Minimizing the doxorubicin-Induced gonadotoxicity by sphingosine-1-phosphate analogue FTY720

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Abstract: Preserving future fertility in young women undergoing chemotherapy is an important issue that has gained recent ubiquitous interest. Despite various options being available, such as oocyte/embryos cryopreservation, ovarian tissue cryopreservation or gonadotropin-releasing hormone agonists’ cotreatment, more work must be done in improving efficacy and availability. A newer alternative, Fingolimod (FTY720), an agonist of Sphingosine-1-phosphate, is under investigation as a possible non-invasive, fertility sparing therapy. Sphingosine-1-phosphate is an antagonist to the pro-apoptotic molecule, ceramide. By inhibiting chemotherapy induced apoptosis, a woman’s fertility can be spared. When administered alongside systemic doxorubicin, this study measured the ability of FTY720 to prevent doxorubicin induced gonadotoxicity in juvenile female rats, determined by ovarian follicle counts. Eight juvenile female rats (2-3 months of age) were divided into 4 pairs, representing 3 experimental and 1 control group. Two rats received systemic doxorubicin (DOXO) and intra-ovarian FTY720 injection in the left ovary; 2 rats received systemic doxorubicin and vehicle in the right ovary; 2 rats received vehicle vector in the right ovary, and FTY720 in the left ovary. Vehicle vector and FTY720 were administered 5 days prior to sacrifice, and doxorubicin was administered 4 days prior to sacrifice. Primordial, primary, secondary, antral, and Graafian follicles as well as corpora lutea were counted. There was a significant difference (P<0.05) in the number of small follicles (primordial and primary) between the 4 groups. There was no significant difference in any follicle counts between the control and the FTY720 + doxorubicin groups. Primordial, primary, secondary, antral/Graafian and corpora lutea counts did not differ between the FTY720 treated groups and the control. Our preliminary results suggest FTY720’s may minimize follicle destruction despite chemotherapy, and implicate on a possible future use of FTY720 in the preservation of chemotherapy-induced gonadotoxicity.

Keywords: Premature ovarian failure, doxorubicin, fertility preservation, gonadotoxicity, FTY720, fingolimod

Introduction

New developments in cancer treatment have significantly increased survival in young cancer patients. However, this survival does not guarantee quality of life. Young women undergoing treatment for malignancies or autoimmune diseases face a potential threat of premature ovarian failure [POF] and infertility from the gonadotoxicity of chemotherapeutic agents [1]. Regarding infertility, many women receive poor counseling outlining their fertility-sparing options. The American Society of Clinical Oncology recommends fertility counseling for young patients [2]. Every year, one out of 49 women under the age of 40 is diagnosed with a malignancy [3]. The most commonly encountered malignancies of this age group are lymphomas, leukemias, melanoma, breast cancer, cervical cancer and sarcomas [4, 5].

Not all chemotherapeutic agents are equally damaging, and can be categorized into three general groups: high risk, medium risk and low risk. The high risk agents include busulphan, chlorambucil, melphalan, procarbazine, ifosfamide, cyclophosphamide, and chlorambucil. Medium risk agents include doxorubicin, paclitaxel, cisplatin and carboplatin. Low risk agents include bleomycin, methotrexate, vincristine, mercaptopurine and 5-fluorouracil [3]. Chemotherapy causes insult to germ cells,
either directly or indirectly, accelerating the depletion process and puts a young woman at risk of POF. Alkylating agents, such as cyclophosphamide, are particularly destructive to gonadal tissue. Acting by a not entirely known mechanism, the gonadotoxic chemotherapy can bring about POF resulting in early onset of menopause and subsequent infertility. Many chemotherapeutic agents target rapidly dividing cycling cells such as in the gastrointestinal tract and bone marrow. However, the female gonads are particularly sensitive and vulnerable. Human females are endowed with roughly two million follicles, at birth. Due to the natural atretic processes within the ovary, the reserve is depleted with advancing age, with the final outcome being menopause [6]. Thus, the age during which chemotherapy is started is very influential in the extent of the destructive process. There are additional comorbidities associated with the chemotherapy-induced hypoestrogenic state, such as bone demineralization, osteoporosis, increased risk of coronary artery disease and sexual dysfunction [5-8]. Chemotherapeutic agents are also applied to women suffering from non-neoplastic conditions, such as Systemic Lupus Erythematosus (SLE), systemic sclerosis, Wegener’s granulomatosis, and polyarteritis nodosa. The drug of choice in these cases is cyclophosphamide, one of the most gonadotoxic drugs [1, 8]. 25-50% of young, reproductive aged women treated with cyclophosphamide for SLE develop POF [8].

Currently, few measures are available to preserve future reproductive capability. Ovarian cortical transplantation is one option. However, it is experimental, invasive, requiring a surgical procedure shortly before beginning treatment. In certain patients this measure may be dangerous, in particular, leukemia patients, as there may be ovarian infiltration with malignant cells [9, 10]. Reintroduction of transplanted ovarian tissue could result in disease relapse because of undetected minimal residual disease [8, 10, 11]. About 40 deliveries have been reported so far using this method. Given its invasive nature, risks, and limited availability, it may not be a ubiquitous option [2]. Oocyte retrieval is another option for eventual use by in-vitro fertilization. However, in this modality, a treatment delay of almost 2 weeks may be necessary [8, 10, 11]. For minimizing chemotherapy delay, ovarian stimulation may begin anytime during the menstrual cycle and retrieval of immature oocytes and in vitro maturation is optional [8, 10, 11]. Besides the more invasive procedures, concurrent pharmacotherapy, with gonadotropin releasing hormone agonists (Gn-RH-a) is a promising option [8, 11]. A follow-up study of children under 15 years of age who received MOPP therapy for Hodgkin’s Lymphoma revealed that only 13% of girls suffered from POF whereas 83% of their male counterparts suffered azoospermia [6, 13]. One of the standard chemotherapy regimens administered for Hodgkin’s Lymphoma is Adriamycin (Doxorubicin), Bleomycin, Vinristine, Dacarbazine (ABVD). Another gonadotoxic protocol followed is Bleomycin, Etoposide, Adriamycin, Cyclophosphamide, Oncovin, Procarbazine, and Prednisone (BEACOPP). GnRH agonists, have been shown to have protective effect on ovarian function against gonadotoxic chemotherapy [11, 14]. Only one histological study has been completed in primates, on Rhesus monkeys, revealing this protective effect. GnRH agonists administered in tandem with cyclophosphamide showed significantly less follicular decline than cyclophosphamide administered alone [15]. Even in the subset of patients who have undergone stem cell transplantation, some have successfully conceived after receiving GnRH agonist during treatment [3]. In addition to the induction of a prepubertal milieu, GnRH agonists may act by reducing the utero-ovarian perfusion, decreasing the total amount of cytotoxic drugs delivered to the ovaries and possibly up regulate an anti-apoptotic molecule, Sphingosine-1-Phosphate (S-1-P) [1, 8, 11], a lipid molecule that functions as an antagonist to the pro-apoptotic molecule, ceramide. Morita et al. were able to show the inhibitory effect of S-1-P on apoptosis in mice oocytes following the administration of doxorubicin and radiotherapy [16]. Doxorubicin is another important chemotherapeutic agent used in the treatment of malignancies that afflict young women. In vitro, young female mice that were deficient in acid sphingomyelinase or treated with S-1-P were resistant to the gonadotoxic effects of doxorubicin [16]. Another component of the apoptotic pathway, acted on by doxorubicin, is the Bax gene, which is a member cellular apoptotic pathway [8]. In mice, the disruption of the Bax gene inhibits doxorubicin gonadotoxicity [1, 17]. In both mouse and human ovaries, doxorubicin was shown to induce caspase apoptotic pathways by the accumulation of double-stranded DNA breaks (DSBs) by γH2AX. γH2AX
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is a marker for DNA damage and is a trigger for the cell to enter apoptosis [18, 19]. Other studies indicate that doxorubicin may upregulate the production of p53, resulting in activation of apoptosis [5, 20].

One of the future endeavors of fertility sparing treatment is the possible use of S-1-P agonists, such as FTY720 (Fingolimod, 2-amino-2-[2-(4-octylphenyl)ethyl] propare-1,3-diol hydrochloride). FTY720 is a more recently approved second line therapy for the treatment of Multiple Sclerosis, and acts as an immune modulator by reducing the number of peripherally circulating CD4 and CD8 cells [21, 22]. Additionally, FTY720 has been successful in preventing renal and liver allograft transplant rejection, by a similar immune modulation process [23]. In vivo, FTY720 is phosphorylated by sphingosine kinase, transforming it into a potent agonist of S-1-P, particularly for the S-1-P1, 3, 4 and 5 receptors [18, 24]. We attempted to evaluate the possible application of FTY720 for minimizing chemotherapy induced gonadotoxicity. Zelinski et al. [25] compared FTY720 with S-1-P in the prevention gonadal insult in Rhesus monkeys following intensive and targeted ovarian radiation. FTY720 was introduced prior to the radiation via intraovarian catheters. Within 4 months post-irradiation, all 3 females treated with FTY720 resumed menstrual cyclicity, and 2/3 became pregnant and delivered healthy offspring. Histological analysis showed significantly more primordial, primary and secondary follicles in the ovaries of females treated with FTY720. This possibly indicates a protective effect when using FTY720 as a fertility sparing method [25].

In this study, we aimed to evaluate FTY720 efficacy as an inhibitor of doxorubicin induced gonadotoxicity in rat ovaries. We intended to examine if FTY720 may exert an observable, histological protective effect on the ovaries of chemotherapy-exposed rats.

**Materials and methods**

**Animals**

This study was conducted using 8 juvenile virgin female Sprague-Dawley rats (Harlan, Jerusalem Israel), (F1-F8), aged 2-3 months. All experimentation was conducted under the compliance of the animal care laws, ethics number IL-0460310. The animals were housed 2-3 to a cage, and water and food were provided *ad libitum*. The 8 rats were randomly divided into 4 groups (3 experimental groups, and 1 control group, each containing two rats).

Group 1 (F3 and F6) received intra-ovarian injection of 10 mM FTY720 (Selleck Chemicals, Houston TX) diluted in 0.1 mL vehicle vector of polyethylene glycol, ethanol and Tween 20 (PET) (Biological Industries, Beit-Haemek, Israel) in the left ovary, and 0.1 mL PET in the right ovary.

Group 2 (F2 and F7) received intra-ovarian injection of 0.1 mL PET in the right ovary, nothing in the left ovary and systemic (intra-peritoneal) doxorubicin (7.5 mg/kg) (Pharmachemie BV, Haarlem, Holland).

Group 3 (F4 and F8) received intra-ovarian injection of 0.1 mL PET in the right ovary, 10 mM FTY720 diluted in 0.1 mL PET in the left ovary and systemic doxorubicin (7.5 mg/kg).

Group 4 (F1 and F5) received intra-ovarian injection of 0.1 mL PET in the right ovary, and nothing in the left ovary.

**Treatment**

The PET and FTY720 were injected one week prior to sacrificing the rats, and the doxorubicin was administered one day after FTY720 or vehicle injections. Following the treatments, the animals were monitored for general health, and signs of deterioration. On the day of sacrifice, the animals were sedated with ketamine and xylazine, and the ovaries and other organs (liver, kidney, lung, heart and skeletal muscle) were excised. The rats were killed by cardiac exsanguination. The additional organs were immersed in formaldehyde [formalin] for future toxicology studies.

**Histology**

The ovaries were carefully dissected and fixed in formalin. The ovaries were then embedded in paraffin-paraplast plus (Leica Biosystems Richmond, IL, USA) blocks for histology. 7 μm serial sections were cut using a microtome, applied to microscope slides, and stained with Mayer’s Hematoxylin and Eosin (both from Bio Optica Milano, Milano Italy).

**Follicle counts**

Follicle counts were performed using every 7th section on the slides. The follicles considered...
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Figure 1. A representation of the follicle counts performed following the effects of each treatment (Control, FTY720 + DOXO, DOXO, and FTY720). The columns represent the mean numbers of follicles with standard error bars. *P<0.03 between DOXO and the other groups regarding small follicles, but no difference between groups regarding more developed follicles.

Figure 2. A multi-group comparison of numbers of small follicles (primordial and primary). The columns represent mean follicle count ± standard error. Histological examination of the ovaries showed a significant difference between the numbers of small follicles (primordial and primary) counted in each of the 4 group, *P=0.0149. There were also significantly more small follicles in the control group when compared to the DOXO only group, **P=0.026. Additionally, when the FTY720 + DOXO were compared with the DOXO group, a statistically significant ***P=0.0136 was calculated, using the Student’s t-test. However, when the Mann Whitney test was used, a non-significant *P=0.0714 was calculated.

Figure 3. A comparison of the control and FTY720 + DOXO group. Columns represent follicle counts ± standard error. Difference is non-significant (P>0.4). This preliminary data suggests the potential ability for FTY720 to minimize DOXO induced gonadotoxicity.

Figure 4. In a comparison of the FTY720 + DOXO and the pure DOXO group, Using parametric testing (Student’s t-test) a significant P=0.0136 was calculated. The columns represent mean follicle counts ± standard error.

In this study were primordial, primary, secondary, antral, Graafian and corpora lutea. Only small, preantral follicles containing a visible oocyte were counted as such. The number of fields per slide was also counted. The classification of the follicles was as follows: primordial follicles consist of few or a layer of very flat granulosa cells surrounding the oocyte, primary follicles consist of a single layer of cuboidal granulosa cells surrounding an oocyte, secondary follicles consist of several layers of cuboidal granulosa cells surrounding an oocyte without
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a fluid-filled antrum, and antral follicles consist of a fluid pocket surrounding the oocyte. Additionally, all corpora lutea were counted. In order to avoid any observer bias, the slides were coded by a different individual.

**Statistical analysis**

Animals treated with PET or nothing (−) were combined into one group since there was no difference between these subgroups. Nonparametric testing (Mann-Whitney) was performed to ensure there was no statistical difference. The calculated P-values were 0.8, 0.26-67, 0.2 and 0.2 for small, secondary, antral/ Graafian and corpora lutea, respectively. Animals treated with either doxorubicin or doxorubicin + PET were combined into a single group, using the same calculations mentioned previously. Differences between the four groups and follicle counts were assessed using the Kruskal-Wallis ANOVA Test. Multiple pairwise comparisons (of the four groups) were made using the Mann-Whitney U-Test. Parametric test was performed using the Student’s T-test on one occasion, when comparing the DOXO and FTY720+DOXO groups. Results were considered statistically significant when P<0.05. All calculations were carried out using GraphPad Prism 6 ® San Diego, CA USA).

**Results**

In this study, we were mainly interested in whether FTY720 had a protective effect on the ovaries following exposure to doxorubicin. The protective effect was assessed by counting the number of small follicles (primordial and primary), as these most closely represent remaining follicular reserve than the other follicle types. When a comparison of small follicle counts was
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made with all four groups, a statistically significant difference ($p=0.0149$) was found (Figure 1). Additional statistical testing of the remaining follicle groups (secondary, antral/Graafian and corpora lutea) showed no significant differences between each other. Individual comparisons between pairs of groups (considering all follicle types) were performed. When compared with the control, FTY720 treated animals did not possess significantly different numbers of small follicles (112.5±64.50 vs. 136.7±51.32, $P=0.9286$). This suggests FTY720 may not offer a benefit to the natural degeneration of follicular reserve in healthy ovaries. The control group had significantly more small follicles than the group treated with doxorubicin (136.7±51.32 vs. 15.50±8.217, $P=0.026$) (Figure 2). In the DOX0+FTY720 group compared with the control, no significant difference in number of small follicles was demonstrated (268.5±16.50 vs. 136.7±51.32, $P=0.4286$) (Figure 3). However, when the DOX0+FTY720 group was compared with animals receiving only doxorubicin, a significant difference was noticed (268.5±16.50 vs. 15.50±8.217, $P=0.0136$ with the Student’s t-test, but not when the Mann Whitney test was used, $P=0.0714$), even though the DOX0+FTY720 group clearly appeared to have more small follicles (Figure 4).

The follicles in the doxorubicin treated animals looked poorer and less distinct in comparison to the control group or the doxorubicin + FTY720 treated animals, in most [but not all slides] (Figures 5–8). There was no morphological difference between the control group and the doxorubicin + FTY720 group, in respect to any follicle type (Figures 5–8). However, it was not possible, to a “blinded” observer, to tell exactly the treatment by looking at the slides, suggesting that the morphological appearance per-se is not diagnostic.
**Discussion**

Preservation of future fertility in young female cancer patients is a crucial issue to address, especially in a world of increased survival and successful treatment. Unfortunately, the gonadotoxicity of many chemotherapeutic agents leaves many women with POF and subsequent infertility. The need to prevent these late effects is crucial. With limited options available to women undergoing chemotherapy and radiotherapy, the medical community is now looking to turn to less invasive, fertility sparing agents. The only current, non-invasive fertility sparing therapy is GnRH agonist, and there is controversy over its efficacy, despite recent publications supporting this modality [1, 3, 8, 26-30]. Some groups have questioned the reason for creating a pre-pubertal ovarian milieu, as young children requiring bone marrow transplants may suffer POF. Existing methods, in particular ovarian cryopreservation, may enable for future pregnancies following chemotherapy and neglect to address the detrimental effects of POF. Previously, studies utilizing mice, rats and Rhesus monkeys have shown that the most important follicles for preserving fertility are small follicles, such as primordial and primary follicles. Recent studies in Rhesus monkeys demonstrated FTY720's fertility sparing effects despite intensive ovarian irradiation [25]. Radiation and high dose alkylating agents' chemotherapy are both extremely gonadotoxic, and act in different ways to cause the damage, but the end results are comparable. Although this recent study [25] was without visualization of the ovarian histology, fertility was quantified by the monkeys' reproductive success. Studies using doxorubicin treated mouse models have successfully shown the protective effects of S-1-P. Given that FTY720 is a long-acting S-1-P mimetic, it is conceivable that its behavior in vivo is similar [16, 23-25].

We found that FTY720 may have a visible protective effect on the follicular reserve in rat ovaries. The conclusion can most properly be made qualitatively, given that the study was underpowered due to the samples' size. The ovaries treated with FTY720+ DOXO had similar numbers of primordial follicles as the control. This suggests that FTY720 may be able to prevent gonadotoxicity and preserve small follicles. When the FTY720+DOXO group was compared with the DOXO alone group, no statistical significance was found using nonparametric testing, \( P=0.0714 \). However, when compared using the Student's t-test, a significant difference was found, \( P=0.0136 \). The discrepancies in the significance are due to the low power of the study. However, the results do suggest that FTY720 may prevent chemotherapy induced gonadotoxicity. The study must be repeated and validated with larger group samples. The differences in the follicle counts (of the secondary, antral, Graafian and corpora lutea) between the control group and the FTY720 + DOXO were not significant. The effects of FTY720 were only tested on one dose of doxorubicin (7.5 mg/kg). Future experimentation must include greater sample numbers. Additionally, testing must be done to make sure that FTY720 does not negatively impact the course of the chemotherapeutic treatment. The smaller sample sizes in this study contributed to the low power, but descriptively, we were able to see a potential benefit of using FTY720. An area that must be explored in the future is if FTY720 can be similarly fertility sparing when offered alongside the most gonadotoxic, alkylating agents, like cyclophosphamide or combination therapies such as BEACOOP or BuCy [Busulfan and Cyclophosphamide].

One of the largest challenges is being able to extrapolate these results to future use in humans. Rat ovaries are surrounded by a bursal sac which allows any therapy to be in close contact with the target tissue. Future work must be completed to determine how FTY720 can be effectively, and minimally invasively introduced into human ovaries, without possibly diminishing the chemotherapeutic effect on the malignant cells [25].

If future, larger studies will validate our preliminary observations, the great endeavour remains to exploit the possible beneficial effects of FTY720 cotreatment with gonadotoxic chemotherapy towards fertility preservation in human. The systemic co-administration of FTY720 or S1P before and in parallel to chemotherapy is possibly dangerous, since the anti-apoptotic effect of these agents may decrease the efficiency of chemotherapy on malignant cells. The other possibility is targeted delivery to the ovaries. However, since human ovaries do not possess an ovarian bursa, like in rodents, repetitive
administration may be necessary, raising the risks of infection and bleeding in patients who are immunocompromised and thrombocytopenic, anemic, and leukopenic due to chemotherapy. Therefore, continuous S1P or FTY720 infusion through intraovarian catheters, like what has been experimentally done in monkeys [25], is also not practical and possibly dangerous. Furthermore, in case of ovarian malignancy and in systemic diseases such as leukemias, where ovarian involvement is not an infrequent occurrence, [27] one has to be careful not to diminish the effect of chemotherapy to successfully combat the ovarian metastases. In such cases even the gonadal targeted delivery may be possibly dangerous. Therefore modalities to safely rule out ovarian involvement, such as PCR of markers of leukemic cells and other possible markers, on ovarian biopsies at laparoscopy done for ovarian tissue cryopreservation, should be accomplished [31].

In cases where the ovarian involvement is unlikely or apparently ruled out, the targeted delivery of FTY720 to the gonads may necessitate a vector which will bind FTY720 and release it to the ovaries only, without systemic contamination, or intraovarian injection. The development of these modalities, and the practical extrapolation of these theoretical possibilities is an endeavour of high technological and scientific merit.

Raising this glove is obviously a great challenge

In summary, addressing the issue of the gonadotoxicity of chemotherapeutic agents is of utmost importance to the enhanced quality of life of the young woman after cancer treatment. There may be benefits to using multiple methods to preserve fertility, as the existing options have not been proven 100% successful [27]. Therefore, to maximize the chances of conceiving, multiple modalities should be offered. The availability of convenient or minimally invasive options is limited, so exploring the potential benefits of FTY720 are valuable. Preliminary data from this study suggests the further exploration of this therapy for fertility preservation. Objectively, FTY720 was able to minimize the gonadotoxicity of doxorubicin, mainly by focusing on the ability to preserve small (primordial and primary) follicles.

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