

# Protective effect of gonadotropin-releasing hormone analog on the ovarian reserve in rats receiving cyclophosphamide treatment

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**Objective:** The aim of the study reported here was to investigate the protective effect of gonadotropin-releasing hormone analog (GnRHa) against cyclophosphamide (CTX)-induced gonadotoxicity.

**Methods:** Eighty Fischer 344 rats were divided randomly into four groups (20 per group). One group received normal saline, one GnRHa, one CTX, and one GnRHa+CTX. Several parameters were used to observe the ovarian reserve, including ovary weight, follicle number and diameter, concentrations of estradiol (E<sub>2</sub>) and follicle-stimulating hormone (FSH), and expressions of sex hormone receptors.

**Results:** When treatment was finished, the number of small follicles in the GnRHa+CTX group was significantly higher than in the CTX-alone group. Thirty days after treatment, the ovary weight, percentage of small follicles, mean follicular diameter, and serum concentrations of E<sub>2</sub> and FSH in the GnRHa+CTX group all recovered, approaching normal levels. Sex hormone receptors did not show significant differences between the four groups.

**Conclusion:** Combination treatment with GnRHa could prevent CTX-induced damage to ovarian reserve.

**Keywords:** gonadotoxicity, ovarian reserve, GnRHa, CTX, premature ovarian failure

## Introduction

Chemotherapy is commonly used for treating various malignancies and can improve the survival rate for cancer patients.<sup>1</sup> Unfortunately, many young survivors unknowingly face reproductive compromise, and the quality of life of these patients deserves more attention.<sup>2,3</sup>

“Cyclophosphamide” (CTX), an alkylating agent widely used in cancer treatment, has long been reported to cause progressive and irreversible damages to ovarian germ cells and supporting stromal cells while exerting its cytotoxic effects.<sup>4</sup> Potential adverse effects of chemotherapy with CTX are a decrease in the number of ovarian follicles, disruption of the menstrual cycle, the induction of infertility, and irreversible premature ovarian failure, which is associated with risks of cardiovascular disease, osteoporosis, and psychiatric diseases such as depression.<sup>5</sup> New strategies have been developed to prevent the adverse effects of chemotherapy on ovarian function. Studies have shown that gonadotropin-releasing hormone analog (GnRHa) can protect the ovary from damage caused by anticancer drugs through inhibiting the hypothalamic–pituitary–ovarian axis and reducing the rate of oogenesis, thereby rendering the germinal epithelium less susceptible to the effects of chemotherapy.<sup>6</sup> Nevertheless, the mechanisms involved in the protective effects of GnRHa against CTX-induced ovarian damage are still not well understood.

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In our research reported here, we explored the protective effects of GnRHa on ovarian reserve through establishing models of CTX-induced ovarian impairment in rats of healthy status.

## Materials and methods

### Chemicals

CTX (Hualin Pharm, Jiujiang, People's Republic of China) was prepared daily in sterile 0.9% NaCl solution at a concentration of 2 mg/mL. GnRHa (Takeda Pharmaceutical Company Limited, Osaka, Japan) was dissolved in 0.9% NaCl solution for injection.

### Animals

Adult female Fischer 344 rats, aged 12 weeks, about 140–210 g in weight, were obtained from the Institute of Laboratory Animals of the Chinese Academy of Medical Sciences, Beijing, People's Republic of China, and maintained under specific pathogen-free conditions. Vaginal smears were collected daily to confirm their sexual cycles. Only rats demonstrating at least two consecutive normal 4-day vaginal estrus cycles were included in the experiments. All the procedures in rats were approved, monitored, and reviewed by the Experimental Animal Committee of Peking Union Medical College Hospital, Chinese Academy of Medical Sciences.

### Treatment regimens

#### Experiment 1

Twelve rats were divided into three groups. Group 1 ( $n=4$ ) received subcutaneous (sc) injection of GnRHa 0.125 mg/rat, Group 2 ( $n=4$ ) received sc injection of GnRHa 0.25 mg/rat, and Group 3 ( $n=4$ ) received sc injection of GnRHa 0.50 mg/rat. A single bolus of GnRHa was administered, and the initial dose of GnRHa 0.125 mg/rat was adopted according to a previous research conducted by Ataya et al.<sup>7</sup> Blood of each rat was collected on the day of metestrus in the first sexual cycle before GnRHa injection and in the 2–7 sexual cycles after GnRHa injection. Time–concentration curves for serum estradiol ( $E_2$ ) and follicle-stimulating hormone (FSH) were plotted.

#### Experiment 2

##### Grouping

Eighty rats were randomly divided into four groups of 20 animals each. The experimental observation duration was 60 days. Group 1 (control) received sc injection of 0.3 mL of 0.9% NaCl solution at intervals of every 30 days, on the first day and 31st day, twice in total. Group 2 (GnRHa) received sc injection of 0.25 mg GnRHa at intervals of every

30 days, on the first day and 31st day, twice in total. Group 3 (CTX) received an initial intraperitoneal (ip) loading dose of CTX (50 mg/kg) on the 31st day, followed by daily ip injection of 5 mg/kg for another consecutive 29 days. Group 4 (GnRHa+CTX) received sc injection of 0.25 mg GnRHa on the first day and 31st day, and an initial ip loading dose of CTX (50 mg/kg) on the 31st day followed by daily ip injection of 5 mg/kg for another consecutive 29 days. The regimen of CTX therapy is very variable, and we selected a regimen that has been used in previous research; that is, a loading dose of 50 mg/kg and maintenance dose of 5 mg/kg daily for 30 days.<sup>7</sup>

Half of the rats were sacrificed when the CTX treatment was finished (60th day), and the other half were sacrificed 30 days later (90th day) for observation of recovery without any other treatment. Both the weight and follicles of the ovaries were examined. Blood was collected on the day of metestrus every 30 days, to detect the concentrations of serum  $E_2$  and FSH.

##### Histological examination

Ovaries of rats were fixed in formalin for 24 hours and prepared for serial sectioning (10  $\mu$ m thickness). Every tenth section was stained with hematoxylin and eosin, and examined under light microscope. The number of follicles with a nucleolus in the oocyte was counted. The average diameter was determined as the mean of the longest and shortest diameters of each follicle, measured as straight-line distances between opposite points on the basement membrane. Follicles less than 30  $\mu$ m were considered small to represent the primordial follicles, characterized by one layer of flattened granulosa cells surrounding the oocyte.<sup>7</sup> Follicles over 30  $\mu$ m in diameter were grouped into medium-large and represented the growing follicles.<sup>7</sup> “Follicle distribution” referred to the percentage of small primordial follicles and the medium-large follicles in all the follicles.

Immunohistochemistry was performed to detect the expression of estrogen receptor alpha ( $ER\alpha$ ), estrogen receptor beta ( $ER\beta$ ), the progesterone receptor (PR), and the androgen receptor (AR). Following deparaffinization, endogenous peroxidase activity was inhibited by incubation with 0.3%  $H_2O_2$  for 15 minutes. The antigens were retrieved by microwave treatment in citrate buffer (10 mM, pH 6). The slides were then washed with phosphate-buffered saline and blocked with 5% bovine serum albumin at room temperature for 20 minutes. The sections were incubated with primary antibodies (Abcam plc, Cambridge, UK) at 4°C overnight, followed by 1 hour's incubation with secondary antibodies (Santa Cruz, CA, USA). Immunostaining was performed

using the 3,3'-Diaminobenzidine (DAB) method. A negative control was obtained by normal nonimmune immunoglobulin (Ig) G. Known immunostaining-positive slides were used as positive controls. "Positive expression" was defined as the presence of yellow-brown granules in the nuclei. Staining results were evaluated by a semiquantitative scoring criterion evaluating both the intensity and proportion of immunopositive cells. A staining index was calculated by multiplying the staining intensity (negative =1, primrose yellow =2, yellow-brown =3, or dark brown =4) and staining proportion ( $\leq 25\%$  =1, 26%–50% =2, 50%–75% =3,  $> 75\%$  =4). Results were assessed by two independent pathologists without knowing the sample information.

#### Semiquantitative analysis of messenger RNA expression through real-time polymerase chain reaction

Total RNA was extracted from ovarian tissues using Life Technologies TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the instructions. After quantification, complementary DNA (cDNA) was synthesized using an Invitrogen reverse transcription kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. The primers for reverse transcription are shown in Table 1. Real-time polymerase chain reaction was performed on an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific). The results were analyzed using a comparative method of  $2^{-\Delta\Delta CT}$ . All amplifications were performed in triplicate.

#### Statistical analysis

Data were analyzed using the SPSS software package (v 19.0; IBM Corp, Armonk, NY, USA). One-way analysis of variance was adopted for statistical comparisons in different groups.  $P < 0.05$  was considered statistically significant.

**Table 1** Primers used for real-time polymerase chain reaction (RT-PCR) analysis of sex steroid receptors

mRNA	RT-PCR primers (5'→3')	Product size (bp)
ER $\alpha$	GATCCTTCTAGACCTTCAGTG TCTTCCAGAGACTTCAAGGTGCT	419
ER $\beta$	AGAGTCCTTGGTGTGAAGCA GGCTGGACAGATATAGTCAT	251
PR	CCATGTGGCAAATCCCACAGGAGT CGGAAATTCACAGCCAGTGCC	320
AR	CCATGGGGTTGGGTGTGGAA TCCCAGAGCTACCTGCTTCA	276
GAPDH	CGGCAAGTTCAATGGCACAGT TCATACTTGGCAGGTTTCTCC	609

**Abbreviations:** AR, androgen receptor; ER $\alpha$ , estrogen receptor alpha; ER $\beta$ , estrogen receptor beta; PR, progesterone receptor; GAPDH, glyceraldehyde phosphate dehydrogenase.

## Results

### Experiment 1

The reproductive cycle of the Fischer 344 rats lasted for 4 days. The serum E<sub>2</sub> and FSH in rats receiving 0.125 mg/rat started to decrease on the fifth day, but elevated again on the 17th day and reached original levels on the 25th day (Figure 1A and B). Continual decline was observed in E<sub>2</sub> and FSH levels in rats receiving 0.25 mg/rat and 0.50 mg/rat, with a minimum of 1.0 pg/mL and 1.0 mIU/mL on the 25th day, indicating that both regimens had obvious inhibitive effects on ovarian functions (Figure 1A and B). Since there was no significant difference between these two regimens, GnRHa at 0.25 mg/rat was used in the next research.

### Experiment 2

#### Ovary weight

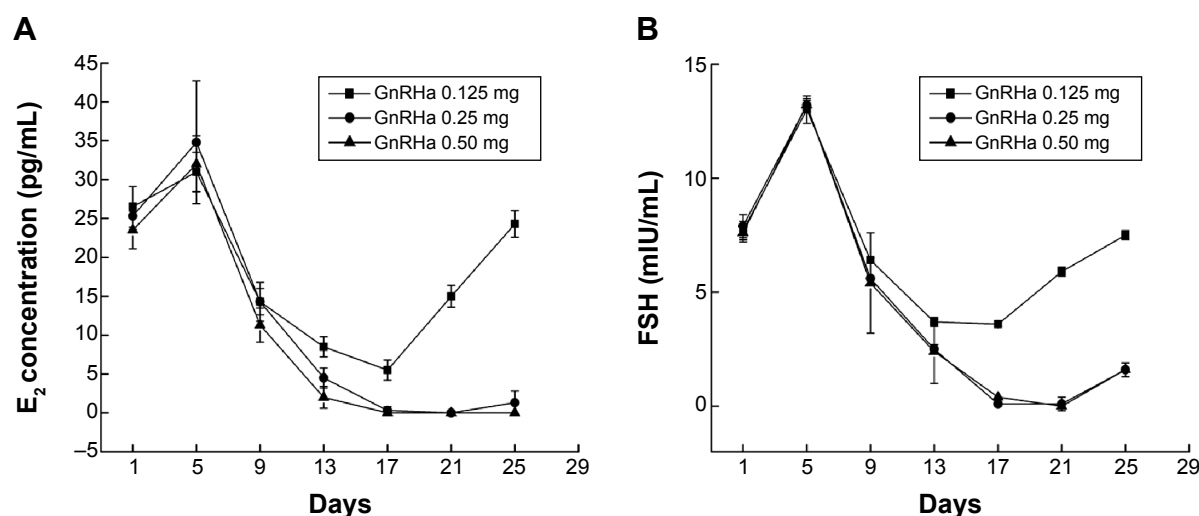
In three treated groups (treatment with GnRHa, CTX, or GnRHa+CTX), the ovary weight had significantly reduced, compared with control group treated with normal saline ( $P < 0.001$ ), on the 60th day when treatment had finished. Groups receiving GnRHa either alone or in combination with CTX, however, showed a significant weight gain 30 days later, while, in the CTX-alone group, the ovary weight remained at a very low level (Figure 2A).

#### Follicle number, diameter, and percentage

On the 60th day and 30 days post-treatment, CTX-alone treatment significantly reduced the total number of follicles compared with saline-only treatment (ie, the control group;  $P < 0.01$ ,  $P < 0.001$ ). The mean follicular diameter in the CTX-alone group was significantly smaller ( $P < 0.001$ ) and the percentage of small follicles was significantly higher ( $P < 0.01$ ) than in the control group (Figure 2B–D).

Administration of GnRHa alone was associated with a higher number of total follicles relative to the control group on the 60th day and 30 days post-treatment ( $P < 0.01$ ). The mean follicular diameter was markedly reduced on the 60th day compared with the control group ( $P < 0.001$ ), but recovered 30 days later. The percentage of small follicles was significantly higher on the 60th day ( $P < 0.001$ ), but decreased 30 days post-treatment (Figure 2B–D).

GnRHa administered with CTX appeared to prevent CTX-induced follicle loss, since the total number of follicles was apparently higher in the GnRHa-CTX group than in the CTX-alone group on both the 60th day and 30 days after treatment ( $P < 0.01$  and  $P < 0.001$ , respectively). The mean follicular diameter was markedly lower in the GnRHa+CTX group compared with in the CTX-alone group on the 60th



**Figure 1** Serum levels of estradiol ( $E_2$ ) and follicle-stimulating hormone (FSH) in rats receiving different regimens of gonadotropin-releasing hormone analog (GnRHa). **Notes:** (A)  $E_2$  and (B) FSH showed continual decline in rats receiving 0.25 mg/rat or 0.50 mg/rat without significant difference between these two regimens.

day ( $P < 0.001$ ), but the situation was reversed 30 days later. The percentage of small follicles in the GnRHa+CTX group was higher on the 60th day ( $P < 0.01$ ), but at 30 days post-treatment, it had decreased to a level lower than that in the CTX-alone group (Figure 2B–D)

#### Serum concentrations of estradiol and follicle-stimulating hormone

Compared with in the control group, the CTX-alone group showed a continual increase of serum concentrations of  $E_2$  and FSH, reaching a maximum on the 90th day (ie, 30 days post-treatment;  $P < 0.01$ ). The serum  $E_2$  and FSH in the GnRHa-alone group and the GnRHa+CTX group showed significant decrease on the 30th and 60th day relative to the control group ( $P < 0.001$ ), but had recovered to a normal level 30 days later (Figure 2E and F)

#### Expression of estrogen receptor alpha, estrogen receptor beta, progesterone receptor, and androgen receptor

Expression of ER $\alpha$ , ER $\beta$ , PR, and AR was detected at both mRNA and protein levels. The ER $\beta$  and AR showed the highest level of expression, followed by PR, and then by ER $\alpha$  (Figure 3). No significant differences in the four hormone receptors were found between the four groups (Table 2).

## Discussion

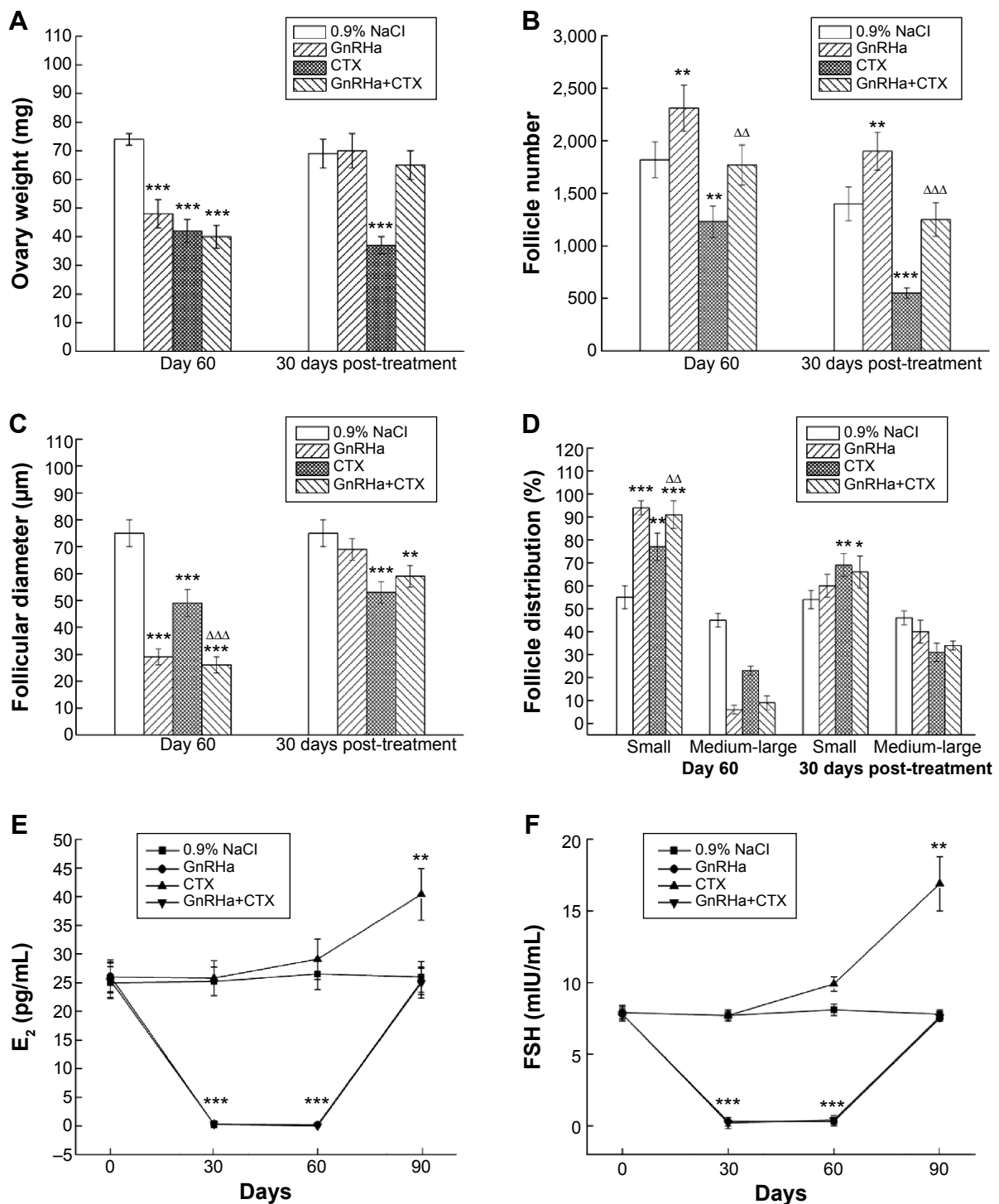
Advances in chemotherapy have resulted in the effective treatment and cure of multiple malignancies. The 2007 Surveillance, Epidemiology, and End Results program estimated

that there were over 275,000 female cancer survivors of reproductive age in the USA.<sup>8</sup> However, impairment of reproductive potential after chemotherapy, which can involve premature ovarian failure, premature menopause, and infertility, can significantly impact the quality of life of young female survivors long after their cancer treatment.

CTX, a widely used chemotherapeutic agent, carries great risk for ovarian failure.<sup>9,10</sup> Therefore, we utilized a standard model of CTX-induced ovarian damage to explore the protective effects of GnRHa on reproductive potential. GnRHa treatment with 0.25 mg/rat and 0.5 mg/rat resulted in strong inhibition on the pituitary gland and ovary in mature cycling rats. There was no significant difference between these two regimens, thus the regimen of 0.25 mg GnRHa per rat was used in the subsequent experiment. The CTX therapy regimen is very variable, and we selected a regimen used in previous research – a loading dose of 50 mg/kg and maintenance dose of 5 mg/kg daily for 30 days.<sup>7</sup>

The ovary weights of rats in both the GnRHa+CTX and CTX-alone groups had significantly decreased when the treatment was finished, but 1 month later, the weight had gradually recovered in the former, while that in the latter remained at a low level, indicating that GnRHa does have protective effects on ovarian reserve.

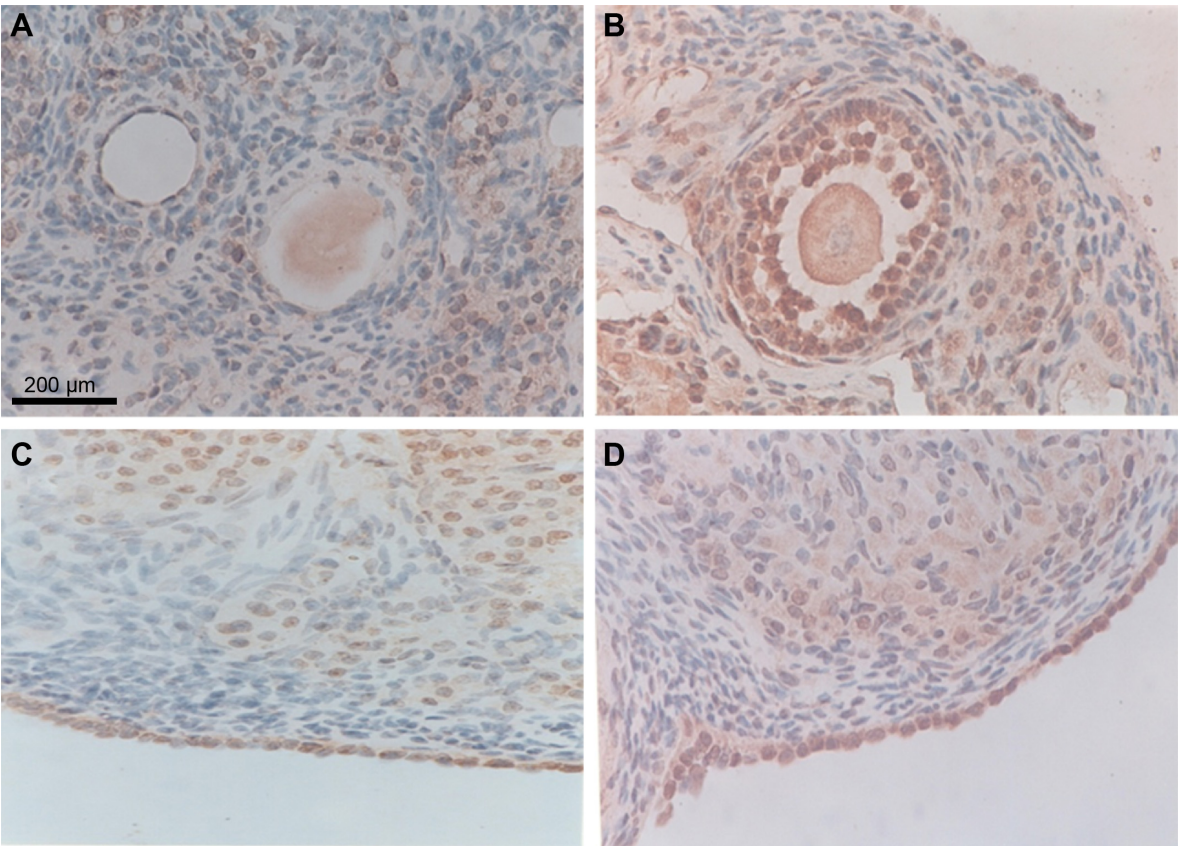
The number of follicles, especially quiescent primordial follicles, is a more direct parameter allowing accurate estimation of ovarian reserve.<sup>11</sup> In our research, we determined that small follicles represented primordial follicles and medium-large follicles represented follicles in development. Analyses of the number and percentage of small and medium-large follicles



**Figure 2** Assessment of ovarian reserve in terms of ovary weight, follicular number, diameter, and percentage, and serum levels of estradiol ( $E_2$ ) and follicle-stimulating hormone (FSH). (A) Ovary weight in the three treated groups was significantly reduced compared with in the control group at treatment end (Day 60). One month later, groups receiving gonadotropin-releasing hormone analog (GnRHa; alone or in combination with cyclophosphamide [CTX]) showed a significant weight gain, while weight was maintained at a very low level in the CTX-alone group. (B) The total number of follicles in the GnRHa+CTX group was significantly higher than in the CTX-alone group at the end of the treatment period (Day 60) and 30 days post-treatment. (C) Follicular diameter in the GnRHa+CTX group was significantly lower than in the CTX-alone group at Day 60, but had recovered to a higher level 30 days post-treatment. (D) The percentage of small follicles in the GnRHa+CTX group was higher than that in the CTX-alone group at Day 60, but had decreased markedly 30 days post-treatment. Serum levels of (E)  $E_2$  and (F) FSH in the GnRHa+CTX group showed significant decrease on Days 30 and 60 relative to the control group ( $P<0.001$ ) and had recovered to a normal level 30 days post-treatment, while in the CTX-alone group, a continual increase of serum  $E_2$  and FSH could be observed.

**Notes:** Asterisks (\*) indicate comparisons with control group: \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ . The change symbol ( $\Delta$ ) refers to comparisons between the GnRHa+CTX group and the CTX-alone group:  $\Delta\Delta P<0.01$ ;  $\Delta\Delta\Delta P<0.001$ .





**Figure 3** Representative immunohistochemical staining of (A) estrogen receptor alpha, (B) estrogen receptor beta, (C) progesterone receptor, and (D) androgen receptor in rat ovaries.

**Note:** No significant differences in the four hormone receptors were found among the four groups.

indicate that CTX mainly destroys the medium-large follicles. Our finding is consistent with the hypothesis that CTX leads to follicular death by damaging rapidly dividing granulosa cells.<sup>12</sup> The consequent decrease in gonadal steroid secretion stimulates a negative feedback, enhancing the recruitment of primordial follicles into the pool of growing follicles.<sup>12</sup> These newly formed larger follicles are then destroyed by CTX, and the vicious cycle results in the depletion of primordial follicles and eventually premature ovarian failure.<sup>13</sup>

On the 60th day, when the treatment had finished, the total number of follicles and the percentage of small follicles in the GnRHa+CTX group were significantly higher than those in the CTX-alone group, while the mean follicular diameter was consistently smaller, demonstrating that GnRHa is indeed capable of preventing CTX-induced follicular loss. Through suppression of follicular development, GnRHa inhibits the recruitment process, preventing the primordial follicles reaching the CTX-sensitive stage.

**Table 2** Expressions of estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), progesterone receptor (PR), and androgen receptor (AR) of rat ovaries at both messenger (mRNA) and protein levels

Group	Rats, n	mRNA intensity				TISS results of IHC			
		ERα	ERβ	PR	AR	ERα	ERβ	PR	AR
1	20	0.24±0.18	1.27±0.51	0.88±0.34	1.57±0.59	5.1±1.0	15.1±1.2	8.6±0.5	18.4±2.0
2	20	0.29±0.21	1.35±0.47	0.76±0.31	1.39±0.51	4.9±1.0	15.5±1.4	8.5±1.2	19.0±1.9
3	20	0.27±0.15	1.13±0.46	0.85±0.40	1.63±0.57	4.9±1.3	15.2±1.8	9.1±1.5	17.9±2.1
4	20	0.24±0.19	1.26±0.49	0.68±0.32	1.46±0.54	4.7±1.4	15.2±1.0	8.5±1.2	18.4±2.0

**Notes:** No significant differences in the four hormone receptors were found among the four groups. "Positive expression" was defined as the presence of yellow-brown granules in nuclei. Staining results were evaluated by a semiquantitative scoring criterion evaluating both the intensity and proportion of immuno-positive cells. A staining index was calculated by multiplying the staining intensity (negative =1, primrose yellow =2, yellow-brown =3, or dark brown =4) and staining proportion (≤25%=1, 26%–50%=2, 50%–75%=3, >75%=4).

**Abbreviations:** IHC, immunohistochemistry; TISS, total immune-staining score.

Thirty days post-treatment, the total number of follicles was still significantly higher in the co-treatment group, while the proportion of small follicles had declined and the follicular diameter increased. This indicates that the inhibitive effects of GnRHa on follicle development are reversible and ovarian functions could return to normal after treatment with GnRHa.

In clinic, the “day 3 FSH test” is the most commonly used test to assess ovarian reserve. Studies have shown that the number of potential useful oocytes in women whose basal follicle-stimulating hormone (bFSH) is equal to or greater than 10 mIU/mL is significantly lower than that in women whose bFSH is equal to or less than 6 mIU/mL.<sup>14</sup> For some women, the  $E_2$  level has already elevated on the third day of their menstrual cycle. Even though the ovarian reserve has diminished, the high level of  $E_2$  may suppress the FSH level to a normal range. Thus, the corresponding  $E_2$  level is also determined. In our study, the levels of serum bFSH and basal  $E_2$  in rats in the GnRHa+CTX group were significantly lower than the rats in the CTX-alone group, further confirming that GnRHa could help protect the ovarian potential from CTX-induced gonadotoxicity.

## Conclusion

Combination treatment with GnRHa could prevent CTX-induced damage to the ovarian reserve and would not attenuate the curative effects of CTX on ovarian carcinomas.

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## Disclosure

The authors declare no conflicts of interest in this work.

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