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Short Communication

Long-term adverse effects of cyclophosphamide on follicular growth and angiogenesis in mouse ovaries

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ABSTRACT

The adverse effects of the anti-cancer agent cyclophosphamide (CTX) on follicular growth and ovarian angiogenesis were investigated in mice. CTX treatment irreversibly induced a loss of follicles through apoptosis and decreased microvascularization of the corpora lutea and follicles in a dose-dependent manner. Our findings demonstrated that CTX adversely affected the ovaries indicating the need to support an awareness of fertility preservation before chemotherapy is initiated.

1. Introduction

Current advances in treatment have substantially increased the survival of women of reproductive age with cancer, but their quality of life deserves more attention. Chemotherapy may cause a loss of fertility due to premature ovarian failure [1,2], and it has been well documented that this failure can be immediate [3,4]. Alkylating agents such as cyclophosphamide (CTX) are a mainstay of chemotherapy regimens used to treat cancer and autoimmune diseases; however, CTX treatment can lead to impaired fertility or ovarian failure as a result of follicular destruction [5,6]. Previous studies have shown that follicular decline in the CTX-treated ovaries occurred in a dose-dependent manner, and apoptotic changes in granulosa and theca cells have been identified as the mechanism leading to the follicle loss [7,8]. Furthermore, the apoptotic death of the cells began immediately after the exposure to CTX [9]. Despite many efforts to understand this phenomenon, details of the mechanisms underlying the CTX-induced ovarian failure, and whether the failure can be reversed with time, are still unclear. Therefore, the current study was designed to examine the long-term effects of various doses of CTX on number of follicles and apoptotic ovarian cells at different time-points following the treatment. We also investigated whether ovarian angiogenesis, assessed as a number of capillary blood vessels, was impaired after the CTX treatment.

2. Materials and methods

C57BL/6 female mice (Japan SLC Inc., Japan) were housed under a 12 h light:dark cycle and were fed *ad libitum*. Three

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hundred female mice that had experienced at least two 4-day reproductive cycles were selected and randomly divided into six groups. Group 1 (control group) was treated with a single intraperitoneal (ip) injection of saline. Groups 2–5 received a single ip dose of 200, 300, 400 or 500 mg/kg of CTX (Sigma Aldrich, St. Louis, MO, USA), respectively. Fifteen mice from each group were sacrificed three days after the injection to collect the ovaries. Other sixty mice treated with the lowest (Group 2) and second highest (Group 4) dose of CTX were sacrificed additionally one, two, four or eight weeks after the CTX treatment. The ovaries were collected from Group 4 because the dosage administered to Group 5 was lethal to more than 50% of mice. In addition, to evaluate a metronomic treatment regimen, Group 6 received eight injections of 50 mg/kg CTX on alternate days. The ovaries were removed three days or one week after the last injection and they were either frozen for RNA extraction or fixed in Bouin's solution and embedded in paraffin wax. The embedded ovaries were serially sliced into 5-μm sections and mounted on slides before staining with hematoxylin and eosin. Ovarian follicles were classified as primordial, primary, secondary or antral follicle, and the number of each stage follicles per square millimeter of ovarian tissue was evaluated.

DNA damage was assessed by performing terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) and staining with the ApopTag-peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA, USA). In brief, the slides were deparaffinized in xylene and re-hydrated in a graded ethanol series. The slides were treated with proteinase K at room temperature (RT) for 15 min followed by incubation in 3% hydrogen peroxide for 15 min to inactivate the endogenous peroxidases. After brief exposure in equilibrium buffer, the slides were incubated in terminal deoxynucleotidyl transferase buffer at 37 °C for 1 h. The terminal transferase was detected by staining with streptavidin/biotin/peroxidase at 37 °C for 30 min followed by diaminobenzidine (DAB) for 5 min. Ovarian sections on the slides were counterstained with hematoxylin and dehydrated prior to microscopic examination.

Immunohistochemical analysis was used to detect the expression and cellular distribution of CD34, a marker for newly formed vascular endothelial cells, in the ovarian sections [10,11]. The sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and blocked with 3% bovine serum albumin (BSA) in Ca^{2+} and Mg^{2+}-free Dulbecco phosphate-buffered saline. The sections were incubated with rat anti-mouse CD34 antibody (1:50, abcam, Cambridge, MA, USA) overnight at 4 °C, washed, and incubated with goat anti-rat secondary antibody conjugated to Alexa Fluor 594 (1:400, Invitrogen, Carlsbad, CA, USA) for 30 min at RT. The sections were counterstained with Hoechst 33342 and examined with confocal microscopy (Keyence BZ-9000) to determine the distribution of CD34 positive cells around the follicles. Furthermore, the number of cells expressing CD34 per mm^2 in the corpora lutea was also calculated.

Expression of mouse *vasa homologue* (*MVH*) and *vascular endothelial growth factor* (*VEGF*) was evaluated by quantitative real-time reverse-transcribed polymerase chain reaction (qRT-PCR). Total RNA was extracted from ovaries with TRIzol (Invitrogen) and purified using the PureLink™ RNA mini kit from Ambion (Carlsbad, CA, USA). cDNA was obtained by reverse transcription of 5 μg of total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in 20 μL reactions using primers from the TaqMan® Gene Expression assays for *MVH/DDX4* (Applied Biosystems) and *VEGF* (Applied Biosystems) with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) in a StepOnePlus™ Real-time PCR System (Applied Biosystems). Each sample was assayed in duplicate and results were normalized to glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*). Relative quantification of gene expression was analyzed by the standard curve method using StepOne version 2.1 software. The data are presented as means ± SEM. One-way analysis of variance followed by the Fisher *post hoc* test or Student's *t*-test were used to evaluate differences between groups. p-Values <0.05 were considered significant.

3. Results and discussion

The ovaries of all groups, examined three days after the CTX treatment, had the same follicular populations; however, the total number of follicles per mm^2 of ovarian tissue was significantly reduced in the CTX groups in comparison with the control group, regardless of the CTX dosage (Fig. 1A and B). The number of atretic follicles was higher in the ovaries treated with CTX than in control ovaries (Fig. 1A and C). All CTX-treated groups had a significantly lower number of cells expressing CD34 in follicles and corpora lutea (Fig. 1A and D). Moreover, apoptotic vascular endothelial cells were observed in corpora lutea (Suppl. Fig. 1). The presented results suggest that CTX induced destruction of ovarian follicles *via* apoptosis of follicular cells and inhibition of follicular microvascularization. In addition, the inhibition of microvascularization was also observed in the corpora lutea. Angiogenesis plays a critical role in the female reproductive system and is associated with cyclic changes characteristic for the ovaries [12], *i.e.*, ovarian follicular development [13] and formation of the corpus luteum [14]. The newly formed ovarian blood vessels likely secure an increased supply of resources such as oxygen, steroid precursors gonadotropins and growth factors [15]. We, therefore, hypothesize that the primary cause of follicular reduction in the ovary after CTX chemotherapy is the CTX-induced malfunction of microvascularization.

Supplementary Figure 1 related to this article can be found, in the online version, at doi:10.1016/j.repbio.2014.04.007.

In the current study, we also evaluated the long-term effects (up to eight weeks) of the CTX treatment (Groups 2 and 4) on the number of follicles (Fig. 2A), number of CD34 positive cells in corpora lutea (Fig. 2B) and gene expression of *VEGF* and *MVH* (Suppl. Fig. 3) in the ovaries. The number of follicles per mm^2 of ovarian tissue in control mice was not affected by maternal aging (Fig. 2A). In contrast, the CTX treatment significantly reduced, in a dose-dependent manner, the number of follicles in the both groups at all time points. In Group 2, a consistent gradual decline in follicular number was observed from week 1 to week 8. Two major reductions, after 3 days and 4 weeks, respectively, were observed in Group 4. The CTX-induced reduction in the follicle number was not reversed