

GnRH Agonist Acts as Ovarian Protection in Chemotherapy Induced Gonadotoxicity: An Experiment Using a Rat Model

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Received 18 December 2006, accepted 3 August 2007

Edited by KENTARO YAMADA

Summary: To reduce chemotherapy induced gonadotoxicity, co-treatment with gonadotropin releasing hormone against analogue (GnRHa) was tested using rat model. Leuprorelin acetate (Leuplin) with or without cisplatin (CDDP) was given subcutaneously at a dose of 9.4 $\mu\text{g/ml}$ to Wistar strain female rats. The total number of follicles was counted and the maturation of follicles was evaluated at the largest section of the ovary on the 5th and 10th day after administration. Leuplin led the ovary to a resting phase in which primordial follicle occupied 80% of all follicles in only 5 days after administration. The serum E2 level was also down by the 5th day and maintained a low level to the 10th day. In co-treatment with GnRHa and CDDP rats, the primordial follicle occupied 90% of all follicles and the total number of follicles was higher than in CDDP alone rats. This rat model verified that GnRHa co-treatment well minimized CDDP induced gonadotoxicity by desensitization of the ovary. These results were promising for the clinical application introducing GnRHa co-treatment as ovarian protection in cancer chemotherapy in young women.

Key words GnRH agonist, chemotherapy related amenorrhea, ovarian protection, apoptosis

INTRODUCTION

Recent advances in cancer chemotherapy have helped cancer survivors achieve long disease free interval. Young male and female patients with germ cell tumor, genital cancer, hematologic malignancies, or pediatric malignancies suffer from sexual dysfunction resulting in infertility. Chemotherapy induced ovarian toxicity by cyclophosphamide was introduced to breast cancer patients in the late 70's [1]. Unlikely the situation in other organs, heavy ovarian toxicity is irreversible, because the number of follicles is limited. Whereas dose intensive chemotherapy including hematopoietic or stemcell transplantation has been widely accepted for young patients, the risk of chemotherapy-related

amenorrhea (CRA) has increased with the age at the time of treatment, in a dose-dependent manner [2]. Even in adolescence, survivors had a risk of menopause four times greater than that of control healthy girls between the ages of 21 and 25 [3]. Nevertheless, ovarian function was often preserved in survivors who were treated prepubertally [3,4]. Gonadotropin releasing hormone agonist analogue (GnRHa) is widely used for endometriosis, and is also available for estrogen dependent tumors, such as leiomyoma, and breast cancer, as medical castration. The aim of GnRHa application in young patients is to inactivate their ovarian function temporarily, and may protect them from CRA. In this report, we tested co-treatment GnRHa with cisplatin (CDDP) using the rat model and inves-

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Abbreviations: CDDP, cisplatin; CRA, chemotherapy related amenorrhea; GnRHa, gonadotropin releasing hormone agonist analogue; RIA, radioimmunoassay.

tigated whether or not it would reduce chemotherapy-induced gonadotoxicity.

MATERIALS AND METHODS

Animals

Five-week old matured female Wistar strain rats were used for this experiment.

Chemicals

Leuprorelin Acetate (Leuprin, Takeda Pharmacia Company, Tokyo, Japan) was prepared with an attached suspension at a concentration of 9.4 $\mu\text{g/ml}$, corresponding to the 50 $\mu\text{g/kg}$ conventional dose for humans. CDDP (Randa, Nippon Kayaku Ltd., Tokyo, Japan) solution (0.5 mg/ml) was prepared at a concentration of 10.1 mg/kg corresponding to 1/2 LD50 of the rat, which was divided over 5 days of administration. Each solution was diluted to 2 ml with 0.9% saline.

Treatment schedule

Experiment 1: To evaluate the gonadal effect of leuprin on untreated rat ovary: Leuprin was injected subcutaneously to 28 rats. Eighteen rats were sacrificed at day 5 (1 menstrual cycle: GnRH α 1 cycle rats) and 10 rats were sacrificed at day 10 (2 menstrual cycles: GnRH α 2 cycle rats). Bilateral ovaries were removed under ether anesthesia on sacrifice. Eight untreated rats were prepared as control, and were sacrificed on the same days and their ovaries also removed.

Experiment 2: To examine the effect of co-treatment of CDDP and GnRH α : Leuprin was injected into 13 rats, in the same way as in experiment 1. CDDP was administered intraperitoneally for 5 days, 5 days after leuprin injection (CDDP+GnRH α rats). The total dose of CDDP was 10.1 mg/kg. The rats were sacrificed 24 hrs after the final CDDP administration. CDDP solution alone was injected into 19 rats for 5 days and they were then sacrificed to remove their ovaries (CDDP rats). Bilateral ovaries were removed in both groups. Only normal saline was injected into 8 rats in the control group, on the same schedule as CDDP rats.

The body weights of the rats were examined at the beginning and the end of the experiment for all groups.

Histological examination

Excised ovaries were fixed in formalin and paraf-

TABLE 1.
Classification of follicles for maturation evaluation

Classification	Subtype	Number of layer*	Diameter (μm)**
Small	type 1	none	~6
	type 2	incomplete	~10
	type 3a	single	~15
Medium	type 3b	single	~30
	type 4	two	~50
	type 5a	three	~60
Large	type 5b	four	~80
	type 6	five	~120
	type 7	cumulus	~200
	type 8	well formed cumulus	~350

* Number of layers of granulosa cells

** Maximum diameter of follicles

fin embedded and stained with hematoxylin and eosin. They were sectioned into 4 μm sliced continuous sections. The number of follicles was counted by a light microscope at maximum diameter. The maturation of follicles was estimated by modified Pederson and Peters classification (Table 1) [5]. Precisely, follicles were classified into 8 subgroups by the number of layers of granulosa cells and the maximum diameter of follicles. Furthermore, the total number of follicles in each group was calculated, and once again divided into 3 groups, small (immature), medium, and large (matured) follicle groups.

Biochemical Analysis

Serum levels of estradiol (E2) were examined by RIA using the rat E2 ^{125}I kit (Amersham) in experiment 1.

Statistics

Data was presented as mean \pm SD. Statistical significant was analysis by the Mann & Whitney test for the comparison of the number of follicles in each group. A value of $P < 0.05$ was considered significant.

RESULTS

Experiment 1

The total number of follicles was counted and the mean number of follicles was compared among the three groups. Significant loss of follicles was found in GnRH α 1 cycle and 2 cycle rats. There was a statistical

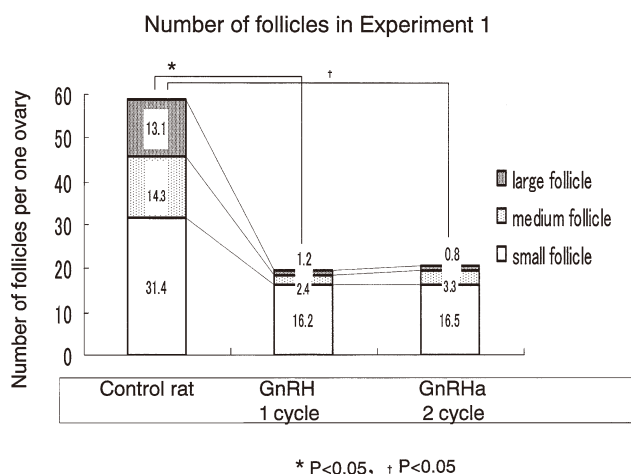


Fig. 1. Total number of follicles significantly decreased in GnRHa rats. Medium and large follicles were lost in GnRHa rats. No difference was found between GnRHa 1 cycle and 2 cycle treatment.

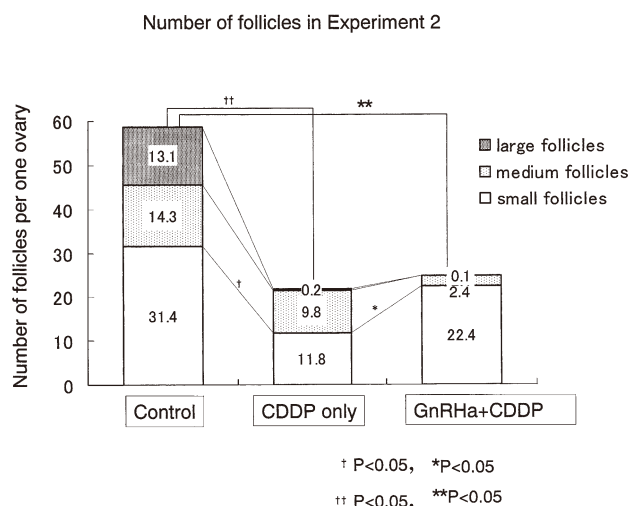


Fig. 3. The number of total follicles significantly decreased in CDDP rats and GnRHa+CDDP rats. A larger number of small follicles were preserved in GnRHa+CDDP rats than CDDP rats.

Serum E2 levels of Control and GnRHa rats

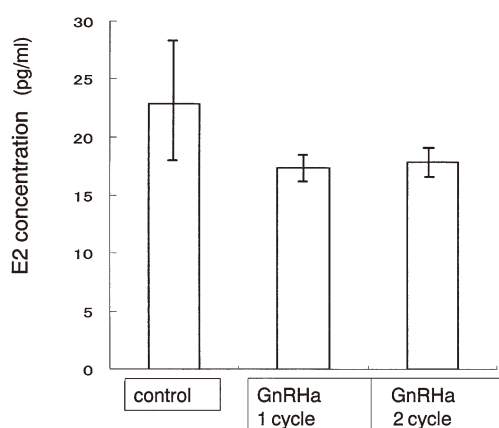


Fig. 2. GnRHa rats showed lower tendency of serum E2 levels than control rats. No difference was found between GnRHa 1 cycle and 2 cycle treatment.

difference in the loss of follicles between the control rats and GnRHa 1 cycle and 2 cycle rats ($P < 0.05$). The mean numbers of follicles in each group were 58.8, 19.8, and 20.6 per ovary, in the control, GnRHa 1 cycle and 2 cycle rats, respectively. Also, there was a difference in the distribution of follicle subtypes between the control and GnRHa rats. Medium and large follicles were significantly lost in GnRHa rats. As a result, the rate of small follicles in GnRHa rats was higher than that in control rats. The rate of small follicles among the total number of follicles in control

rats, GnRHa 1 cycle rats and GnRHa 2 cycle rats were 54.4%, 80.0% and 80.1%, respectively. There was no difference in the total number of follicles and the rate of small follicles between GnRHa 1 cycle and 2 cycle rats (Fig. 1). The serum levels of E2 in GnRHa 1 cycle and 2 cycle rats showed a similar tendency (not significant) of decline from that in the control rats (Fig. 2).

Experiment 2

A significant loss of follicles was found in GnRHa+CDDP rats and CDDP rats comparing to control rats. Although, there was no difference in the total number of follicles between GnRHa+CDDP rats and CDDP rats, a larger number of small follicles were preserved in GnRHa+CDDP rats than CDDP rats (GnRHa+CDDP rats : 22.4 ± 6.3 vs. CDDP rats: 11.8 ± 6.3). The rate of small follicles among the total follicles in CDDP rats and GnRHa+CDDP rats were 54.1% ($11.8/21.8$) and 90.0% ($22.4/24.9$) respectively (Fig. 3). There were no rats having body weight loss exceeding 20% of their original weight.

DISCUSSION

Chemotherapy-induced gonadotoxicity is a state of hypergonadotropic hypogonadism. It caused CRA and finally irreversible ovarian failure in women. Histologically, focal or diffuse cortical fibrosis, reduction in follicle numbers, and impaired follicular maturation were observed in the ovary obtained from autopsy

cases, and those findings were independent of pubertal age [6]. The risk of gonadal damage is directly related to the age of the patient. Chapman et al. [7] reported a higher incidence of ovarian failure in patients older than 30 years of age after completion of chemotherapy in Hodgkin lymphoma. In breast cancer, no patients under 30 years of age showed CRA for doxorubicin-containing regimen, 33% of patients between 30 and 39 years showed CRA, and 96% of patients between 40 and 49 years showed CRA [8]. Shinagawa [9] demonstrated antral (large) follicle was more sensitive to cytotoxic chemotherapeutic agent than primordial (small) follicle in rat models. In prepubertal girls, less than 10% had ovarian dysfunction due to alkylating agents [10]. The large number of primordial follicles in the prepubertal ovary may contribute to resistance to cytotoxic chemotherapy [11]. Ataya et al. [12] tested co-treatment of cyclophosphamide and GnRHa in rat models. GnRHa inhibited follicle maturation from primordial follicles to antral follicles. As a result, primordial follicle dominant ovary was well protected from cyclophosphamide-induced gonadotoxicity. Co-treatment of GnRHa with anticancer agent seems to be favorable as ovarian protection from gonadotoxicity in cancer chemotherapy.

In clinical application of ovarian protection by GnRHa, Waxman et al. [13] failed to verify the effectiveness of co-treatment of buserelin with anticancer drugs in the treatment of Hodgkin lymphoma. Blumenfeld et al. [14], however, reported a higher incidence of lymphoma patients who resumed spontaneous ovulation among patients treated with GnRHa and anticancer agents than patients with chemotherapy alone (96% versus 35%). There are several conceivable reasons for this discrepancy. In our experiments, only a 5-day interval from GnRHa administration was enough to introduce the resting phase to the rat ovary in which primordial follicles were dominant. It demonstrated that pituitary-ovarian desensitization by GnRHa administration was completed quickly in the rat, because the rat menstrual cycle is very short. This was also verified by a prompt decline in serum E2 level. Nevertheless, significance of decreasing of E2 was not shown in this experiment. Small amount of preserved medium follicle in GnRHa rats could become a source of E2 production.

Direct suppression of GnRH receptors in rat ovary was helpful in this rapid completion of desensitization [15]. Meanwhile, human ovary has a much lower concentration of GnRH receptor than rat ovary [14]. It is supposed to take a longer interval to desensitize human ovary. Waxman's study failed to protect ovarian

function, because patients in that study did not have enough time for desensitization before initiating chemotherapy. For a better result of ovarian protection, GnRHa administration should be started as soon as possible to protect antral follicles during the first course of chemotherapy.

GnRHa induced apoptosis in rat granulosa cells [16]. By this GnRHa direct effect on the ovary, loss of follicles was observed in GnRHa treated rats in our experiment. Nevertheless, in the human ovary, apoptosis by GnRHa will not be a serious problem. It should appear less frequently because of lower concentrations of GnRH receptor in human ovary.

Among many anticancer agents, cyclophosphamide was the most frequently tested drug for gonadal toxicity in animal models. Although CDDP is one of the most available drugs in gynecologic cancer, there have been few reports on gonadal toxicity. Wallace et al. [17] reported that a higher dose of CDDP revealed higher rates of gonadotoxicity in chemotherapy for human osteosarcoma. Maneschi et al. [18] reported only 2 cycles of relatively high dose CDDP plus bleomycin induced CRA in advanced cervical cancer. So CDDP induced gonadotoxicity is worthy of discussion. Shinagawa [9] reported CDDP induced ovarian toxicity occurred in a dose and time dependent manner in rat models, and we demonstrated co-treatment with GnRHa worked well as ovarian protection.

Chemotherapy related ovarian failure may cause the patient to lose quality of life, with vasomotor symptoms, vaginal dryness, body weight gain, rapid bone loss and risk of subsequent fracture [19]. The effectiveness of goserelin (zoradex) as medical castration was indicated in chemotherapy for premenopausal breast cancer patients. Goserelin and cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy showed similar survival to the patients treated by CMF alone. Co-treatment of goserelin showed not only an additive effect of chemotherapy but also that it worked as ovarian protection. Ovarian function was reversible in patients under 40 years of age [20]. As the number of younger patients who receive chemotherapy increases, clinical application of ovarian protection becomes more and more interesting. Based on our experiments, the protective effects of GnRHa against CDDP induced gonadotoxicity were verified in rat model. These results encourage us to introduce some clinical trials as a pilot study. In order to obtain a long time follow up of ovarian function, collaboration with other institutions should be initiated.

ACKNOWLEDGMENTS: The authors thank Dr. Takashi

Nishida for instruction for research and Ms Yaeko Namatsu for the technical assistance of animal experiments.

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