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Influence of gonadotropin-releasing hormone agonist on the effect of chemotherapy upon ovarian cancer and the prevention of chemotherapy-induced ovarian damage: an experimental study with nu/nu athymic mice*

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Abstract: Background and objective: Gonadotropin-releasing hormone (GnRH) plays an important role in the regulation of ovarian function and ovarian cancer cell growth. In this study, we determined whether administration of the GnRH agonist (GnRHa), triporelin, prior to cisplatin treatment affects cisplatin and/or prevents cisplatin-induced ovarian damage. Methods: nu/nu mice were injected with ovarian cancer OVCAR-3 cells intraperitoneally. After two weeks, the mice were treated with saline (control), cisplatin, GnRHa, or cisplatin plus GnRHa for four weeks. At the end of the experimental protocol, blood, tumor, ovary, and uterine tissues were resected for hematoxylin and eosin (H&E) staining, immunohistochemical analyses of Ki67, nuclear factor-κB (NF-κB), and caspase-3, transmission electron microscopy of apoptosis, or enzyme-linked immunosorbent assay (ELISA) analyses of anti-Mullerian hormone (AMH). Results: Cisplatin treatment effectively inhibited tumor growth in mice treated with human ovarian cancer cells; however the treatment also induced considerable toxicity. Immunohistochemical analyses showed that Ki67 expression was reduced in cisplatin-treated mice compared to control (P<0.05), but there was no statistically significant differences between cisplatin-treated mice and cisplatin plus GnRHa-treated mice (P>0.05), while expressions of NF-κB and caspase-3 were reduced and induced, respectively, in cisplatin-treated mice and cisplatin plus GnRHa-treated mice. Apoptosis occurred in the GnRHa, cisplatin, and cisplatin plus GnRHa-treated mice, but not in control mice. Ovaries exposed to GnRHa in both GnRHa mice and cisplatin-treated mice (combination group) had significantly more primordial and growth follicles and serum levels of AMH than those in the control mice and cisplatin-treated mice (P<0.05). Conclusions: Administration of GnRHa to mice significantly decreased the extent of ovarian damage induced by cisplatin, but did not affect the anti-tumor activity of cisplatin.

Key words: Gonadotrophin-releasing hormone, Cisplatin, Ovarian cancer, Animal experiment
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1 Introduction

Ovarian cancer remains the most fatal disease affecting the female reproductive system (Jemal *et al*., 2008). Unless diagnosed early, prognosis of ovarian cancer is extremely poor. Early ovarian cancer causes non-specific symptoms, making early detection very difficult. Furthermore, the exact cause of ovarian cancer is unknown and the risk factors for ovarian cancer include hormones, genetic mutations (such as *BRCA1* (breast cancer 1, early onset) and *BRCA2*),

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and alcohol consumption, while other factors may show a protective effect, e.g., giving birth to multiple children, early age at the first pregnancy, and use of low dose hormonal contraception. To date, surgery is the preferred treatment choice for ovarian cancer and chemotherapy is usually administered after surgery to treat any residual disease. To effectively control ovarian cancer, improve patient survival, and reduce disease incidence, novel approaches for the early detection, effective treatment, and prevention are urgently needed.

Gonadotropin-releasing hormone (GnRH) plays a key role in the regulation of ovarian functions and mammalian reproduction. GnRH is normally synthesized and released from the hypothalamus. Recent studies have shown that GnRH is also produced by ovarian cancer cells (Imai *et al*., 1994; Emons *et al*., 2000). GnRH and its receptor have been detected in most endometrial and ovarian cancer cell lines and in over 80% of biopsy specimens of these cancers (Imai *et al*., 1994; Emons *et al*., 2000). GnRH receptor expression in ovarian cancer cells directly mediates the antiproliferative effects of GnRH agonist (GnRHa) (Gründker *et al*., 2002b). Other studies have demonstrated the differential effects of GnRHa on the proliferation of human ovarian cancer cells. For example, Arencibia and Schally (2000) reported that GnRH-I produced by human ovarian cancer cells acts as a negative autocrine regulator of proliferation. In contrast, Emons *et al*. (2000) found that the GnRH-I agonist, triptorelin, stimulated proliferation of human ovarian cancer cells after 48 h culture at a dose of 10 ng/ml, but inhibited tumor cell growth after 72 h culture at a concentration of 1000 ng/ml. Other studies have shown that GnRHa can inhibit the proliferation of various human tumor cells, including ovarian cancer in a dose- and time-dependent manner (Emons and Schally, 1994; Shibata *et al*., 1997; Gründker *et al*., 2002a). The anti-proliferative effects of GnRH-I are mediated by the suppression of an extracellular signal-regulated protein kinase 1/2 pathway (Kim *et al*., 2006). However, it is unclear whether GnRHa can protect the ovaries from chemotherapy-induced toxic effects and alter chemotherapeutic responses in ovarian cancer.

In the current study, we used a mouse model of ovarian cancer to demonstrate GnRHa-induced effects on the regulation of ovarian cancer chemotherapy and prevention of chemotherapy-induced ovarian damage, as well as the signaling mechanisms involved.

2 Materials and methods

2.1 Cell line and culture

The human ovarian serous papillary cystadenocarcinoma cell line, OVCAR-3, was obtained from the Academia Sinica Cell Bank (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose with 10% fetal bovine serum, 100 U/ml ampicillin and streptomycin (all from Gibco, Grand Island, NY, USA) at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2.

2.2 Animal experiments

Female nu/nu athymic mice (4–6 weeks old) were obtained from the Guangdong Provincial Research Center for Laboratory Animal Medicine (China). For producing the mouse model of ovarian cancer, OVCAR-3 cells were grown and harvested with 0.25% trypsin-phosphate buffered saline (PBS)-ethylenediaminetetraacetic acid (EDTA), and then washed with DMEM and PBS, resuspended in PBS at a concentration of 1×10^6 per 200 μl, and intraperitoneally injected into these mice (n=24). Two weeks following tumor cell inoculation, these mice were randomly divided into four groups (six mice per group). Group A mice were intraperitoneally injected once with saline and maintained for six weeks, Group B mice were given a subcutaneous injection of saline once and maintained for two weeks and then an intraperitoneal injection of cisplatin (5 mg/kg) (Qilu pharmaceutical Company, Shandong, China) was administered once a week for four weeks. Group C was treated with a subcutaneous injection of triporelin (0.3 mg) (Beaufour-Ipsen Pharmaceutical Co., Ltd., Tianjin, China) once and maintained for two weeks, and then an intraperitoneal injection of saline was given weekly for four weeks, and Group D was treated with a subcutaneous injection of triporelin (0.3 mg) once and maintained for two weeks and then an intraperitoneal injection of cisplatin (5 mg/kg) was administered weekly for four weeks. Mouse body

weight was measured weekly and at the end of the experiment the mice were then sacrificed for the collection of tumor tissues and ovaries. These tissues were weighed using an electronic balance. These tissues were excised and fixed in 4% formaldehyde and embedded in paraffin for histology and immunohistochemical analyses.

2.3 Immunohistochemistry

Immunohistochemical staining was performed to detect the expression of Ki67, nuclear factor-κB (NF-κB), and caspase protein 3 in paraffin-embedded tissue sections of ovaries using a standard protocol of horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG described previously (Gründke *et al*., 2000). Mouse anti-Ki67 and anti-NF-κB antibodies were obtained from Beijing Zhongshan Golden Bridge Biotechnology (China). Mouse anti-caspase-3 antibody was obtained from Guangzhou Jet Way Biotech Co., Ltd. (China). Immunohistochemically stained tissue sections were reviewed and scored by a pathologist without any knowledge of the tissue. Briefly, immunohistochemical staining for Ki67 protein in tissue sections were reviewed and then five random fields at 400× magnification were selected and counted for positively stained tumor cells vs. the total number of cells per field. Staining intensity of these tumor cells was also recorded: 1, negative; 2, weakly positive; 3, positive; or 4, strong positive. The index of total cell immunostaining score (TISS) was then calculated: percentage of positive cells×staining intensity, and then scored a percentile of 0% 1, 0–25% 2, 25%–50% 3, or 50%–75% 4. We then performed image analyses using Image-pro plus 6.0 software to analyze the average optical density of NF-κB and caspase-3 staining.

2.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to detect anti-Mullerian hormone (AMH) levels in blood samples of the nude mice with or without drug treatments. Briefly, the blood samples were obtained from the mouse eyes and serum was isolated through centrifugation for ELISA analyses of AMH expression using a kit from Uscn Life Science Inc. (Wuhan, China) according to the manufacturer's protocol. Optical densities were read at 405 nm and AMH concentrations were determined by comparison with standard curves.

2.5 Histological evaluation of ovarian tissues

At the end of the animal experiments, left and right ovaries were randomly assigned to be fixed in buffered formaldehyde and then embedded in paraffin. Paraffin-embedded ovarian tissues (one per animal) were serially sectioned at 5 μm for hematoxylin and eosin (H&E) staining and evaluation. Briefly, follicles were classified as primordial if they contained an oocyte surrounded by a partial or complete layer of squamous granulosa cells. Primary follicles showed a single layer of cuboidal granulose cells. Follicles were classed as secondary if they possessed more than one layer of granulose cells with no visible antrum. Early antral follicles possessed generally only one or two small areas of follicular fluid (antrum), while antral follicles possessed a single large antral space. Preovulatory follicles had a rim of cumulus cells surrounding the oocyte. Primary, secondary, early antral, antral, and preovulatory follicles were classed as growth follicles. The number of primordial and growth follicles were counted and compared according to previous studies (Myers *et al*., 2004; Danforth *et al*., 2005).

2.6 Transmission electron microscopic evaluation of apoptosis

At the end of the animal experiments, tumor tissues from the mice were resected and fixed in 4% (0.04 g/ml) glutaric acid and then in 1% (0.01 g/ml) osmium tetroxide. The tissue samples were then embedded, polymerized, ultrathin-sectioned for double staining with uranyl acetate and lead citrate, and reviewed using a transmission electron microscope (JELO100CX, JEOL, Japan). One hundred cells were reviewed from each sample and then summarized and compared to each group.

2.7 Statistical analysis

All data are expressed as mean±standard error of the mean (SEM). Statistical analysis was carried out using one-way analysis of variance (ANOVA). A P-value <0.05 was considered to be statistically significant.

3 Results

3.1 Effects of cisplatin alone and combination with GnRHa on suppression of ovarian cancer growth

We produced a mouse model of human ovarian cancer and treated these mice with cisplatin, GnRHa, or a combination of the drugs for six weeks, with mouse weight recorded weekly (Fig. 1a). At the end of the experiments, the mice were sacrificed and tumors, ovaries, and uteri from these mice were resected and analyzed. Data showed that the mouse body weight and total weight of the ovaries, uteri, and tumor tissues decreased in the cisplatin and the combined cisplatin and GnRHa groups (Fig. 1). Histological examination showed that human ovarian cancer OVCAR-3 cells grew well in the mouse peritoneal cavity (Fig. 2). At the molecular level, Ki67 expression was significantly reduced in cisplatin-treated mice compared to the control mice (P<0.05), but there was no statistically significant difference between the cisplatin-treated mice and cisplatin plus GnRHa-treated mice (P>0.05) (Fig. 3). In contrast, expressions of NF-κB and caspase-3 were reduced and induced, respectively, in cisplatin-treated mice and combined cisplatin and GnRHa-treated mice (Fig. 4). In addition, transmission electron microscopic analysis showed that apoptosis occurred in the GnRHa, cisplatin, and cisplatin plus GnRHa-treated mice, but not in control mice (Fig. 5). These data indicate that cisplatin treatment was able to effectively inhibit tumor growth in this mouse model of human ovarian cancer but the treatment also created considerable toxicity. However, pretreatment with GnRHa did not affect cisplatin-induced changes in tumor growth, ovarian or uterine weights, and gene expression compared to the cisplatin alone treated mice.

3.2 Prevention of cisplatin-induced ovarian damage using GnRHa

We evaluated the effects of cisplatin alone and in combination with GnRHa on mouse ovaries and uteri. Compared with the control group, the weights of the ovaries and uteri in the cisplatin, GnRHa, and cisplatin plus GnRHa treated mice were significantly reduced (P<0.05) (Figs. 1b and 1c). Furthermore, cisplatin treatment showed fewer primordial follicles and

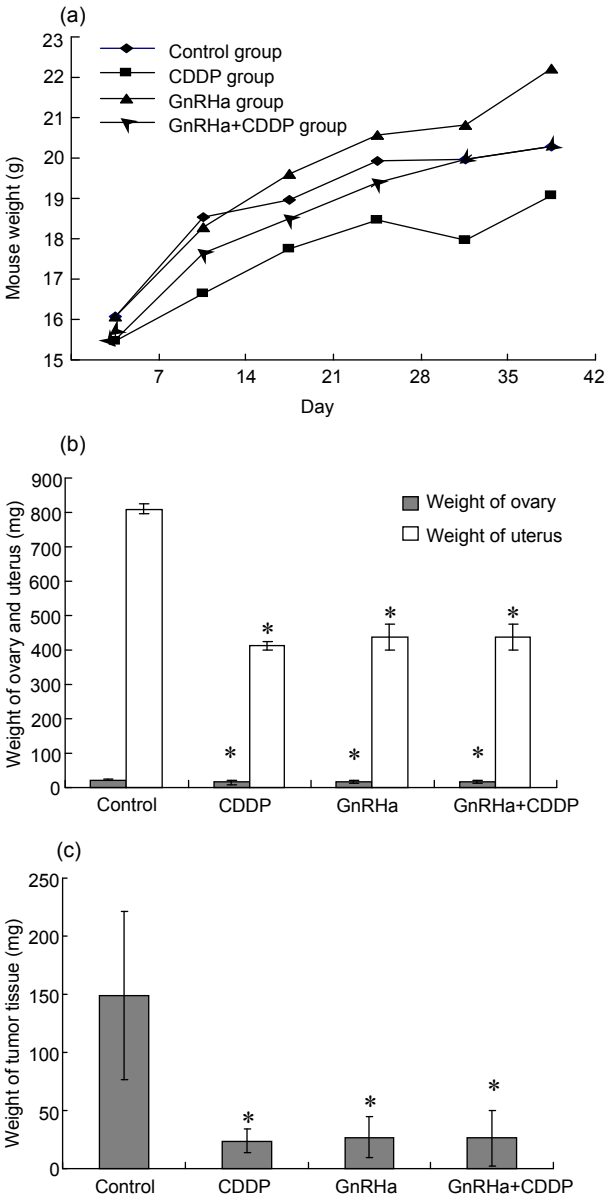

Fig. 1 Weight changes in mice

(a) Mouse weight growth curve. The athymic nude mice were intraperitoneally injected with OVCAR-3 cells. Two weeks following tumor cell inoculation, the mice were randomly placed into four groups for six-week treatment of saline (control group), or a week of saline followed by four weeks of cisplatin (CDDP), two weeks of GnRHa triporelin and four weeks of saline, or two weeks of GnRHa and then four weeks of CDDP treatment. The mouse body weight was measured weekly; (b) Weights of the ovary and uterus. At the end of the experiments, the mice were sacrificed and the ovary and uterus were resected and weighed. * P<0.05 (vs. control group); (c) Tumor weight. At the end of experiments, the tumor tissues were resected and weighed. * P<0.05 (vs. control group)

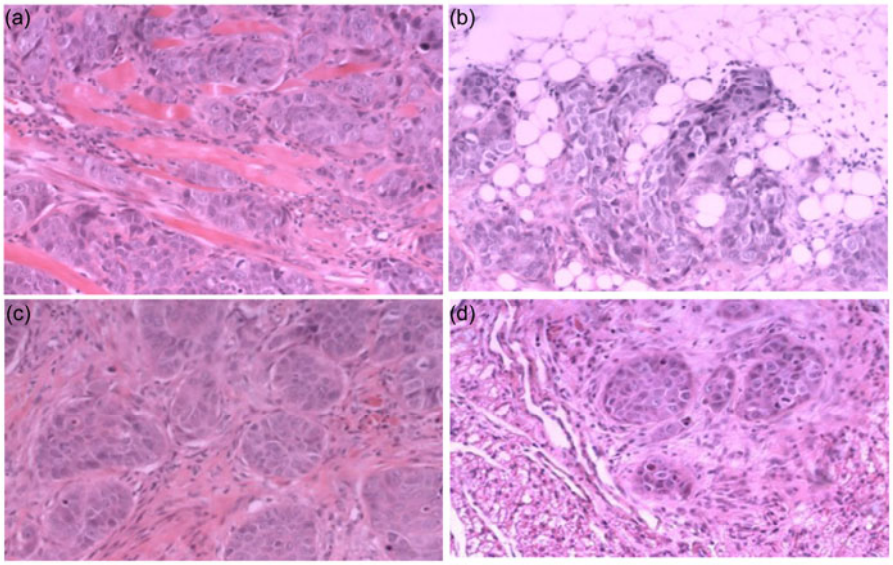

Fig. 2 H&E staining of tumor tissues

At the end of experiments, the mice were sacrificed and tumor tissues were resected and processed for H&E staining. (a) Control group; (b) Cisplatin (CDDP) group; (c) GnRHa group; (d) GnRHa+CDDP group (400× magnification)

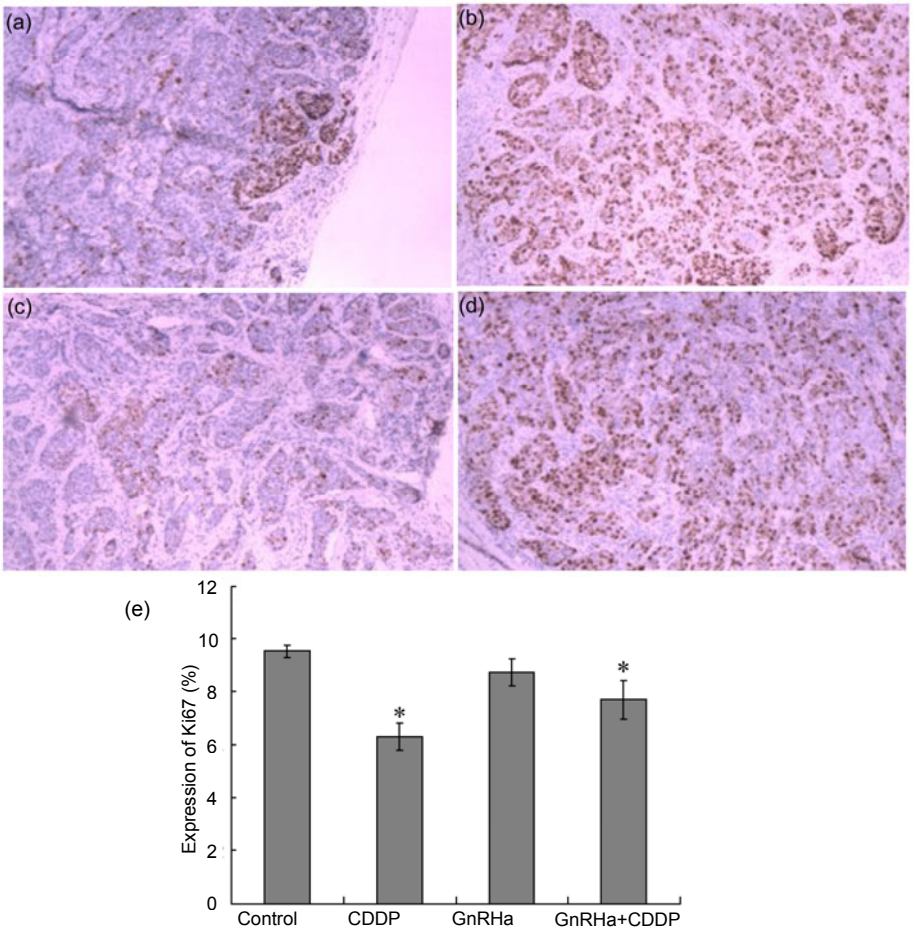

Fig. 3 Expression of the proliferation marker Ki67 in tumor tissues

Tumor tissue sections were immunostained with Ki67 antibody (a–d), reviewed, scored, and summarized (e). * $P<0.05$ (vs. control group). (a) Control group; (b) Cisplatin (CDDP) group; (c) GnRHa group; (d) GnRHa+CDDP group (400× magnification)

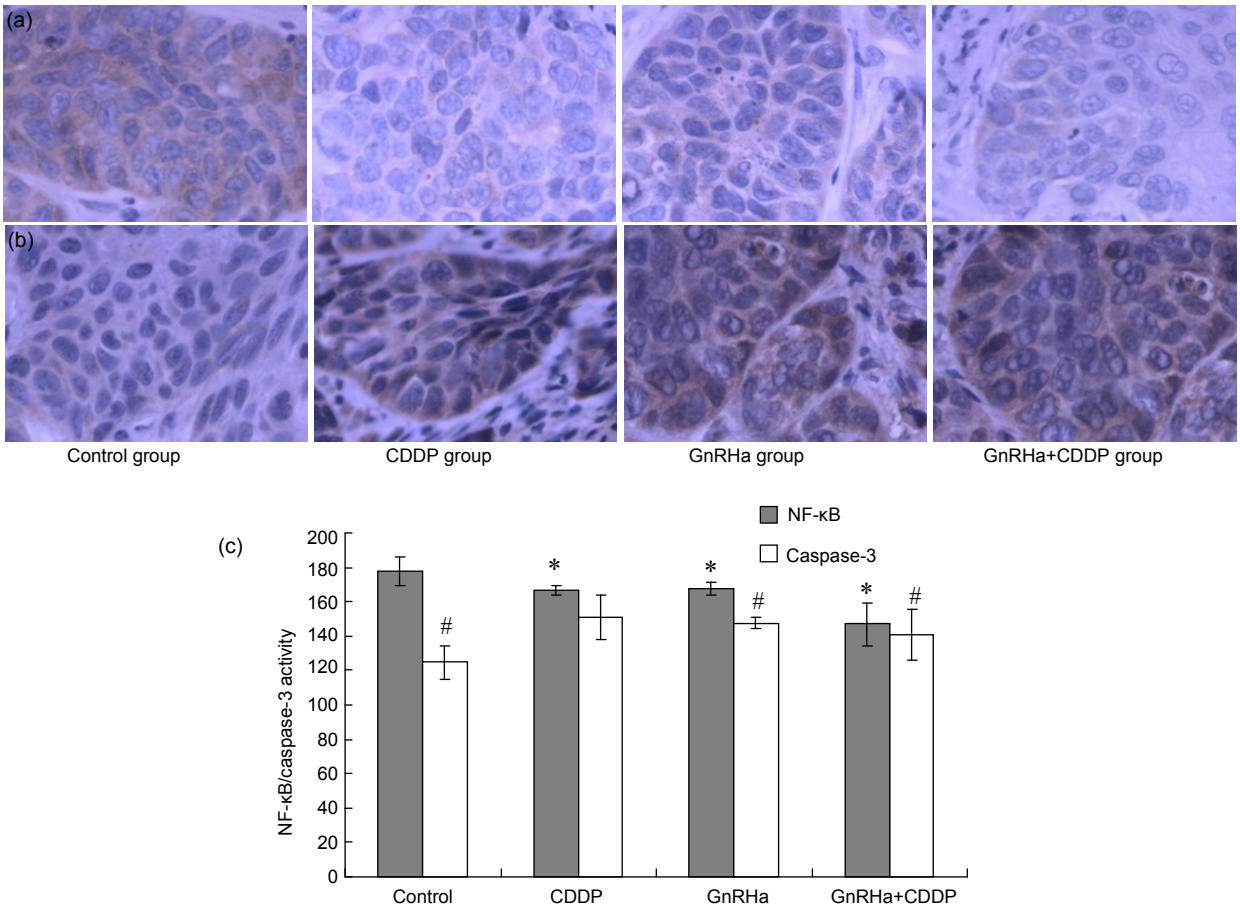

Fig. 4 Expressions of NF-κB and caspase-3 in tumor tissues using immunohistochemistry

(a) Expression of NF-κB; (b) Expression of caspase-3; (c) Quantified data of NF-κB and caspase-3. $^{*}P<0.05$ (vs. control group); $^{\#}P<0.05$ (vs. cisplatin (CDDP) group) (400× magnification)

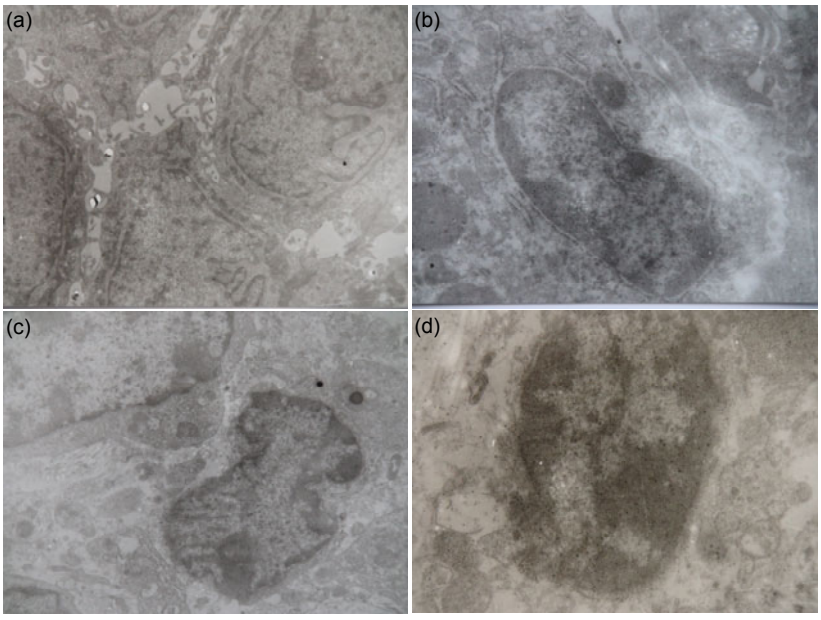

Fig. 5 Transmission electron microscopic (TEM) analysis of tumor cell apoptosis

Tumor tissues were subjected to TEM tissue processing and TEM evaluation and scoring. Representative photo images are shown. Briefly, the control mice did not have many apoptotic ovarian cancer cells, e.g., the cell membrane, mitochondria, nucleus, and other organelles were intact, whereas cisplatin-treated mice showed early apoptotic cells, e.g., chromosome edging in the nuclei, nuclear shape not structured. In contrast, GnRHa treated mice showed both apoptotic and necrotic cells. The cisplatin and GnRHa combination group showed cell membrane budding to form vesicles and apoptotic bodies. (a) Control group; (b) Cisplatin (CDDP) group; (c) GnRHa group; (d) GnRHa+CDDP group

follicular growth in the ovaries than that of the cisplatin combined with GnRHa treated mice (P<0.05) (Fig. 6a). In contrast, there was no significant difference in the primordial and follicular growth between the combination group and the control group (Fig. 6a). In addition, serum levels of AMH concentration significantly decreased in the cisplatin-treated group as compared to the other three groups, while there were no significant differences among the control, GnRHa, and combination groups (Fig. 6b).

4 Discussion

In the current study, we investigated the effect of the GnRHa triporelin on its ability to regulate the effect of cisplatin on ovarian cancer growth in mice and on preventing cisplatin-induced ovarian damage. We found that cisplatin treatment effectively inhibited tumor growth in this mouse model of human ovarian cancer; however, cisplatin also induced considerable toxicity. In contrast, the GnRHa triporelin did not affect the ability of cisplatin to inhibit ovarian cancer growth and apoptosis as well as gene expression, whereas the addition of triporelin to cisplatin-treated mice had significantly more primordial and follicular growth and serum levels of AMH than those in the cisplatin-treated mice.

Ovarian primordial follicles, which constitute the vast majority of ovarian follicles and represent the 'ovarian follicular reserve', are not under gonadotrophic control. Since GnRH receptors are characterized in the ovaries of different species (Janssens *et al*., 2000), a decrease in pituitary gonadotrophins probably does not directly protect the primordial follicles. Alternatively, a protective effect of GnRHa on murine ovaries exposed to chemotherapy could also be mediated by direct ovarian effects. Furthermore, GnRHa reduce pituitary gonadotrophin secretion through receptor down-regulation. They cause an initial flare-up effect on pituitary follicle stimulating hormone (FSH) and luteinzing hormone (LH) secretion, which results in a flare-up of follicular growth. Previous studies have reported that chemotherapy increases the growth follicles causing them to 'burn out', whereas the addition of GnRHa increases the primordial follicles but decreases the growth follicles ration (Meirow *et al*., 2004). Further research and clinical studies are needed in order to confirm these results.

This study demonstrated that triporelin significantly decreased the extent of ovarian damage induced by cisplatin, but did not affect the anti-tumor activity of cisplatin.

This mouse model of human ovarian cancer is frequently used to assess mechanisms of ovarian cancer development and preclinical evaluation of chemotherapeutical agents. Ovarian cancer growth and progression is regulated by many factors. For example, the presence of GnRH messenger RNA (mRNA) and GnRH receptors has been reported in ovarian cancers (Gründker *et al*., 2002a; Völker *et al*.,

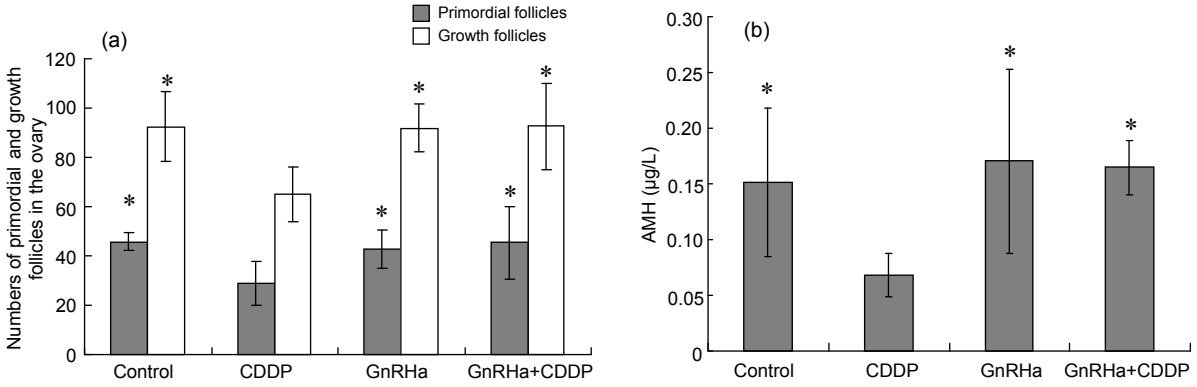

Fig. 6 Functions of the ovary

(a) Morphology of the ovary. The ovarian tissues were resected from the mice and processed and stained with H&E and reviewed under the microscope. The data were summarized for primordial and growth follicles in the ovary; (b) ELISA analysis of AMH. The blood from these mice was obtained for serum isolation and the latter was analyzed for AMH expression using ELISA. * P<0.05 (vs. cisplatin (CDDP) group)

2002), although their functional significance in ovarian cancers is not yet known. Several in vitro studies have suggested that GnRH exerts a direct inhibitory effect on the growth of normal and malignant ovarian epithelial cells in a dose- and time-dependent manner through the up-regulation of p53 and p21 protein levels which lead to cell cycle arrest (Kang *et al*., 2000; Völker *et al*., 2002). The anti-proliferative effects of GnRHa on ovarian cancer cells can be mediated by activation of the GnRH receptor (Kim *et al*., 2006) and the induction of apoptosis by GnRH was mediated by activation of the Fas signaling pathway (Imai *et al*., 1998; Kang *et al*., 2000; Tang *et al*., 2002). Our current data suggest that GnRHa was able to induce ovarian cancer cells to undergo apoptosis, but prevented ovarian damage by cisplatin. The reason why GnRHa did not synergistically affect cisplatin on ovarian cancer cells and ovary damage but prevented ovary damage is unknown. However, clinical data have failed to demonstrate any anti-tumor effects of GnRHa in ovarian cancer (Emons *et al*., 1996).

Furthermore, the current study demonstrated that cisplatin was able to reduce NF-κB and induce caspase-3 expression in ovarian cancer cells. NF-κB is a protein complex that controls the transcription of various genes for various biological activities of the genes, such as genetic regulation, immune response, cell adhesion, differentiation, proliferation, angiogenesis, and apoptosis (Stoffel *et al*., 2004; Pereira and Oakley, 2008). The imbalance between NF-κB and its inhibitor, IκB, is closely related to the development of many diseases, including cancer (Sun and Zhang, 2007). Some studies have implied that inhibition of NF-κB expression can promote apoptosis in certain cells, such as neurons (Grilli *et al*., 1996), Schwann cells (Carter *et al*., 1996), and embryonic kidney cells (Grimm *et al*., 1996). Several other ovarian cancer cell lines have been found to express NF-κB, including OVCAR-3 (Bours *et al*., 1994), CAOV-3 (Asschert *et al*., 1999), and UT-OC-5 (Seppänen *et al*., 1998). This is due to NF-κB regulation of anti-apoptotic genes, including *TRAF1* and *TRAF2*, to in turn monitor the activity of the caspase family of enzymes, which are central to most apoptotic processes. Thus, NF-κB plays an important role in inhibiting chemotherapy-induced apoptosis (Wu *et al*., 1996; Gründker *et al*., 2000). Moreover, the activated caspase-9 will digest other effector caspases, such as caspase-3, and the latter will activate (ADP-ribose) polymerase PARP (poly AD-ribose polymerase) to stimulate cells to undergo apoptosis (Zou *et al*., 1997; Enari *et al*., 1998). NF-κB activation blocks caspase cleavage and cytochrome c release, indicating that NF-κB suppresses the earliest signaling components of the caspase cascade (Wang *et al*., 1998).

In addition, previous data have shown that activation of GnRH receptors in the pituitary leads to a decline in gonadotropin secretion and subsequent reduction of gonadal steroids. GnRH receptors in tumor cells not only serve as tumor growth factors (Kang *et al*., 2000), but also protect the function of the ovary. For example, Meirow *et al*. (1999) showed that the number of primordial follicles in the ovary is the most sensitive index to reflect ovarian reserve functions in their animal experiment. In our current study, the primordial and growth follicles decreased in cisplatin-treated mice, but the combination of cisplatin with GnRHa protected the ovary, e.g., the number of primordial and growth follicles remained constant. Moreover, AMH is expressed by granulosa cells in the ovary during the reproductive years in female subjects; thus, detection of serum AMH levels serves as a functional test for the ovary. In addition, we examined the morphological changes of the ovary following treatment. Through all of these assessments, we have demonstrated that pretreatment with GnRHa prevented ovarian damage from cisplatin treatment. These data suggest that future studies should focus on a clinical Phase I trial using a combination of GnRHa with cisplatin therapy in ovarian cancer patients.

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