Sphingosine-1-phosphate prevents chemotherapy-induced human primordial follicle death

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STUDY QUESTION: Can Sphingosine-1-phosphate (SIP), a ceramide-induced death pathway inhibitor, prevent cyclophosphamide (Cy) or doxorubicin (Doxo) induced apoptotic follicle death in human ovarian xenografts?

SUMMARY ANSWER: SIP can block human apoptotic follicle death induced by both drugs, which have differing mechanisms of cytotoxicity.

WHAT IS KNOWN ALREADY: SIP has been shown to decrease the impact of chemotherapy and radiation on germinal vesicle oocytes in animal studies but no human translational data exist.

STUDY DESIGN, SIZE, DURATION: Experimental human ovarian xenografting to test the in vivo protective effect of SIP on primordial follicle survival in the chemotherapy setting. The data were validated by assessing the same protective effect in the ovaries of xenografted mice in parallel.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Xenografted mice were treated with Cy (75 mg/kg), Cy+SIP (200 μM), Doxo (10 mg/kg), Doxo+SIP or vehicle only (Control). SIP was administered via continuous infusion using a mini-osmotic pump beginning 24 h prior to and ending 72 h post-chemotherapy. Grafts were then recovered and stained with anti-caspase 3 antibody for the detection of apoptosis in primordial follicles. The percentage of apoptotic to total primordial follicles was calculated in each group.

MAIN RESULTS AND THE ROLE OF CHANCE: Both Cy and Doxo resulted in a significant increase in apoptotic follicle death in human ovarian xenografts compared with controls (62.0 ± 3.9% versus 25.7 ± 7.4%, P < 0.01 and 76.7 ± 7.4% versus 25.7 ± 7.4%, P < 0.01, respectively). This chemotherapy-induced apoptotic death was reduced both in the Cy+SIP (32.7 ± 4.4%, P < 0.01) and the Doxo+SIP group (27.1 ± 7.6%, P < 0.01) compared with Cy and Doxo groups, respectively. In the Doxo+SIP and Cy+SIP groups, the percentages of apoptotic follicles were similar to those of vehicle-treated controls (P > 0.05). The findings from the ovaries of the severe combined immunodeficient mice mirrored the findings with human tissue.

LIMITATIONS, REASONS FOR CAUTION: The functionality of the rescued human ovarian follicles needs to be evaluated in future studies though the studies in rodents showed that rescued oocytes can result in healthy offspring. In addition, the impact of SIP on cancer cells should be further studied.

WIDER IMPLICATIONS OF THE FINDINGS: SIP and its future analogs hold promise for preserving fertility by pharmacological means for patients undergoing chemotherapy.

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Key words: Sphingosine-1-phosphate / cyclophosphamide / doxorubicin / apoptosis / human ovarian xenograft

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Introduction

With the advent of early cancer diagnosis and more effective individualized cancer treatments, a growing number of young cancer patients become long-term survivors during their reproductive years (Loren et al., 2013). However, many cancer drugs have non-specific effects on healthy tissue, one of the most significant of which is gonadotoxicity. The pool of primordial follicles present in each ovary is irreplaceable and its accelerated depletion induced by chemotherapy leads to premature ovarian failure and infertility. A great majority of cancer survivors have to face ovarian failure and infertility induced by chemotherapeutic drugs (Partidge et al., 2008; Azim et al., 2011). Reproductive concerns account for 72% of the variance in scoring the quality of life in one study, showing how devastating the potential loss of fertility to patients can be (Wenzel et al., 2005).

In the last 15 years, a wide range of options became available for fertility preservation in females facing loss of ovarian function due to chemotherapy. Among those, embryo and oocyte cryopreservation are now considered established methods and the IVF success rates with cryopreserved oocytes appear to have caught up with IVF success rates using fresh oocytes (Cil et al., 2013). Cryopreservation of oocytes and embryos may resolve the issue of chemotherapy-induced infertility, but they do not address the issue of impaired ovarian endocrine function. Given the significant quality-of-life consequences of premature ovarian failure (POF) and the fact that chemotherapy-induced menopause may have even further endocrine consequences due to stromal damage (Soleimani et al., 2011b), preservation of ovarian function may offer additional advantages. Auto-transplantation of previously cryopreserved ovarian tissue is the only fertility preservation approach that can address these reproductive endocrine complications of chemotherapy. In addition, it may be the only option for those who have limited time before chemotherapy (apart from in vitro maturation), or those who already began chemotherapy (and hence cannot undergo oocyte retrieval because of the possibility that DNA damage is already present in oocytes in antral follicles). Furthermore, ovarian freezing is the only option for prepubertal and young children. Nevertheless, ovarian transplantation with cryopreserved tissue is invasive. It still remains experimental due to the novelty of the procedure (Oktay and Karlikaya, 2000) and because of the limited number of pregnancies resulting from this approach (Jeong et al., 2012; Burmeister et al., 2013; Isachenko et al., 2013). A much simpler and non-invasive option would be the administration of a pharmacological agent during chemotherapy to block the apoptotic effects of these agents on ovarian follicles to preserve ovarian function. Unfortunately the preponderance of evidence suggests that ovarian suppression does not achieve this goal (Loren et al., 2013).

Cyclophosphamide (Cy) is an alkylating agent which induces cytotoxicity by creating DNA cross-links, which in turn result in DNA breaks. Doxorubicin (Doxo) is an intercalating agent that damages DNA by inserting itself between DNA bases. Cy (Oktay and Oktay, 2007) and Doxo (Soleimani et al., 2011b) are two common chemotherapeutic agents that have been shown to cause human primordial follicle apoptosis and ovarian reserve depletion (Oktay and Oktay, 2007; Soleimani et al., 2011b). Sphingosine-1-phosphate (SIP) is a naturally occurring sphingolipid, which is an inhibitor of the ceramide-induced death pathway (Bonnaud et al., 2007). As an anti-apoptotic agent, SIP has been shown to decrease the impact of radiation and chemotherapy on the oocyte population in animal models (Paris et al., 2002; Hancke et al., 2007). We have previously shown that SIP improves the success of human ovarian tissue xenografting by enhancing and accelerating angiogenesis and stromal cell proliferation (Soleimani et al., 2011a). However, there is no study showing that SIP can protect human primordial follicles against chemotherapy-induced ovarian follicle death. Therefore, we conducted this study to evaluate the effects of SIP on human ovarian tissue during exposure to chemotherapeutic agents.

Materials and Methods

Ovarian tissue fragments were obtained from consenting female patients and organ donor cadavers (n = 5, age range 16–37) undergoing ovarian tissue slow freezing. The study protocol was approved by the institutional review board of New York Medical College and written informed consent was obtained from all living subjects involved in the study. Animal studies were approved by the Institutional Animal Care and Use Committee at New York Medical College.

Human ovarian cortical graft preparation and xenografting

Human ovarian cortical pieces were prepared as 3 mm³ (3 × 1 × 1 mm) pieces and were transplanted into the dorsal muscles of non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice (Taconic, USA) as previously described (Oktay et al., 1998; Soleimani et al., 2011a). This day was denoted as Day 1. Briefly, ovarian tissue fragments were collected in a modified M199 medium (Gibco, Invitrogen, Carlsbad, CA, USA) while the recipient animals were being anesthetized by Ketamine 75 mg/kg, intraperitoneally (i.p.) (Ketathesia, Dublin, OH, USA) and Xylazine 1 mg/kg, i.p. (AnaSed, Shanandoah, IA, USA). A small incision was made in the skin on the dorsal midline of the recipient animal. Human ovarian tissue pieces were selected randomly and transplanted into the dorsal muscles of NOD-SCID (n = 3/animal) recipient mice with fine watchmakers’ forceps. The skin incision was closed under aseptic conditions. Animals were allowed to recover for 10 days to allow for the xenografts to vascularize maximally (Soleimani et al., 2011a). The xenografted mice then received 10 mg/kg Doxo or 75 mg/kg Cy intravenously. The doses were selected based on previous studies (Oktay and Oktay, 2007; Soleimani et al., 2011a). Human ovarian cortical grafts were recovered 72 h later and analyzed by activated caspase 3 (AC3) immunohistochemistry (IHC) to assess the activation of apoptotic cell death pathways.

Assessing the preventive effect of SIP on Cy and Doxo-induced apoptotic death

SCID mice (n = 3 in each group) xenotransplanted with human ovarian cortical pieces received either SIP (200 µM) or vehicle using Alzet mini-osmotic pumps (Durect, Cupertino, CA, USA). Mini-osmotic pumps were used because of the very short plasma half-life of SIP. A mini-osmotic pump with a flow rate of 8 ml/h was utilized to administer SIP continuously beginning 24 h prior to xenografting and continuing for 72 h after chemotherapy exposure (Days 9–12). Control animals received the vehicle via the identical route.

As a validation step and to determine whether local delivery will result in a systemic effect, the in vivo impact of Doxo and Cy on primordial follicle death was also confirmed by the assessment of AC3 expression in the ovaries of xenografted mice.
Sphingosine-1-phosphate protects ovarian follicles

Immunohistochemistry
Ovarian samples were recovered 72 h after chemotherapy exposure and processed for IHC with activated caspase-3 (AF-835, R&D Systems) antibodies for the detection of apoptosis.

The paraffin-embedded tissue blocks were serially sectioned at 4 μm thickness. The first 10 serial sections were placed in order on glass microscope slides numbered 1–10, the next 10 serial sections were placed on slides numbered 1–10 following the above section and so on, until all sections were placed on a glass slide. Therefore, the adjacent sections on a single slide represented serial sections that are 40 μm apart. Only follicles with a visible nucleus in the oocytes were included in the counts.

For assessing immunohistochemistry results, we used a quasi-quantification scale with: no staining, 0; faint staining, 1; medium staining, 2; and strong staining, 3. Only follicles with oocyte staining at the intensity of 2–3 were counted as positive stained. The follicles with positive granulosa cells only but without positive oocytes were not considered to be apoptotic follicles. The percentage of apoptotic follicles was expressed as 100 × the ratio of apoptotic to total follicle numbers on each slide.

Statistical analysis
The SPSS 17.0 (SPSS Inc., Chicago, IL) software was used for statistical analysis. Continuous data (presented as mean ± SD) were analyzed by one-way analysis of variance followed by the LSD post hoc test. The difference was considered statistically significant if the P-value was <0.05.

Results

Impact of S1P co-treatment on Cy-induced human and mouse ovarian follicle death
Evaluation of the percentage of primordial follicles positive for AC3 expression indicated that Cy causes a significant increase in apoptotic follicle death in human ovarian tissue compared with the controls (Fig. 1A, 62.0 ± 3.9% versus 25.7 ± 7.4%, P < 0.01). S1P co-treatment with Cy was associated with a significant decrease in the percentage of apoptotic follicles compared with the Cy treatment-only group (32.7 ± 4.4%, P < 0.01). To confirm the impact of Cy and the protective effect of S1P on mouse ovarian follicles, and to validate our findings, we analyzed anti-caspase 3 expression in the ovaries of the xenografted mice in a parallel design. In agreement with the findings from human ovarian follicles, Cy resulted in a significant increase in apoptotic follicle death in mouse ovarian tissue compared with the control group (Fig. 1B, 48.2 ± 11.6% versus 28.2 ± 8.9%, P < 0.05), while S1P protected mouse primordial ovarian follicles from Cy-induced apoptotic death (25.6 ± 3.5%, P < 0.05).

Impact of S1P co-treatment on Doxorubicin-induced human and mouse ovarian follicle death
In the Doxo treatment-only group, the percentage of apoptotic follicles increased significantly compared with the controls (Fig. 2A, 76.7 ± 7.4% versus 25.7 ± 7.4%, P < 0.01). S1P co-treatment with Doxo resulted in a significant decrease in the percentage of apoptotic follicles in human ovarian tissue compared with the group receiving Doxo treatment only (27.1 ± 7.6%, P < 0.01).

In parallel, Doxo also resulted in a significant increase in apoptotic follicle death in the ovaries of xenografted mice compared with the controls (Fig. 2B, 46.4 ± 5.4% versus 24.8 ± 5.2%, P < 0.05). Furthermore, S1P co-treatment with Doxo resulted in a significant reduction of mouse ovarian follicle death (32.2 ± 10.8%, P < 0.05 compared with Doxo only).

S1P treatment abrogates chemotherapy-induced human ovarian follicle death
To determine whether S1P co-treatment eliminates chemotherapy-induced apoptotic follicle death, we compared percentages of apoptotic human ovarian follicles in the S1P-treated group to the untreated controls. In the S1P-treated groups receiving either Cy or Doxo, the percentages of apoptotic follicles were similar to those in untreated controls. This results indicated that S1P completely blocked Cy and Doxo-induced apoptotic follicle death (32.7 ± 4.4% versus 25.7 ± 7.4% with P = 0.4 in Cy groups; 27.1 ± 7.6% versus 25.7 ± 7.4% with P = 0.9 in Doxo groups). Similar results were obtained with the concurrent mouse ovary analysis (25.6 ± 3.5% versus 28.2 ± 8.9% with P = 0.6 in Cy groups; 32.2 ± 10.8% versus 24.8 ± 5.2% with P = 0.06 in Doxo groups), validating our results.

Discussion
It is well known that chemotherapy with alkylating agents results in very high rates of POF depending on the dose and the age of the recipient (Oktem and Oktay, 2007; van der Kaaij et al., 2011a). In one study, it has been shown that the risk of POF increases by 23% per year of age at the time of treatment (van der Kaaij et al., 2012).

Apoptotic oocyte death has been identified as the main mechanism behind the large loss of germ cells and related POF in rodent studies (Tilly and Kolesnick, 2002) and in human ovarian xenograft models (Oktem and Oktay, 2007; Soleimani et al., 2011a; Jeong et al., 2012). Previous research in our lab has shown that both Cy (Oktem and Oktay, 2007) and Doxo induce apoptosis in mouse and human ovarian tissue (Soleimani et al., 2011b), and do so mainly by inducing double-strand DNA breaks in primordial follicle oocytes. Doxo also induces significant stromal and microvascular damage and resultant ischemia (Soleimani et al., 2011a) but the contribution of this process to overall follicle loss process has not been quantified.

Fertility preservation for female cancer patients usually focuses only on cryopreservation of eggs, embryos and ovarian tissue. However, long-term health issues related to POF are also major concerns to those patients. While transplantation of cryopreserved ovarian tissue can restore ovarian endocrine function (Oktay and Karlikaya, 2000; Oktay et al., 2001, 2004; Oktay and Tilly, 2004), ovarian transplantation is usually performed only when there is a desire to have children as the life span of these grafts maybe limited (Soleimani et al., 2011a). As the only proposed pharmacological method for protecting the ovarian endocrine at present, the effect of gonadotrophin-releasing hormone analog therapy during chemotherapy is still controversial (Oktay et al., 2007; Munster et al., 2012; Partridge, 2012) and ineffective according to numerous recent studies (Ayensu-Coker et al., 2012; Yang et al., 2013). Thus the development of a pharmacological agent that can both preserve fertility and gonadal function would be a breakthrough in Reproductive Medicine.
Figure 1 Expression of activated caspase 3 (AC3) in human and mouse ovarian tissue treated with cyclophosphamide and sphingosine-1-phosphate (S1P). (A) Bar graph shows higher percentage of AC3-positive follicles in human ovarian tissue treated with cyclophosphamide and S1P compared with the control group. The photomicrographs represent AC3 staining of human ovarian tissue in different groups. (B) Bar graph shows higher percentage of AC3-positive follicles in mouse ovarian tissue treated with cyclophosphamide and S1P, compared with the control group. The photomicrographs represent AC3 staining of human ovarian tissue in different groups. Black arrow, AC3-negative follicle; red arrow, AC3-positive follicle; Cy, cyclophosphamide.
S1P suppresses ceramide-induced apoptosis probably by activating Akt via a PI3K-dependent pathway (Nakahara et al., 2012). The activated Akt phosphorylates GSK-3 and suppresses caspase-3 activity (Greenspon et al., 2009). Thus, the balance between the levels of ceramide and S1P determines the fate of the cell (Cuvillier et al., 1996). Many studies in rodent models in the past decade and one study in a primate model have confirmed the efficacy of S1P in protecting oocytes against chemotherapy and radiotherapy-induced death (Revel and Laufer, 2002; Hancke et al., 2007; Peng et al., 2012). However, human applications have remained limited, and the S1P's potential protective effect on human ovarian tissue in response to chemotherapy was uncertain until the performance of our study.

In this study, we showed that controlled long-term delivery of S1P to xenografted human ovarian tissue rescues ovarian follicles from

\[\text{Figure 2} \text{ Expression of activated caspase 3 (AC3) in human and mouse ovarian tissue treated with doxorubicin and sphingosine-1-phosphate (S1P).} \]

(A) Bar graph shows higher percentage of AC3-positive follicles in human ovarian tissue treated with doxorubicin and S1P compared with the control group. The photomicrographs represent AC3 staining of human ovarian tissue in different groups. (B) Bar graph shows higher percentage of AC3-positive follicles in mouse ovarian tissue treated with doxorubicin and S1P compared with the control group. The photomicrographs represent AC3 staining of human ovarian tissue in different groups. Black arrow, AC3-negative follicle; red arrow, AC3-positive follicle; Doxo, doxorubicin.
chemotherapy-induced cell death. Cy and Doxo caused apoptotic primordial follicle death in accordance with our previously published reports (Soleiman et al., 2011b). Initiating S1P 24 h prior to chemotherapy and continuing it for 72 h post exposure effectively eliminated the apoptosis induced by these chemotherapeutic reagents.

The strength of our study is its use of human ovarian tissue in a well-established xenograft model and parallel validation of the results with the mouse ovarian tissue, though the number of mice used in the validation study was relatively small. While our results are highly encouraging, combined with previous rodent data showed that S1P-rescued oocytes can result in healthy newborns and do not propagate genetic damage in the offspring (Paris et al., 2002; Hancke et al., 2007), even though the functionality, chromosomal and genomic integrity of the rescued human ovarian follicles needs to be evaluated in future studies. Furthermore, the influence of S1P on existing tumor cells has to be evaluated. Even though we administered S1P locally, it had systemic effects as shown by its protective effect on the in situ ovaries of the xenografted mice. Providing assurance however, a recent study showed that S1P treatment did not reduce the effectiveness of chemotherapy on S180 sarcoma cells in a rodent model (Peng et al., 2012). However, no similar data are available in a human ovarian xenograft model. Future studies should focus on the impact of S1P on existing human tumor cells in the host and developing long-acting S1P analogs that can be targeted to human oocytes. It must be added that gonadotoxic chemotherapy agents are increasingly commonly used in the treatment of non-cancer conditions especially in the context of bone marrow transplantation agents that act through differing mechanisms against cancer cells. This finding may eventually lead to the development of pharmacological strategies to preserve ovarian function and fertility in women undergoing cancer treatments.

Authors’ roles
K.O. conceived the idea, designed the experiments, interpreted the data, wrote and revised the manuscript and obtained the initial funding. F.L. conducted the experiments, analyzed and interpreted the data and wrote and revised the manuscript. V.T. conducted the experiments and participated in manuscript writing. S.L. participated in the experiments and contributed to the discussion. C.C. obtained supplementary funding and participated in the experiments. P.D.S. participated in manuscript writing, data interpretation, and obtained supplementary funding.

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Conflict of interest
The authors declare that there is no conflict of interest that could prejudice the researchers in the impartiality of the research reported.

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