

# Transient impact of paclitaxel on mouse fertility and protective effect of gonadotropin-releasing hormone agonist

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**Abstract.** Paclitaxel (PXL) is a chemotherapeutic agent widely used in solid tumors. However, whether PXL causes premature ovarian insufficiency in women of reproductive age remains controversial. The aim of the present study was to answer how and for how long PXL affects fertility, and to identify the protective effect of gonadotropin-releasing hormone agonist (GnRHa) in mice. A single dose of PXL was administered to 7-week-old female ICR mice. Mice were treated with GnRHa for 1 estrous cycle prior to chemotherapy, and for another following chemotherapy. On the days 1, 6, 11 and 16 following the administration of PXL, mice were assessed by ovarian histology, ovarian stimulation and mating experiment. Multiple doses of PXL were also administered to verify the duration of the gonadotoxicity of PXL. It was determined that PXL only destroyed antral follicles on day 1 following chemotherapy without reducing primordial follicles. *In vitro* experiments revealed that PXL impaired oocytes in metaphase, excluding those at the germinal vesicle stage. The number and quality of retrieved metaphaseII(MII) oocytes in PXL-exposed mice were reduced on day 1 following chemotherapy, which was recovered on day 11. MII oocytes from mice pretreated with GnRHa recovered on day 6 following chemotherapy. Following 3 estrous cycles in mice after the last dose of the 3-dose paclitaxel administration, follicles in all stages and retrieved MII oocytes were recovered. It was

concluded that the impairment caused by PXL on follicles and oocytes in mice lasted for <3 estrous cycles, which was shortened by pretreatment of GnRHa.

## Introduction

Cancer is a major public health problem worldwide. Notably, increasing incidences of certain types of cancer, such as cervical (1-3), breast (4) and lung cancer (5), have been reported in young women. Although cancer mortality is continuously declining due to progress in treatment (6), improving cancer-associated outcomes and quality of life in these patients remains imperative. Chemotherapeutic agents are well known for their side effects, such as leukopenia, hepatic insufficiency and premature ovarian insufficiency (7). Endocrine dysfunction and infertility induced by ovarian function impairment in patients of reproductive age can seriously affect their quality of life. The increasing demand for ovarian function preservation has therefore become a major challenge for onco-fertility specialists (8).

According to Information Management System (IMS<sup>TM</sup>) data, the global best-selling chemotherapeutic agent is paclitaxel (PXL) (9), which is widely used in cervical (10), breast (11) and lung cancers (12). Mechanistically, PXL functions as an antineoplastic drug by inhibiting guanosine triphosphate hydrolysis in the microtubule lattice, inducing microtubule stabilization (13). Although PXL had been used for decades, the few studies revealing its effect on the ovaries of animals have had conflicting results (14-18). The results of certain clinical trials and meta-analyses that focused on whether PXL diminishes human fertility were not reliable, due to lack of an accurate ovarian reserve marker (19-22). Therefore, it is important to elucidate how PXL affects ovaries and how long its gonadotoxicity lasts.

Currently, gonadotropin-releasing hormone analogues/agonists (GnRHa) are the first and most widely used agents for ovarian protection during chemotherapy (7). The main mechanism of the protective effect of GnRHa is reducing follicle-stimulating hormone (FSH) levels and suppressing follicle growth to maintain ovaries in a relatively dormant state (7). However, the evidence of fertility preservation by GnRHa remains insufficient, since controversial results were reported in several meta-analyses (23-25). Different

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chemotherapy regimens used in each clinical trial were probably responsible for the contrasting results. It remains unclear whether GnRHa would be effective in protecting ovaries from the gonadotoxicity caused by PXL.

The aim of the present study was to clarify the phenomena and mechanisms through which PXL impairs rodent ovaries, define the duration of its gonadotoxicity and investigate whether GnRHa can protect ovaries during PXL treatment, in the hope of providing laboratory evidence for the clinical application of PXL and GnRHa more safely in women of reproductive age.

## Materials and methods

**Animals.** Seven-week-old female and 10-week-old male ICR mice were purchased from the Hubei Provincial Center for Disease Control and Prevention, China. Mice were kept for 1 week in the animal husbandry to enable acclimatization to the local conditions in controlled temperature (20–25°C) and light (12-h light/dark cycle) with free access to food and tap water. Three to four female mice were housed in one ventilated cage with wood shavings as bedding which was changed every 3 days. Each male mouse was separately housed. The whole experiment lasted 3 months, including 501 female mice and 12 male mice. All the animals were monitored every day, and weighed every 5 days. Animals were sacrificed by cervical dislocation for sample collection following inhalation of 70% v/v CO<sub>2</sub>. Euthanasia was confirmed by the cessation of a heartbeat. Symptoms meeting the NIH guidelines (26) such as abnormal postures, weight loss, loss of appetite or weakness were set as humane endpoints for the present study. All experiments were approved by the Institutional Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval no. TJ-A20161101).

**PXL and GnRHa treatment.** Female ICR mice, weighing 30–35 g, were randomly assigned to four groups: The vehicle, PXL, GnRHa and PXL+GnRHa groups. Based on our pre-examination and a previous study (27), the estrous cycle of ICR mice was 5 days on average. Therefore, GnRHa (1 mg/kg, triptorelin acetate; Ferring Pharmaceuticals) or normal saline was administered intraperitoneally to mice prior to chemotherapy for 5 days. Next, animals received a single dose of PXL (30 mg/kg; Pfizer, Inc.) or vehicle intraperitoneally. One quarter of the mice in each group were assessed 24 h after chemotherapy. The rest of the mice were continuously administered GnRHa for another estrous cycle (5 days) following chemotherapy, and were assessed on days 6, 11 and 16 following chemotherapy (Fig. 1A). Another set of mice was administered 30 mg/kg PXL every 3 days, for a total of 3 doses. GnRHa (1 mg/kg) was also administered prior to, during and following chemotherapy for a total of 16 days, and mice were then assessed on days 1, 6, 11 and 16 following chemotherapy (Fig. 1B). During the experiment, one mouse in the GnRHa group suffered from weight loss and was euthanized before the endpoint of our experiment.

**Histology and follicle count.** After anesthetizing by 50 mg/kg pentobarbital sodium (Merck KGaA) intraperitoneally, blood was collected from the eye orbit for serum anti-müllerian

hormone (AMH) analysis (n=5/group/time-point). Then these mice were euthanized by cervical dislocation, and mortality was confirmed by the cessation of a heartbeat. Subsequently, the ovaries were collected. One ovary from each mouse was fixed in 4% v/v paraformaldehyde (Wuhan Servicebio Technology Co., Ltd.) for 24 h at 4°C, embedded in paraffin and sectioned at 5- $\mu$ m thickness for histology. Hematoxylin and eosin (H&E) staining was performed using standard methods (28). Follicle counts were conducted on serially cut sections from every sixth section of entire ovaries. The follicles were counted at different stages and the mean count per section was calculated for each stage. The follicle stages were classified as previously described (29).

**AMH measurement.** Blood was collected from the orbit and placed at room temperature for 1 h. Following centrifugation at 3,000 x g, serum AMH levels were determined using a mouse anti-AMH ELISA kit (cat. no. CSB-E13156m; Cusabio Technology LLC), following the manufacturer's instructions.

**Oocyte collection and in vitro maturation.** An intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; Beijing Solarbio Science & Technology Co., Ltd.) was administered, followed by 12 IU human chorionic gonadotrophin (hCG; Lizhu Pharmaceutical Trading Co., Ltd.) 48 h after the induction of ovulation in mice. Subsequently, 14–17 h after hCG, cumulus oocytes were collected in an oviduct ampulla after mice were scarified by cervical dislocation following inhalation of 70% v/v CO<sub>2</sub>. Cumulus cells were removed using 1% hyaluronidase (Merck KGaA), and denuded metaphase II (MII) oocytes were prepared for further study. In total, 265 mice were sacrificed for oocyte collection.

Oocytes with germinal vesicle (GV) were punctured from ovaries using a 0.5-mm syringe 48 h following PMSG administration. GV oocytes were cultured in M199 (GE Healthcare Life Sciences) drops under liquid mineral oil (Vitrolife) at 37°C in an incubator with 5% CO<sub>2</sub> for *in vitro* maturation. Oocytes in MI were retrieved after 6 h, and MII oocytes were retrieved after 12 h. GV, MI and MII oocytes were then cultured in 1  $\mu$ M PXL for 5 min at 37°C. GV and MI oocytes were washed for ongoing culture to gain MII oocytes. The ongoing culture time of GV and MI oocytes was 12 and 6 h, respectively. MII oocytes were washed and cultured in normal M199 drops for 4 h for further study.

**Immunofluorescence and confocal microscopy.** Oocytes from each group at each time-point (n=5) were fixed with 4% v/v paraformaldehyde at 4°C overnight. They were then transferred to 0.5% v/v Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd.) for 5 min. Following blocking in 1% w/v BSA-supplemented (Wuhan Servicebio Technology Co., Ltd.) PBS for 1 h, oocytes were incubated with the monoclonal anti- $\alpha$ -tubulin antibody produced in mice at a dilution of 1:1,000 (cat. no. T5168; Merck KGaA) at 4°C overnight. Then specimens were then incubated with anti-mouse IgG (H+L), F(ab')<sub>2</sub> Fragment (Alexa Fluor® 488 Conjugate) at a dilution of 1:300 (product no. 4408S; Cell Signaling Technology, Inc.) for 1 h at 37°C, and then co-stained with Hoechst 33342 (Merck KGaA) for 5 min at 37°C. Oocytes were mounted onto

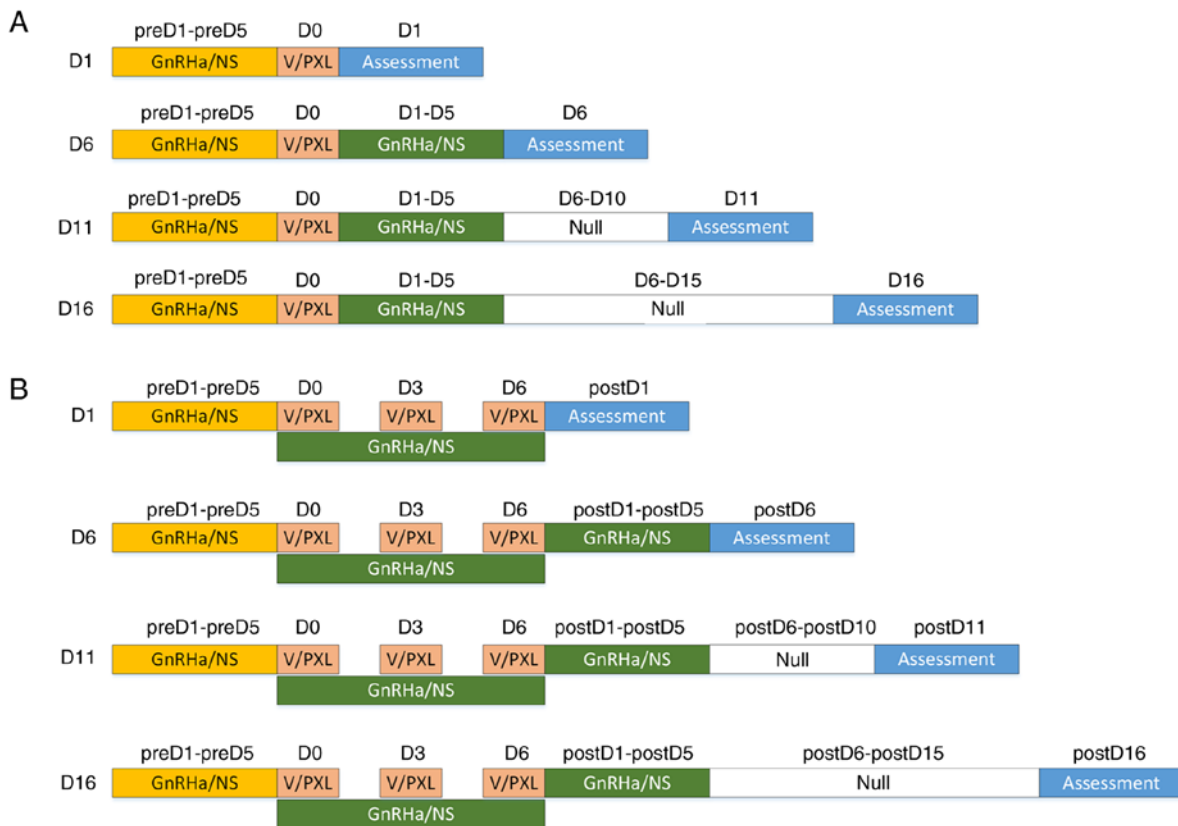


Figure 1. Experimental scheme. (A) Single-dose scheme: Before chemotherapy, mice were pretreated with NS or GnRHa for 5 days (1 estrous cycle). On D0, a single dose of chemotherapy (PXL) or vehicle was administered. On day 1 following chemotherapy, a quarter of the mice were assessed (n=24/group). The rest were continuously administered NS or GnRHa for another 5 days. On day 6, 11 and 16 following chemotherapy, a quarter of the mice were assessed at each time-point. (B) Multiple-dose scheme: Three doses of paclitaxel were administered consecutively every 3 days and GnRHa was administered continuously during chemotherapy. On days 1, 6, 11 and 16 following chemotherapy, a quarter of the mice (n=10/group) were assessed at each time-point. NS, normal saline; GnRHa, gonadotropin-releasing hormone agonist; PXL, paclitaxel; V, vehicle.

glass slides and observed under a confocal laser-scanning microscope (FV1000; Olympus Corporation).

**Chromosome (CH) spread of oocytes.** Oocytes from each group at each time-point (n=4) were placed in hypotonic solution of 0.9% w/v sodium citrate (Wuhan Servicebio Technology Co., Ltd.) for 10 min at 37°C and then exposed to Tyrode's buffer (pH 2.5; Merck KGaA) for ~30 sec at 37°C to remove the zona pellucida. Oocytes were then fixed in a drop of 1% v/v paraformaldehyde with 0.15% v/v Triton X-100 (pH 9.2) on a glass slide. Then slides were dried in a humid chamber for over 2 h at 37°C. CHs were stained with 2% v/v Giemsa (Wuhan Servicebio Technology Co., Ltd.) for 10 min at room temperature and observed under a light microscope at a magnification of x100. The normal number of MII oocyte CHs (univalents) was 20. Aneuploidy oocytes had  $\pm 20$  univalents.

**In vitro fertilization.** The caudae epididymides of healthy 12-week-old male mice scarified by cervical dislocation following inhalation of 70% v/v CO<sub>2</sub> were lanced in G-IVF medium (Vitrolife) to release sperm. Following capacitation, sperm was added to pooled oocytes from 4 mice in each group, at each time-point, in G-IVF medium, for 5 h at 37°C and 5% CO<sub>2</sub>. These oocytes were then moved to drops of G1 (Vitrolife) medium under mineral oil. The presence of two pronuclei was considered as successful fertilization.

**Mating protocol.** Six female mice in the estrous cycle from each group at each time-point mated with healthy male mice which had been demonstrated to be fertile at a ratio of 3:1 for 72 h. No special treatment was administered to male mice. Female mice were separated as soon as a plug was observed or after 72 h of mating.

**Pup CH analysis.** Three pups from each pregnant mouse were selected randomly for CH analysis. Two-week-old pups were intraperitoneally injected with 0.5% w/v 0.1 ml colchicine (Jialin) and sacrificed by cervical dislocation following inhalation of 70% v/v CO<sub>2</sub> 20 min after injection at room temperature. Bone marrow from the thighbones was flushed into 0.075 M potassium chloride (Wuhan Servicebio Technology Co., Ltd.) solution, and then, marrow cells were fixed with methanol-acetic acid (3:1) fixative for 30 min at room temperature. CH slides were conventionally stained with 2% v/v Giemsa solution for 10 min at room temperature. The number of CHs was counted under a light microscope at magnification of x100 to determine aneuploidy.

**Statistical analysis.** The results are presented as the mean  $\pm$  SEM. The data were analyzed by one-way ANOVA followed by LSD post hoc or  $\chi^2$  test using SPSS 17.0 (SPSS, Inc.). A P-value of <0.05 was considered to indicate a statistically significant difference.

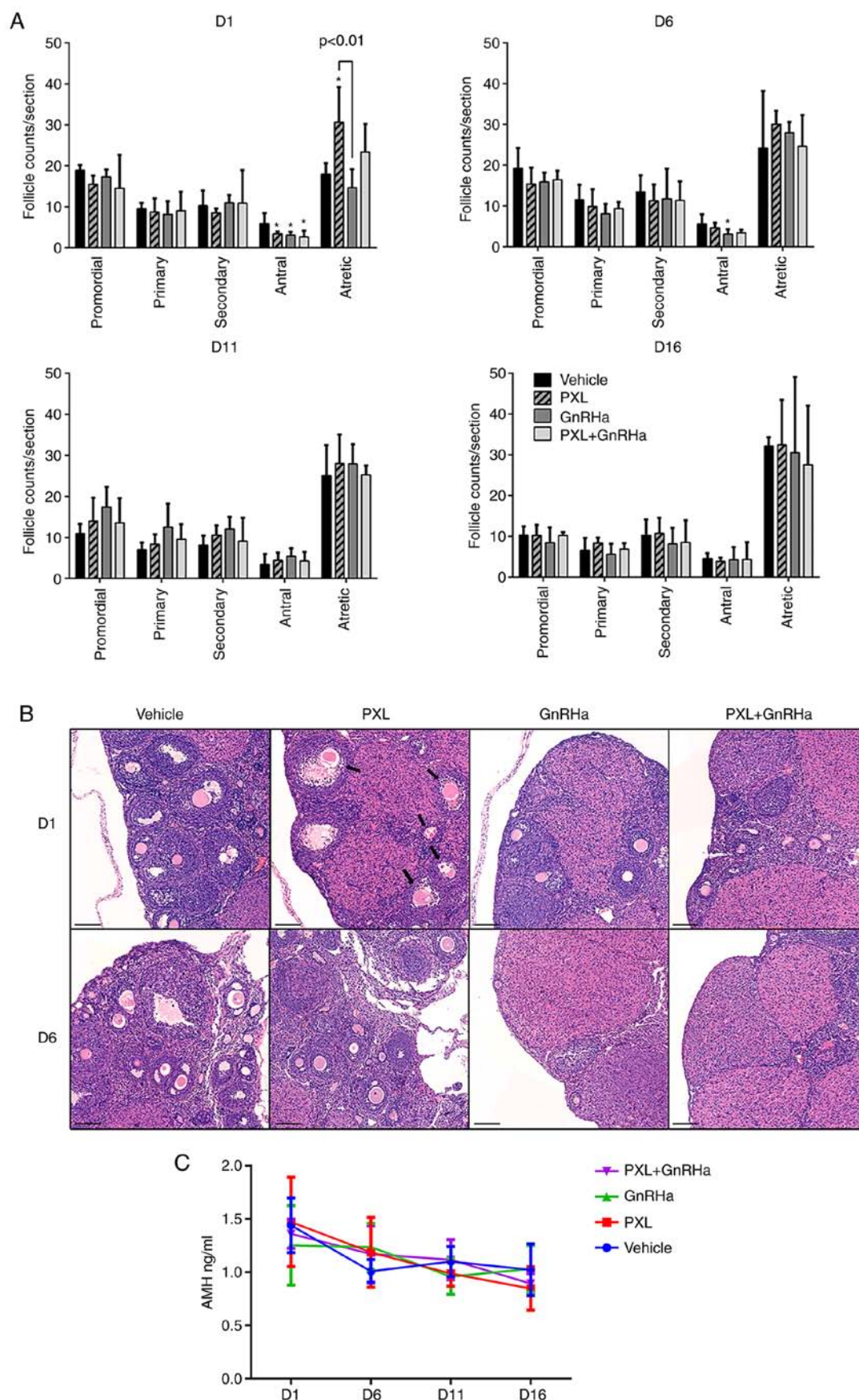


Figure 2. Damage caused by PXL on follicles only lasts for 1 estrous cycle, and GnRH $\alpha$  tends to abrogate this damage. (A) Number of primordial, primary, secondary, antral and atretic follicles on days 1-16 following chemotherapy. Data are expressed as the mean  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA followed by LSD test. \* $P < 0.05$  vs. the control group (vehicle). (B) H&E-stained sections revealing representative histological fields in the four groups on days 1-6 following chemotherapy (original magnification,  $\times 10$ ). The arrows indicate atretic follicles, which were mainly from antral follicles. Scale bars, 100  $\mu$ m. (C) Serum AMH levels in the 4 groups on day 1-16 following chemotherapy. Data are expressed as the mean  $\pm$  SEM. PXL, placlitaxel; GnRH $\alpha$ , gonadotropin-releasing hormone agonist; H&E, hematoxylin and eosin; AMH, anti-müllerian hormone.



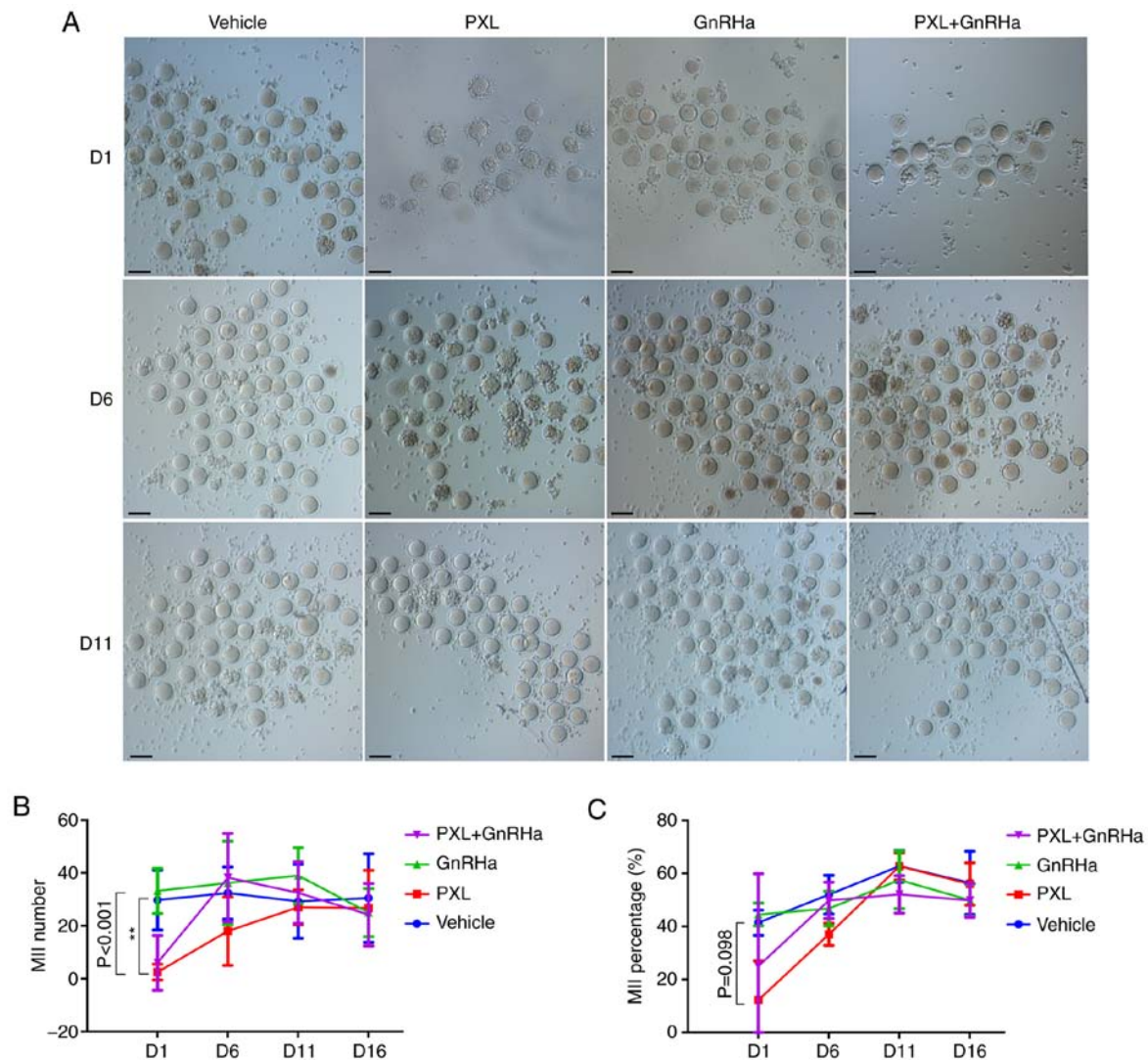


Figure 3. PXL-induced reduction of MII oocytes lasts for <2 estrous cycles, and GnRHa shortens the recovery time. (A) Oocyte morphology under a stereo-microscope (original magnification,  $\times 10$ ). Scale bars,  $100\ \mu\text{m}$ . (B) The number of oocytes in the four groups on days 1-16 following chemotherapy. Data are expressed as the mean  $\pm$  SEM. (C) MII percentage in the four groups on days 1-16 following chemotherapy. Data are expressed as the mean  $\pm$  SEM. Statistical analyses was performed by one-way ANOVA followed by LSD post hoc test. \*\* $P < 0.005$  vs. the control group (vehicle). PXL, paclitaxel; MII, metaphase II; GnRHa, gonadotropin-releasing hormone agonist.

## Results

*PXL only damages antral follicles in a transient manner and does not jeopardize ovarian reserve.* In order to determine the impact of PXL on ovarian reserve, histological analysis of the ovaries was performed and the serum AMH of mice was evaluated. On day 1 following the injection of PXL, only antral follicles were significantly reduced in the PXL group, as compared with the control group ( $3.35 \pm 0.30$  vs.  $5.90 \pm 1.27$ ;  $P < 0.05$ ). The antral follicles in the GnRHa group ( $3.07 \pm 0.47$  vs.  $5.90 \pm 1.27$ ;  $P < 0.05$ ) and PXL+GnRHa ( $2.60 \pm 0.75$  vs.  $5.90 \pm 1.27$ ;  $P < 0.05$ ) groups were also reduced, due to the suppressing effect of GnRHa. Atretic follicles were significantly increased in the PXL group as compared with the control group ( $30.67 \pm 4.27$  vs.  $17.95 \pm 1.35$ ;  $P < 0.01$ ). Co-administration of GnRHa plus PXL tended to abrogate the increase of atretic follicles caused by PXL, but not significantly ( $23.35 \pm 3.44$  vs.  $30.67 \pm 4.27$ ;  $P = 0.09$ ). Primordial, primary and secondary follicles were compared between groups (Fig. 2A and B).

After 1 estrous cycle, on day 6 after chemotherapy, the follicle counts between the four groups had already exhibited no difference in each follicular stage, except that antral follicles in the GnRHa group were still less than the control ( $3.10 \pm 1.09$  vs.  $5.60 \pm 1.53$ ,  $P < 0.05$ ) (Fig. 2A). On days 11 and 16 following chemotherapy, there was no difference in follicle counts between the four groups. Histological analysis revealed that PXL destroyed antral follicles in a transient way and had no effect on primordial follicles. To further confirm the mild effect of PXL on ovarian reserve, serum AMH was assessed in mice, and the result revealed a stationary trend of AMH without any significant difference between the groups at each time-point (Fig. 2C).

*PXL induces meiotic arrest in oocytes in metaphase but not in GV stage in vitro.* The antral follicles were damaged by PXL where oocytes resumed meiosis, experienced germinal vesicle breakdown (GVBD) and M I, and then stopped at MII, waiting for fertilization. Following exposure to PXL and

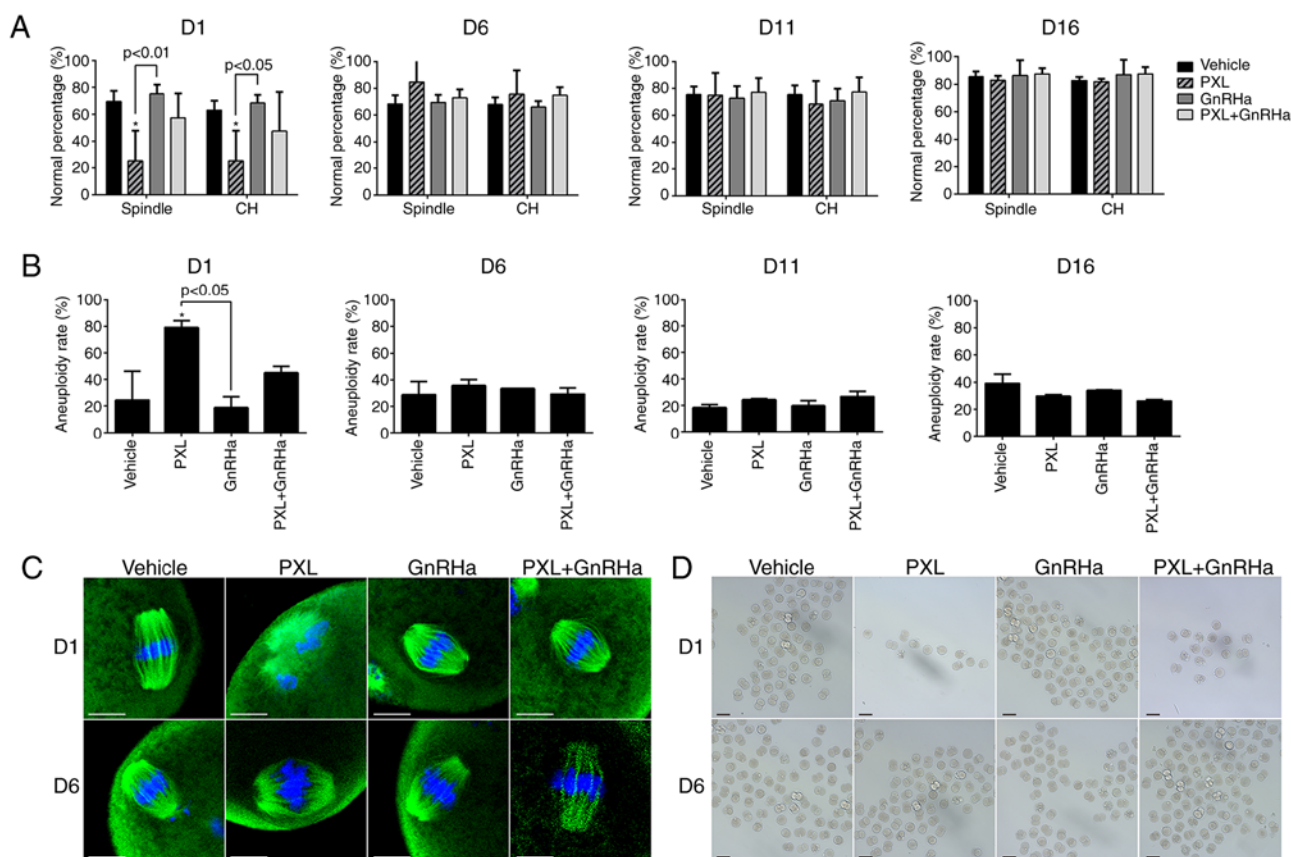


Figure 4. PXL-induced impairment of MII oocytes lasts for 1 estrous cycle, and GnRH $\alpha$  tends to protect against it. (A) Percentage of oocytes with a normal spindle morphology and aligned CHs. (B) Aneuploidy rate of oocytes following oocyte CH spread. Data are expressed as the mean  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA followed by LSD test. \* $P < 0.05$  vs. the control group (vehicle). (C) Representative images of spindles and CHs in MII oocytes. CHs are shown in blue and microtubules in green under a confocal microscope (original magnification,  $\times 100$ ). Scale bars,  $5 \mu\text{m}$ . (D) Embryos at the 2-cell stage 12 h after fertilization under a stereomicroscope (original magnification,  $\times 10$ ). Scale bars,  $100 \mu\text{m}$ . PXL, placlitaxel; MII, metaphase II; GnRH $\alpha$ , gonadotropin-releasing hormone agonist; CH, chromosome.

washing, GV oocytes were continuously cultured. The results revealed that PXL did not affect the GVBD ( $75.17 \pm 1.81\%$  vs.  $77.45 \pm 2.85\%$ ;  $P > 0.05$ ; Fig. S1A) or maturation rate ( $43.65 \pm 4.81\%$  vs.  $41.02 \pm 4.13\%$ ;  $P > 0.05$ ; Fig. S1A), which suggested that GV oocytes were either away from or resistant to the damage produced by PXL. MI oocytes were also administered PXL, which was then washed off. Following ongoing maturation, the oocytes exposed to PXL barely extruded the polar body, and the maturation rate was markedly lower ( $27.84 \pm 10.00\%$  vs.  $86.80 \pm 4.40\%$ ,  $P < 0.005$ ; Fig. S1B). Immunostaining revealed that the spindle apparatuses of these arrested MI oocytes that were exposed to PXL were mostly damaged, while the majority of MI oocytes without PXL administration exhibited normal spindle organization and CH alignment (spindle,  $3.70 \pm 11.33\%$  vs.  $69.84 \pm 20.78\%$ ,  $P < 0.05$ ; CH,  $0.00 \pm 0.00\%$  vs.  $73.01 \pm 27.74\%$ ;  $P < 0.05$ ; Fig. S1B). Similarly, PXL-exposed MII oocytes had a clearly disordered spindle organization and CH alignment, as compared with the controls (spindle,  $29.72 \pm 3.47\%$  vs.  $71.28 \pm 17.99\%$ ; CH,  $26.62 \pm 6.50\%$  vs.  $60.72 \pm 14.97\%$ ;  $P < 0.05$ ; Fig. S1C). As a result, the fertilization rate of PXL-exposed MII oocytes was markedly reduced ( $9.49 \pm 6.41\%$  vs.  $46.35 \pm 8.72\%$ ,  $P < 0.01$ ; Fig. S1C), while nearly half of the control oocytes were able to be fertilized and develop into 2-cell embryos. It was confirmed that only oocytes in metaphase and not earlier-stage oocytes

were affected by PXL through the destruction of spindle apparatuses.

*PXL-induced impairment of MII oocytes lasts for 2 estrous cycles in vivo, and GnRH $\alpha$  can protect oocytes.* To confirm the effect of PXL on mouse oocytes *in vivo*, the amount and morphology of oocytes retrieved following ovarian stimulation in mice was observed. On day 1 following chemotherapy, retrieved MII oocytes in the PXL group were significantly less than those in the vehicle group ( $2.50 \pm 1.50$  vs.  $29.75 \pm 5.65$ ;  $P < 0.005$  Fig. 3B), and the percentage of MII in retrieved oocytes had also decreased ( $12.22 \pm 14.8\%$  vs.  $41.40 \pm 4.7\%$ ;  $P = 0.096$ ; Fig. 3C). At this time-point, GnRH $\alpha$  did not rescue the number of PXL-exposed oocytes, since the MII oocytes retrieved in the PXL+GnRH $\alpha$  group were not more than those retrieved in the PXL group ( $6.0 \pm 6.0$  vs.  $2.50 \pm 1.50$ ;  $P > 0.05$ ). On day 6 following chemotherapy, the MII oocyte number and percentage in the PXL group started to recover, but was still slightly lower than that in the control ( $18.00 \pm 6.49$  vs.  $32.40 \pm 4.39$ ;  $P > 0.05$ ) ( $37.1 \pm 4.2\%$  vs.  $52.00 \pm 7.3\%$ ;  $P > 0.05$ ). In addition, mice co-treated with GnRH $\alpha$  took less time to completely recover. In the PXL+GnRH $\alpha$  group, the number and percentage of MII oocytes were totally similar to those of the controls ( $38.25 \pm 8.35$  vs.  $32.40 \pm 4.39$ ;  $P > 0.05$ ) ( $49.85 \pm 6.9\%$  vs.  $52.00 \pm 7.3\%$ ;  $P > 0.05$ ), and the number of MII oocytes was

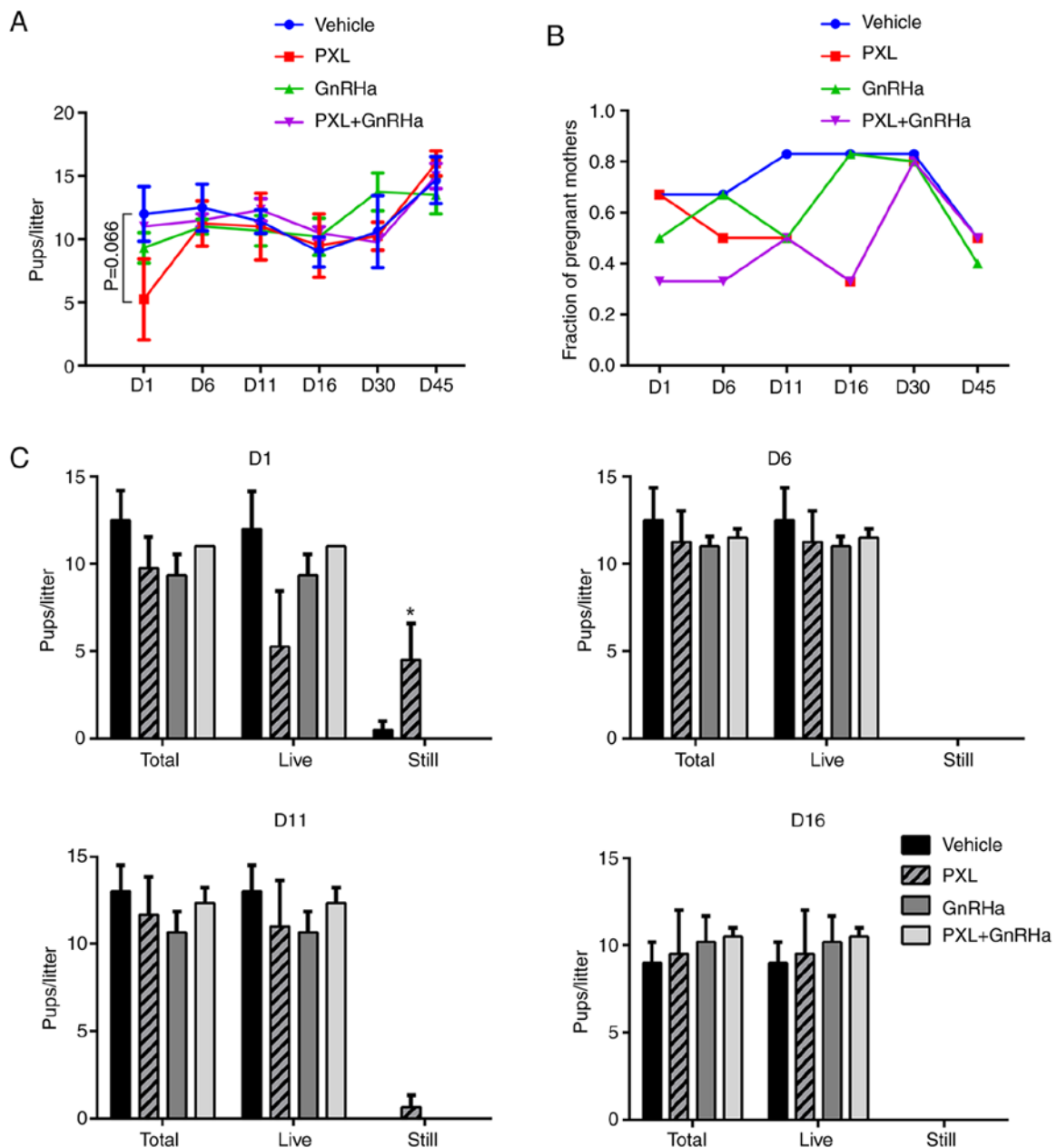


Figure 5. Adverse reproductive outcomes PXL-induced last for 1 estrous cycle, and GnRHa reduces stillbirths. (A) Live pups per litter delivered in the four groups on days 1-45 following chemotherapy. (B) Pregnancy rate in the 4 groups on days 1-45 following chemotherapy. (C) Total pups, live pups and stillbirths per litter delivered in the four groups days 1-16 following chemotherapy. Data are expressed as the mean  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA followed by LSD post-hoc test. \* $P < 0.05$  vs. the control group (vehicle). PXL, paclitaxel; GnRHa, gonadotropin-releasing hormone agonist.

slightly higher than that in the PXL group, although without significance ( $38.25 \pm 8.35$  vs.  $18.00 \pm 6.49$ ;  $P = 0.058$ ). On days 11 and 16, the number and percentage of MII oocytes were similar among the four groups (Fig. 3).

Although some oocytes developed into MII oocytes, we cannot say that all of them were normal. Spindle morphology, CH alignment, karyotype analysis and *in vitro* fertilization were used to evaluate the quality of MII oocytes. Spindle apparatus immunostaining revealed that, on day 1 following chemotherapy, most oocytes collected from the control or GnRHa groups had typical barrel-like spindles with CHs located on the equatorial plate (Fig. 4C). By contrast, a majority of disorganized spindles and misaligned CHs were observed in the PXL group (spindle,  $25.32 \pm 22.69\%$  vs.  $69.64 \pm 7.76\%$ ;

$P < 0.05$ ; CH,  $25.21 \pm 22.69\%$  vs.  $63.21 \pm 6.84\%$ ;  $P < 0.05$ ; Fig. 4A). Co-treatment of GnRHa tended to protect spindle organization, although without significance (spindle,  $57.50 \pm 18.12\%$  vs.  $25.32 \pm 22.69\%$ ;  $P > 0.05$ ; CH,  $47.5 \pm 29.18\%$  vs.  $25.21 \pm 22.69\%$ ;  $P > 0.05$ ; Fig. 4A). A significantly higher frequency of aneuploid oocytes of  $\pm 20$  univalents was found in the PXL group than in the control or GnRHa groups ( $79.17 \pm 5.15\%$  vs.  $24.44 \pm 21.76\%$  vs.  $18.75 \pm 8.11\%$ ;  $P < 0.05$ ; Fig. 4B) on day 1 following chemotherapy. Co-treatment with PXL and GnRHa tended to reduce the aneuploidy rate ( $79.17 \pm 5.15\%$  vs.  $45.00 \pm 5.03\%$ ;  $P > 0.05$ ; Fig. 4B). The fertilization rate was also clearly decreased in the PXL group, as compared with the control ( $27.27\%$  vs.  $75.51\%$ ;  $P < 0.05$ ; Fig. 4D; Table SI) on day 1 following chemotherapy. The fertilization ability of the PXL+GnRHa group was also

Table I. Reproductive outcomes of mice mated during the 1st estrous cycle after chemotherapy.

Groups	N	Litters (%)	Total pups (live pups)	Live pups/litter <sup>a</sup>	P-value	Stillbirths	Stillbirths/litter <sup>a</sup>	P-value
Vehicle	6	4 (66.7)	50(48)	12.00±2.16		2	0.50±0.50	
PXL	6	4 (66.7)	39 (21)	5.25±3.20	0.066	18	4.50±2.10 <sup>b</sup>	0.050
GnRHa	6	3 (50.0)	28 (28)	9.33±1.20	0.464	0	0.00±0.00 <sup>b</sup>	0.799 (0.042)
PXL+GnRHa	6	2 (33.3)	22 (22)	11.00±0.00	0.806	0	0.00±0.00	0.822

Reproductive outcomes of mice mated on the 1st day after chemotherapy. <sup>a</sup>Statistical analyses were performed by one-way ANOVA followed by LSD test. <sup>b</sup>Represents statistically significant difference between PXL and PXL+GnRHa groups, P=0.042.

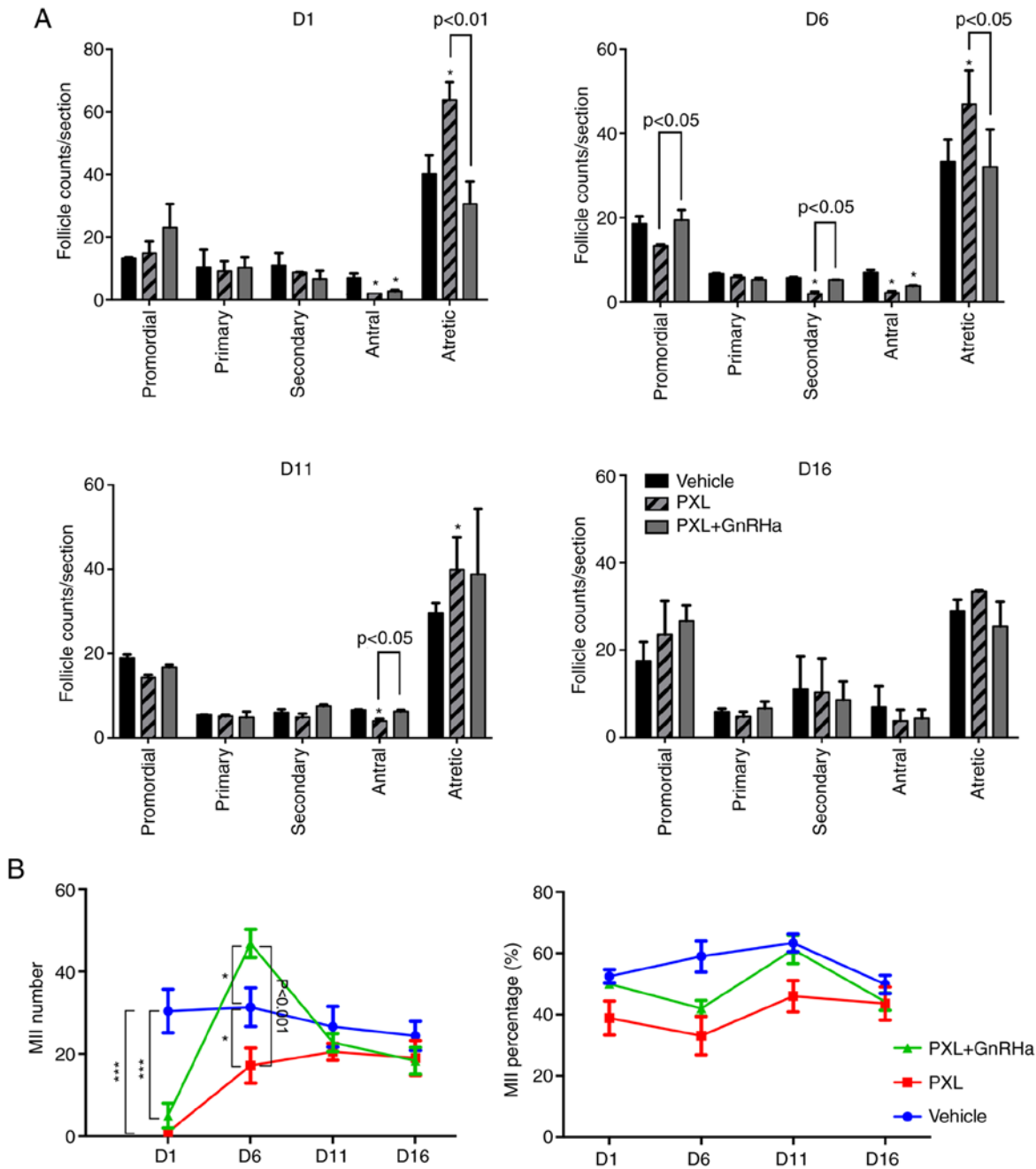


Figure 6. Multiple doses of PXL also damage antral follicles in a transient way. (A) Number of primordial, primary, secondary, antral and atretic follicles and (B) The number and percentage of MII oocytes retrieved in the three groups on days 1-16 after 3 doses of chemotherapy. Data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by LSD test. \*P<0.05, \*\*\*P<0.001 vs. the control group (vehicle). PXL, paclitaxel; MII, metaphase II; GnRHa, gonadotropin-releasing hormone agonist.



partially restored (64.29 vs. 27.27%;  $P>0.05$ ). After 1 estrous cycle, on day 6 following chemotherapy, the normal spindle morphology, as well as CH alignment, aneuploidy and fertilization rates of the oocytes in the PXL group had all reached the same level as those of the control group (Fig. 4).

*PXL-induced adverse reproductive outcomes last for 1 estrous cycle and GnRHa reverses this outcome.* A mating experiment was carried out to study whether damage to oocytes caused by PXL could affect reproductive outcomes. After mating on day 1 following chemotherapy, animals treated with GnRHa produced fewer litters (Table I; Fig. 5B), as 4/6 mice were pregnant in the control and PXL group, but only 2-3 mice in the GnRHa or PXL+GnRHa groups. No significant difference in live pups per litter was observed among the four groups. However, markedly, the PXL group delivered a total of 18 dead pups, which was more than those delivered in the control group ( $P=0.05$ ) and the GnRHa group ( $P=0.046$ ). There was no stillbirth in the PXL+GnRHa group but since only 2 mother mice delivered, no statistical difference was identified. (Table I; Fig. 5A and C). On day 6 and 11 following chemotherapy, the percentage of pregnant mothers in mice treated with GnRHa remained low. But on day 16, the pregnancy rate of mice treated with PXL dropped. As for live pups/litters and stillbirths, 1 estrous cycle after chemotherapy, the live pups/litters delivered in each group were similar, with stillbirth seldom happening (Table I; Fig. 5A and C). To ensure that the offspring of PXL-exposed animals had no potential genetic defects, we examined the karyotypes of 3 random pups from every pregnant mother. The results revealed that every live pup had a euploid karyotype (Tables SII-SV).

*Multiple doses of PXL do not cause permanent damage to mouse fertility.* In order to mimic the clinical usage of PXL, 3 subsequent doses of PXL were administered to mice (1 dose per 3 days). Since it was determined that GnRHa had no adverse effect on mouse fertility in the single-dose experiment, in order to reduce mice sacrificing in our study, we canceled this group in the multiple-dose experiment. The GnRHa was administered 1 estrous cycle before, during and continuing with one more estrous cycle following chemotherapy (Fig. 1B). The follicle counts were similar to those of the single-dose-PXL experiment. On day 1 following 3 doses of chemotherapy, the antral follicles in the PXL and PXL+GnRHa groups were significantly reduced ( $7.00\pm1.00$  vs.  $2.00\pm0.00$  vs.  $2.70\pm0.30$ ;  $P<0.05$ ). The primordial, primary and secondary follicles were all similar between groups. The administration of GnRHa during chemotherapy significantly reduced the PXL-induced atretic follicles ( $30.60\pm5.00$  vs.  $63.80\pm4.00$ ;  $P<0.05$ ). On days 6 and 11 following chemotherapy, antral follicles in the PXL group were still less than those in the control, but co-treatment with PXL and GnRHa helped antral follicles recover on day 11. The increase in atretic follicles in the PXL group also lasted until day 11, but the PXL+GnRHa group maintained a similar amount of atretic follicles to that of the control group. On day 16 following chemotherapy, no significant difference in follicles of all stages was identified among groups (Fig. 6A). To further estimate the duration of the gonadotoxicity caused by multiple doses of PXL administration, ovarian stimulation was also

performed. On days 1 and 6 following the last dose of PXL, significantly less MII oocytes were collected in the PXL group than in the control (D1,  $1.00\pm0.00$  vs.  $30.40\pm5.27$ ;  $P<0.001$ ; D6,  $17.20\pm4.25$  vs.  $31.33\pm4.67$ ;  $P<0.05$ ; Fig. 6B). With the protection of GnRHa, even more MII oocytes were retrieved than in the control ( $46.80\pm3.44$  vs.  $31.33\pm4.67$ ;  $P<0.05$ ; Fig. 6B) on day 6 following chemotherapy. On days 11 and 16, the amount of MII oocytes was similar in all groups.

## Discussion

In the present study, it was determined that PXL only induced loss of antral follicles and increased atretic follicles without causing any loss of primordial, primary or secondary follicles. Notably, after 1-2 estrous cycles for recovery, the follicle counts of each stage tended to be the same among groups. In addition, AMH examination verified that the ovarian reserve was not affected by PXL. PXL only affected antral follicles. That was consistent with the results of former studies, which reported that PXL acted on cells with active division, irrespective of their malignancy (30), since the development from secondary to antral follicles is a process in which granulosa cells, theca cells and vessels proliferate rapidly (31). The transient impact of PXL may be due to its short plasma half-time (32-34) and limited effect on pre-antral follicles. When pre-antral follicles grew into antral follicles in the next estrous cycle, the plasma concentration of PXL had already decreased. As a result, after one estrous cycle, the morphology of follicles was able to recover.

To understand which stage of oocytes in antral-preovulatory follicles would be affected by PXL, an *in vitro* experiment was carried out. The results revealed that, following exposure to PXL, mice GV oocytes were capable of maturation, which suggested that GV oocytes were insensitive to PXL, since microtubules had not assembled into specific forms at this stage (35,36). However, oocytes in MI or MII were very sensitive to PXL, as their development depends on the assembly of microtubules (37,38) and the formation of a spindle apparatus, which were destroyed by exposure to PXL.

To confirm the effect of PXL on oocytes *in vivo*, an animal model was designed to estimate the time mice required to recover normal ovulation. This was measured through ovarian stimulation of mice at different time-points following chemotherapy. It was determined that PXL caused acute oocyte damage. Following the administration of PMSG in mice 1 day after chemotherapy, when oocytes were in antral-preovulatory follicles and directly exposed to PXL, the number and quality of oocytes was markedly decreased. Following 1 estrous cycle, MII oocytes collected in the PXL group had a similar quality to those of the control group. Two estrous cycles after chemotherapy, the MII oocytes in the PXL and control groups were totally comparable. These results were consistent with the transient effect of PXL on antral follicles, and supplemented the findings of a previous study, which stated that PXL caused a meiotic maturation delay and spindle defects in mouse oocytes on hCG trigger day (14).

The mating experiment indicated that, although PXL did not disrupt copulation, it could cause stillbirths, which was consistent with a previous study (18). Following 1 estrous cycle, the recovery of litter size and disappearance of stillbirth

in the PXL group was consistent with the effect of PXL on antral follicles and oocytes, further verifying the transient effect of PXL *in vivo*. Of note, there was 1 stillbirth on day 11 in the PXL group, which may have been a random incident, since there was no other stillbirth in any subsequent mating; 3 stillbirths were recorded in the control group on day 1. The karyotypes of every live pup delivered in all four groups were normal, which indicated that PXL caused lethal impairment in oocytes. On day 16, a slope in the pregnancy rate was observed in the PXL group, following repeated experiments. However, on days 30 and 45 following chemotherapy, the pregnancy rate and litter size in the PXL group were similar to those of the control group. This result suggested that the reduced pregnancy rate in the PXL group on day 16 was not due to the decrease in ovarian reserve, but other unknown reasons that require further exploration.

GnRHa have been studied as protective agents of chemotherapy-induced ovarian failure for decades. The mechanism of GnRHa was considered to be decreasing FSH levels and suppressing follicle growth through the pituitary-gonadal axis (39), as well as upregulating anti-apoptotic molecules (40) and decreasing the exposure of primordial follicles to cytotoxic agents (41). However due to contradictory results of clinical trials (23,42), to date, GnRHa has not been recommended as a regular ovarian protective agent (43,44). In the present study, the follicle count results revealed that GnRHa suppressed follicle maturation effectively. GnRHa reduced atretic follicles in the PXL+GnRHa group, which suggested that pretreatment with GnRHa kept ovaries in a relatively quiescent condition to avoid damage induced by PXL to antral follicles. It was speculated that the growing follicles were suppressed due to the administration of GnRHa before they became sensitive to PXL. During PMSG or mating-induced ovulation when the plasma concentration of PXL decreased rapidly, exposure to PXL at lower concentrations led to the better-quality of oocytes. In the mating experiment, mice that received a GnRHa injection delivered fewer litters, since GnRHa disrupted copulation by suppressing reproductive hormones. In combination, the protective effect of GnRHa was not obvious, possibly due to the short duration of the effect of PXL on ovaries.

Multiple-dose administration of PXL had a similar effect on ovaries to that of the single-dose experiment, suggesting that there was no cumulative effect or follicle exhaustion caused by PXL. This mouse model mimicked the common clinical chemotherapy regimen with PXL included [i.e., TP regimen in ovarian cancer (45) and AC-T regimen in breast cancer (11), in which multiple courses of PXL treatment are required]. Notably, ovulation in the PXL+GnRHa group was higher than that of the control 1 estrous cycle after the last dose of PXL. This may be due to the controlled ovarian hyperstimulation effect of GnRHa followed by gonadotrophin PMSG. This suggested that the temporary destruction of the oocytes was more likely associated with the last dose of PXL, which once again underlined the transient effect of PXL on ovaries.

In clinical practice, physicians may face several challenges, such as what the required duration of contraceptive method administration is following chemotherapy and whether GnRHa should be used to protect ovarian function. Physicians used to recommend 6 months of contraceptives based on experience. The present study revealed that PXL had no impact

on ovarian reserve and only a transient one on oocytes. The present laboratory evidence provided the possibility for shortening contraceptive method administration following PXL chemotherapy. The present study also revealed that GnRHa has a protective effect on ovaries in PXL chemotherapy, and provided a scheme of GnRHa administration for reproductive protection, which suggests that the application of GnRHa should be encouraged in PXL-based chemotherapeutic regimens. Further research should be carried out to determine whether the impact of PXL on human ovaries and oocytes is consistent with its impact on mouse ovaries and oocytes.

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### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

NM performed the experiments, analyzed and interpreted the data and wrote the manuscript. GeC contributed to the execution of the oocyte experiment. JC performed the hormone assays and assisted in data analysis. MC performed the animal experiments and acquired the data. YY performed the histological analyses of ovaries. QL, MT and XF assisted in the animal experiments. XL contributed to the designing and drafting the article and discussed the results. SZ contributed to the analysis and interpretation of data. DM and GaC conceived the study, discussed the results and supervised the study. KL conceived the study, designed the experiments, conducted study, discussed the results, contributed to the drafting of the article, submission and revision. JA conceived the study, designed the experiments, discussed the results and supervised the study. All the authors have read and approved the final article.

### Ethics approval and consent to participate

All experiments were approved by the Institutional Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval no. TJ-A20161101).

### Patient consent for publication

Not applicable.

## Competing interest

The authors declare that they have no competing interests.

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