Accelerated ovarian aging in mice by treatment of busulfan and cyclophosphamide

Yan JIANG§1, Jing ZHAO§1, Hui-jing QI2, Xiao-lin LI1, Shi-rong ZHANG1, Daniel W. SONG3, Chi-yang YU†‡4, Jian-gang GAO†‡1

(1Key Laboratory of the Ministry of Education for Experimental Teratology and School of Life Science, Shandong University, Jinan 250100, China)
(2Jinan First People’s Hospital, Jinan 250011, China)
(3Dulwich College, Legend Garden Campus, Beijing 101300, China)
(4Department of Gynecology and Obstetrics, Southwest Hospital, Chongqing 400038, China)

†E-mail: chiyangyu@hotmail.com; jggao@sdu.edu.cn

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Abstract: Busulfan/cyclophosphamide (Bu/Cy) conditioning regimen has been widely used to treat cancer patients, while their effects on major internal organs in females are not fully understood. We treated female mice with Bu/Cy, and examined the histopathology of major internal organs on Day 30 after the treatment. The results show that Bu/Cy treatment affected the ovaries most extensively, while it had less effect on the spleen, lungs, and kidneys, and no effect on the heart, liver, stomach, and pancreas. To better understand the effect of Bu/Cy on the ovaries, we counted follicles, and determined the levels of ovarian steroids. The Bu/Cy-treated mice showed a reduction of primordial and primary follicles ($P<0.01$) on Day 30 and a marked loss of follicles at all developmental stages ($P<0.01$) on Day 60. Plasma levels of estradiol and progesterone in Bu/Cy-treated mice decreased by 43.9% and 61.4%, respectively. Thus, there was a gradual process of follicle loss and low estradiol in Bu/Cy-treated mice; this is a profile similar to what is found in women with premature ovarian failure (POF). The Bu/Cy-treated mice may serve as a useful animal model to study the dynamics of follicle loss in women undergoing POF.

Key words: Premature ovarian failure, Busulfan, Cyclophosphamide, Chemotherapy, Mouse model
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1 Introduction

Chemotherapy can improve the long-term survival of cancer patients, but has side effects such as ovarian failure, infertility, and liver toxicity. Chemotherapeutic agents can be classified into five classes depending on their mode of action: alkylating agents, antimetabolites, aneuploidy inducers, radiomimetics, and topoisomerase II inhibitors. These drugs are often used in combination for increased anti-tumour effects (Meirow and Nugent, 2001; Maltaris et al., 2007; Jemal et al., 2010).

Both busulfan and cyclophosphamide (Bu/Cy) are alkylating agents; they have been frequently used in combination as a conditioning regimen before hematopoietic stem cell transplantation for patients with leukemia, inherited metabolic diseases, or chronic granulomatous disease. In comparison to other regimens, the toxicities of Bu and Cy are relatively low (Copelan et al., 1993; de Magalhaes-Silverman et al., 1997). However, like other chemotherapeutic agents, Bu/Cy can cause problems in some organs like the ovaries and lungs in patients.
The impairment to the ovaries is the most severe compared with the impact upon other organs (Meirow and Nugent, 2001; Ulrickson et al., 2009).

There are some relevant reports about the effects of Bu and Cy on major internal organs using mice as models. Al-Hashmi et al. (2011) tried to develop a graft-versus-host disease mouse model by treating the mice with Bu/Cy and transplanting with hematopoietic stem cells. They checked the histology of major internal organs of these transplanted mice, and found there were histopathological changes in the liver, pancreas, spleen, lungs, and heart, but not in the kidneys. About the effects of Bu or Cy on ovaries in animals, it was reported that treatment with Bu or Cy alone can cause loss of follicles (Hemsworth and Jackson, 1963; Burkl and Schiechl, 1978; Pelloux et al., 1988; Meirow et al., 1999; Shirota et al., 2003).

Until now, there was no systematic study of the effects of the combination of Bu and Cy on major internal organs in mice, especially upon the ovaries, which seem to be the most sensitive to these two drugs. In this study, we examined the effects of a combination of Bu/Cy on major internal organs using female mice as a model and focused on the ovaries. We not only examined the histological changes of the ovaries, but also checked the hormone levels in Bu/Cy-treated mice in comparison with un-treated mice.

2 Materials and methods

2.1 Animals and treatments

All mice used in these studies were of CD-1 background. Forty female mice at two months of age were treated with a single injection of Bu in dimethyl sulfoxide (DMSO; 12 mg/kg subcutaneously) and Cy in 0.9\% (9 g/L) sterile sodium chloride solution (120 mg/kg intraperitoneally) (Johnson et al., 2005). Bu powder is difficult to dissolve in water and has been reported to cause occasional deaths by intraperitoneal injection (Jopling and Rosendaal, 2001). We dissolved Bu in DMSO at a concentration of 3.6 mg/ml. We weighed the mice (28–32 g) and injected subcutaneously at the dosage mentioned above (about 0.1 ml for each mouse). Twenty untreated age-matched female mice were used as the control group.

On Day 30 after Bu/Cy treatment, twenty mice were weighed, anesthetized with pentobarbital sodium (5 \( \mu \)g/g intraperitoneally) and sacrificed. The major internal organs (the ovary, heart, liver, spleen, lungs, kidneys, stomach, and pancreas) were harvested for histological analysis. We also collected the ovaries of the other twenty Bu/Cy-treated mice on Day 60 to examine their histological changes. All studies were conducted in accordance with the standards of the Shandong University Ethics Committee.

The harvested organs were fixed in 10\% formaldehyde. All the samples were embedded in paraffin, sectioned (5 \( \mu \)m), and stained with hematoxylin and eosin (H&E). Additionally, we counted follicles on ovarian sections. Each ovary produced about 200 sections, and follicles on every 20th section were counted. Follicles were classified as previously assigned (Mayer et al., 2004; Myers et al., 2004). An oocyte, which was surrounded by a single layer of flattened granulosa cells, was defined as primordial follicles. Primary follicles were defined as an oocyte surrounded by a single layer of cuboidal granulosa cells. Secondary follicles possessed an oocyte surrounded by more than one layer of granulosa cells without antral space. Antral follicles were identified as containing an antral space.

2.2 Hormone assay

On Day 30 after Bu/Cy treatment, whole blood samples (1.0–1.2 ml) were harvested after mice were anesthetized. We collected whole blood samples by retro-orbital puncture. The samples were incubated at 37 °C for 1 h. Thereafter, the samples were centrifuged at 3000 r/min for 30 min at room temperature and the supernatant was collected. The plasma levels of estradiol, progesterone, and testosterone were measured as indicators to assess ovarian senescence in animal models (Danilovich and Ram Sairam, 2006).

2.3 Data analyses

Data analyses included calculations of group means and standard error of means (SEM). Data for follicle numbers and plasma hormone concentrations were analyzed by \( t \)-test with significance set at \( P \) value <0.05.