Sphingosine-1-phosphate suppresses cyclophosphamide induced follicle apoptosis in human fetal ovarian xenografts in nude mice☆

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Objective: To investigate the antiapoptosis effect of sphingosine-1-phosphate (S1P) on human fetal ovarian tissue treated by cyclophosphamide (CTX).
Design: Experimental animal study.
Setting: University center for reproductive medicine and IVF unit.
Animal(s): Female immunodeficient BALB/c nude mice, 6 to 8 weeks old.
Intervention(s): Human fetal ovarian tissue slowly cryopreserved then subcutaneously transplanted in immunodeficient mice.
Main Outcome Measure(s): Follicle survival assessed qualitatively and quantitatively using H&E staining, and cellular apoptosis of the ovarian grafts evaluated using transmission electron microscopy and DNA nick end labeling in situ (TUNEL assay).
Result(s): The alkylating agent CTX caused a substantial follicle loss and apoptotic DNA fragmentation in the ovarian grafts in a period of 2 weeks of transplantation. The S1P treatment significantly prevented follicular apoptosis and maintained primordial follicle population in the grafts.
Conclusion(s): This study shows for the first time that S1P protects primordial follicles in human ovarian grafts from a chemotherapy drug treatment via suppressing follicle apoptosis. (Fertil Steril® 2014;102:871–7. ©2014 by American Society for Reproductive Medicine.)
Key Words: Apoptosis, cyclophosphamide, ovarian tissue cryopreservation, ovarian tissue transplantation, sphingosine-1-phosphate

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As progress has been achieved in anticancer treatments, the cure rate of cancer in children has been greatly improved. Accordingly, quality of life, especially with regard to the preservation of fertility after cancer treatment, has become increasingly important. The damage to fertility from some anticancer therapies to ovaries is irreversible. Alkylating agents, for example, the most destructive among all types of chemotherapy drugs, can act on primordial follicles, decreasing the ovarian reserve and resulting in premature ovarian failure (1, 2).

Therefore, it is important to preserve the fertility of cancer patients before initiating their anticancer treatments.

Cyclophosphamide (CTX) is one of the most frequently used alkylating anticancer drugs. Although the mechanisms underlying how the drug induces follicle damage are still unclear, studies have demonstrated that follicle apoptosis occurs after the treatment with CTX in cancer patients (2, 3), which indicates that it may interfere with the cell decision of fate. A cell fate of apoptosis or growth is related to the balance of ceramides and sphingosine-1-phosphate (S1P). Ceramides suppress cell growth and induce cell apoptosis whereas S1P promotes cell growth and mitosis (4, 5). Morita et al. (6) has demonstrated that administration of S1P before radiotherapy prevents premature ovarian failure in mice. In fact, S1P not only improved cell proliferation but also suppressed cell death. In another study, local administration of S1P in mouse ovaries decreased follicle apoptosis and increased pregnancy rates in a dose-dependent manner (7). The effect of S1P on CTX-treated human ovarian follicle survival and development has yet to be reported.

Primordial follicles are formed as early as 13 weeks of gestation in humans and become abundant from around 16 to 20 weeks of gestation (8, 9). Oocytes are arrested in the diplotene stage of meiotic prophase I and are sensitive to the local ovarian signals and metabolic requirements provided by the surrounding granulosa cells. Cell death is quantitatively a major activity of prenatal oocytes (10, 11). At her time of birth, a woman's ovary has a finite reserve of around 700,000 primordial follicles (12). After natural depletion during fetal and postnatal development, these primordial follicles constitute the reserve pool of germ cells for the full duration of a woman's reproductive life span. Premature ovarian failure results from the loss of the primordial follicle pool in female ovaries.

Studies on the preservation of primordial follicles, using fetal ovaries or adult ovaries, have important potential applications in the clinical treatment of cancer with antichemotherapy drugs. In our current study, we examined the antiapoptosis effect of S1P on the survival and developmental capacity of primordial follicles in cryopreserved human fetal ovaries after the CTX treatment in a mouse xenograft mode.

MATERIALS AND METHODS

Animals

Female immunodeficient BALB/c Nude mice aged 6 to 8 weeks old were purchased from the SLAC animal laboratory in Shanghai and were housed at the SPF facility of Animal Center of Wenzhou Medical Collage, at a temperature of 23–25°C with a 12-hour light/dark cycle. Sterilized food and water were available ad libitum. The animal experiments were reviewed and approved by the research ethics committee of Wenzhou Medical College.

Collection of Human Fetal Ovaries

With informed consent from the patients, fetal ovaries were collected at autopsy from 10 aborted fetuses (23.9 $\pm$ 1.4 weeks of gestational age) within 3 hours. The human research ethics committee of the First Affiliated Hospital of Wenzhou Medical College approved the study.

Reagents

We purchased S1P, Leibovitz (L-15), dimethyl sulfoxide (DMSO), sucrose, ethanol, and sodium pentobarbital from Sigma-Aldrich. The CTX was purchased from Jiangsu Heng Rui Medicine, Ltd. Ethylene glycol (EG), fetal bovine serum (FBS), TWEEN-80, and polyethylene glycol were purchased from ZSBIO.

Cryopreservation and Thawing of Human Fetal Ovarian Tissue

Fetal ovaries were immersed in L-15 medium supplemented with 10% FBS and transferred to the laboratory at 4°C within 2 hours after collection. After dissecting the connective tissue, the ovaries were cut into 3 $\times$ 3 $\times$ 3 mm cubes and placed in cryovials (Greiner Bio-one) containing 1 mL of L-15 medium supplemented with 1.5 M DMSO and 10% FBS for cryopreservation, as previously described elsewhere (13). Briefly, the cryovials were loaded in a Planer Cryochamber (Planer PLC) precooled to 0°C. The vials were cooled at 2°C/min to -7°C and held for 10 minutes, seeded manually, held at -7°C for a further 10 minutes, cooled to -40°C at 0.3°C/min, further cooled to -140°C at 10°C/min, and finally transferred to liquid nitrogen for storage. For thawing the ovarian tissue, the cryovials were rapidly transferred to a water bath at 37°C. The thawed tissues were washed in a serial process of L-15 medium supplemented with 10% FBS and 0.25 M, 0.1 M, and 0 M sucrose for 10 minutes each.

Xenotransplantation Procedure

The immunodeficient nude mice were anesthetized by intraperitoneal injection of 100 μL of sodium pentobarbital diluted 1:4 in phosphate-buffered saline (PBS; Sigma-Aldrich). A pocket was made between skin and muscle layer on back of the mice through a dorsal-vertical incision. Frozen-thawed ovarian tissue fragments were preincubated with either S1P, PET (a solvent for S1P, consisting of 5% polyethylene glycol, 2.5% ethanol, and 0.8% TWEEN-80 in PBS), or PBS for 1 minute at room temperature and then were inserted into the pocket. We injected 5 μL of S1P, PET solvent, or PBS into the pocket according to the pretreatment solution. Finally, the skin incisions were closed with 7/0 silk sutures. The CTX (10 mg/mL) was administrated to recipient mice by intraperitoneal injection at a dosage of 0.2 mg/g 1 hour after xenotransplantation. The ovarian grafts were retrieved 2 weeks after grafting for evaluation. Two tissue fragments were inserted into each pocket. A total of 30 tissue fragments were grafted in the recipient nude mice.

Experimental Design and Groups

Five groups (n = 3 recipients in each group) were designed in the experiments according to the solution that the ovarian tissue had been treated with before grafting. Group A: ovarian tissue was preincubated with 2.0 mM S1P and grafted. Group B: