counts per square millimeter in every fifth section. For the determination of apoptosis in paraffin-embedded sections, TUNEL (terminal nucleotidyl transferase–mediated nick end labeling) method was used. PECAM-1 (CD31) staining was used for the assessment of graft vascularization. Germ cell marker MVH and mitosis marker phospho-histone H₃ (Ser¹⁰) were used for the identification of proliferating germ cells.

Statistical analysis. The data were prospectively collected from three experiments. For comparison of follicle counts, ANOVA was used. Graphic bars were created using GraphPad Prism software. A *P* value <0.05 was considered significant.

Results

Comparability of fetal ovarian tissue to postnatal ovarian tissue. The mean number of PFs \pm SE/mm² (follicle density) was 35.6 \pm 3 in fetal ovaries of 24-week gestational age, which was higher than those determined in 17- and 22-year-old patients (18.25 \pm 5.17 and 16.6 \pm 3.5, respectively). Furthermore, adjacent sections of a 24-week-old fetal ovary were stained immunohistochemically with germ cell marker MVH and mitosis marker phospho-histone H₃ (Ser¹⁰) to determine if germ cell mitosis still takes place in fetal ovary at this gestational age. As shown in Fig. 1A and B, rare cells were stained positive for both MVH (Fig. 1A) and phospho-histone H₃ (Fig. 1B).

These data indicate that whereas fetal ovarian tissue contains a larger number of PFs compared with postnatal individuals, it possesses all the elements of a mature ovary with the exception of developing follicles. Although it is not identical to a postnatal ovary, because the target of concern for chemotherapy agents is PFs, fetal ovarian tissue should provide a suitable environment to study the impact of chemotherapeutics on human ovarian reserve.

Graft vascularization and survival. Grafts were well vascularized 2 weeks post-transplantation (Fig. 2A). The microvasculature was visualized by confocal microscopy following tail-vein injections of Evans blue (Fig. 2B) and FITC-labeled lectin B₄ (see Supplementary Figure). Xenografted tissue showed a CD31 staining pattern similar to that of non-xenografted fetal tissue. Furthermore, CD31-positive areas were filled with RBC showing functional competency of the newly formed vessels (Fig. 2C and D). Consistent with the full vascularization, ovarian histology was similar to non-xenografted ovaries with the exception of diminished PF density (35.6 \pm 3 versus 4.8 \pm 0.8, pre- and post-xenografting, respectively;

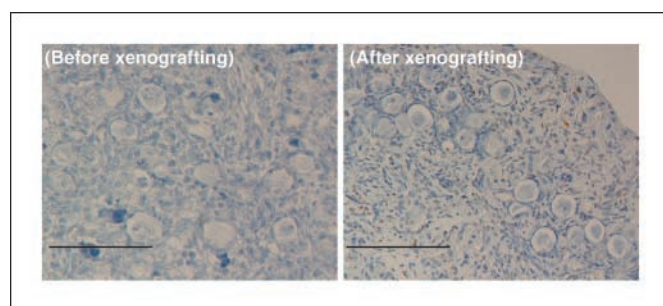


Figure 3. Assessment of graft survival. 24-wk-old fetal ovary comprised mainly of PFs (left). A large number of surviving PFs are visible 2 wk after transplantation (right). Bars, 100 μ m.

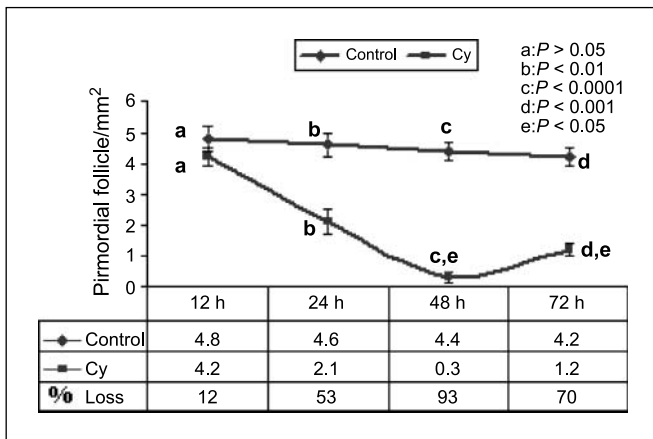


Figure 4. Comparison of PF density between control and cyclophosphamide (CY)-treated xenografts. PF loss starts as early as 12 h postinjection and peaks at 48 h. The follicle loss is only 12% at 12 h ($P > 0.05$), but significantly increases at 24 h (53%, $P < 0.01$) and peaks at 48 h (93%, $P < 0.0001$). Note the moderate increase in PF density from 48 to 72 h and, as a result, attenuation in follicle loss to 70% at 72 h ($P < 0.001$).

Fig. 3). However, post-xenografting follicle density was similar to two adult individuals at age 33, who had 5.66 ± 0.9 and 5.23 ± 1.1 PF/mm², making our model even more comparable to adult individuals.

Chemotherapy-induced follicle destruction. Grafts recovered at 12 h after cyclophosphamide administration had a PF density statistically similar to control grafts (mean \pm SE, 4.2 ± 0.3 versus

4.8 ± 0.8 ; $P > 0.05$). At 24 h, there was a significant decline in follicle density in the treated grafts compared with controls (2.15 ± 0.4 versus 4.62 ± 0.4 ; $P < 0.01$; see Supplementary Table for median values and ranges. At 48 h, follicle density further decreased in the treated grafts in comparison to controls (0.31 ± 0.1 versus 4.46 ± 0.3 ; $P < 0.0001$). At 72 h post-drug injection, the number of PFs in the treated grafts increased to 1.27 ± 0.17 . This increase was statistically significant when compared with the follicles counted in treated grafts at 48 h (1.27 ± 0.17 versus 0.31 ± 0.1 ; $P < 0.05$), but it was still significantly lower than that of the control grafts at 72 h (1.27 ± 0.17 versus 4.22 ± 0.4 ; $P < 0.001$; Fig. 4).

When the follicle loss was expressed as percentage difference between controls and treated animals, there was only a 12% loss 12 h following the treatment ($P > 0.05$). The follicle loss significantly increased at 24 h (53%, $P < 0.01$), peaked at 48 h (93%, $P < 0.0001$), and was reduced at 72 h (70%, $P < 0.001$; Fig. 4).

The intensity and frequency of TUNEL staining peaked earlier than the reduction in PF density. The most extensive staining was seen at 12 h, decreasing at 24 and 48 h, and fading at 72 h. At 12 h, whereas in 100% of the TUNEL-positive follicles, the oocyte had stained, only $63 \pm 11\%$ showed pregranulosa cell staining. All discernible oocytes and pregranulosa cells stained TUNEL positive at 24 and 48 h. Less than 1% of follicles were TUNEL positive in controls (Fig. 5).

Discussion

In this study, we reported a novel xenograft model to test the ovarian toxicity of chemotherapeutics. Because of its use in the

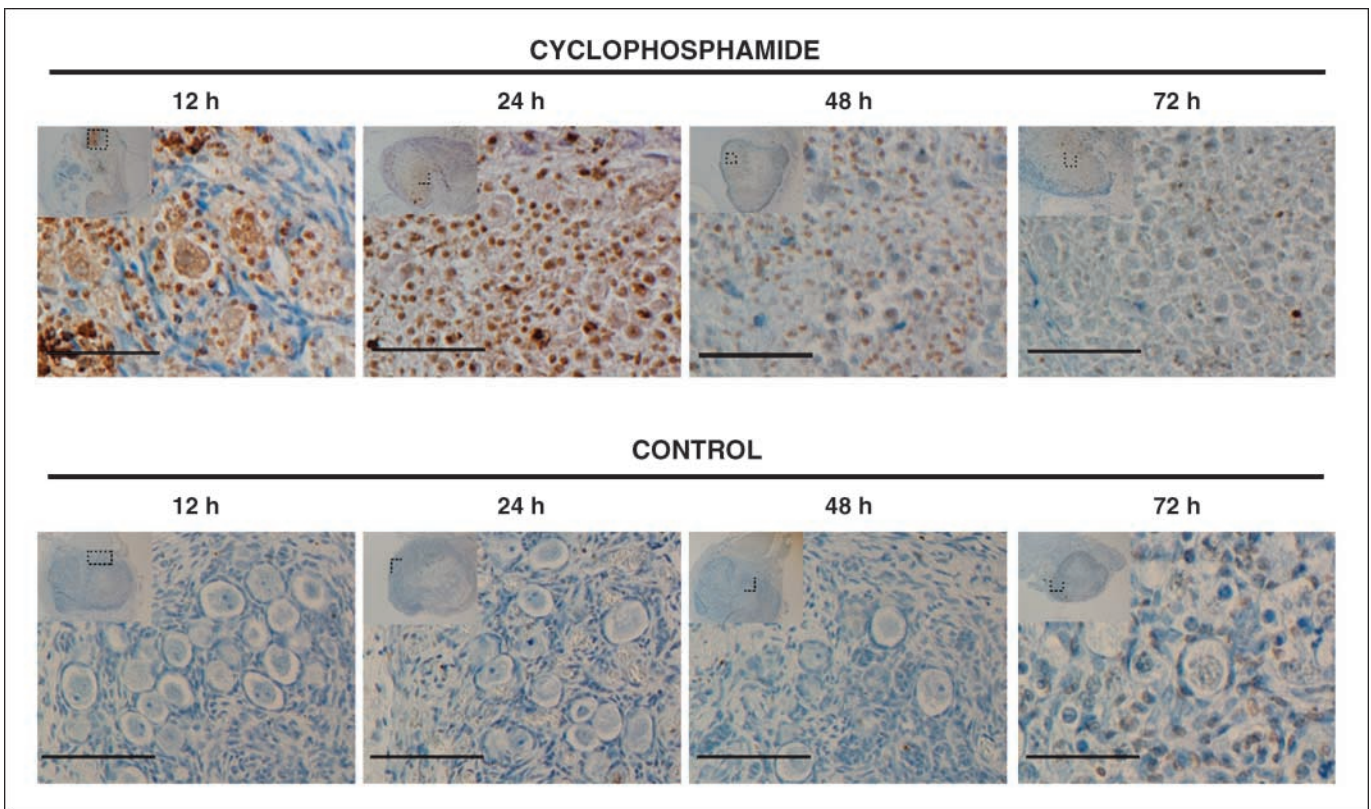


Figure 5. Assessment of cyclophosphamide-induced follicle death by TUNEL assay. Following chemotherapy (top), TUNEL staining peaks at 12 h, decreases in intensity at 24 to 48 h, and fades at 72 h. In control grafts (bottom), <1% of follicles are TUNEL positive. Bar, 100 μ m.

treatment of a wide range of malignancies, including breast cancer, leukemias, lymphomas, and soft tissue tumors, and because there are ample clinical data on its gonadal toxicity (2), we chose cyclophosphamide to validate our model.

Using this model, for the first time, we showed the time course and mechanism of damage of cyclophosphamide in human ovary. In this model, the injection of a single dose of cyclophosphamide caused a 93% reduction in PF density in 48 h, confirming the gonadotoxicity of this drug *in vivo*. Furthermore, we showed by TUNEL assay that cyclophosphamide causes follicle damage by apoptosis. TUNEL staining peaked at 12 h before a significant decline occurred in PF number, indicating that ovarian follicle damage is initiated almost immediately following exposure to cyclophosphamide. As another novel finding, we also showed that the apoptotic cell death affected oocytes before granulosa cells following exposure to cyclophosphamide. Although all of the oocytes were TUNEL positive 12 h after cyclophosphamide exposure, only two-thirds of pregranulosa cells stained TUNEL positive. In the subsequent time points, all granulosa cells and oocytes were stained with TUNEL, indicating that the time course for apoptotic death starts earlier for oocytes. Further research will be needed to determine whether that is the oocyte death, which leads to pregranulosa cell demise, or whether granulosa cells are more resistant to genotoxic insult in humans.

The data that were obtained from our model are not only consistent with the clinical experience based on the cessation of menstruation but are also in keeping with cyclophosphamide's impact on rodent ovaries. At 200-mg/kg dose, cyclophosphamide caused 87% reduction in PF counts 72 h after its administration to mice (10).

Human xenograft models are highly validated in cancer research and played a significant role in drug development in the past 25 years. For example, subcutaneous tumor models predicted clinical response to cancer drugs with 90% certainty and the drug resistance with 97% accuracy (5). Furthermore, a recent study validated the cytotoxic impact of chemotherapeutics on human testicular tissue in a similar xenograft model in nude mice (11). Thus, xenografting seems to be a relevant model to test the impact of cancer drugs on tumors and reproductive tissue.

It has been a dogma that PF reserve is established *in utero* in humans, and that there is no regeneration postnatally (12). However, this dogma has recently been challenged in rodents (13, 14). Moreover, it is a common observation that many women conceive years after experiencing premature menopause as a result of high-dose chemotherapy (15) or even after idiopathic premature ovarian failure (16). In this study, we incidentally observed that the PF counts partially recovered 72 h after chemotherapy. Although this observation raises the possibility of PF regeneration in human ovary, this phenomenon can be specific to fetal ovary. Fetal ovaries have a complete apparatus to function at the adult level at this gestational age, but there can still be remaining mitotic germ cells that can result in the regeneration of follicles. Thus, whereas the use of adult tissue would have been ideal in this model, it would not be practical to obtain healthy human ovarian tissue from young females. Nevertheless, our model can provide a relative assessment of the gonadotoxicity of various chemotherapy agents so that clinicians can choose drugs with lower likelihood of infertility when this is a concern for the patient and when equally effective alternatives exist. There are, however, potential limitations with our model. Fetal ovarian tissue may not be entirely comparable to postnatal tissue because of the presence of mitotic germ cells. Furthermore, access to fetal ovarian tissue from midtrimester abortions may be limited. Our model will also have to be validated using agents other than cyclophosphamide. Overall, fetal ovarian xenografting is a promising model to characterize the gonadotoxic effects of current and emerging cancer drugs without the need for lengthy clinical studies.

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