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Effects of ethephon on serum levels of sex hormone, apoptosis, and cell cycle of ovaries in mice

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Abstract

Introduction: The effects of ethephon on the reproductive systems of mammalian females are still ambiguous. This study was conducted to evaluate the toxic effects of ethephon on the female reproductive system.

Material and methods: Forty female C57 mice were used as experimental subjects and evenly divided into 8 groups, which were fed with mixed ethephon (0, 107.3, 214.5, and 429 mg/kg bw/day) and pure water. After 20 and 40 days of gavage, the mice were weighed and individual organ coefficients of the ovaries were measured. Enzyme-linked immunoassay was used to detect the serum levels of serum sex hormones. The cell cycle distribution and rate of apoptosis of mouse ovarian tissues were examined using flow cytometry.

Results: Ethephon intoxication significantly decreased serum levels of progesterone (P) and oestradiol (E_2) and increased the serum levels of luteinizing hormone (LH). The serum levels of follicle-stimulating hormone (FSH) decreased and then increased over time. In addition, ethephon significantly inhibited the apoptosis rate in the ovary and caused G0/G1 and G2/M arrest.

Conclusion: These results indicate that prolonged exposure to ethephon may have negative effects on the female reproductive system.
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Key words: ethephon; female mice; cell cycle; apoptosis; serum sex hormone

Introduction

Organophosphate pesticides (OPs) are widely used for agricultural production, resulting in annual exposure to 2–3 million people, mostly in the developing world [1]. It has several advantages, including shortening the production cycles and increasing cost effectiveness. However, excessive exposure to OPs is a global threat to human health and causes environmental pollution. Organophosphate pesticides are found as residue in soil, rivers, and the ocean [2]. Approximately 250,000 people die from pesticide poisoning annually, and OPs are the most commonly implicated pesticides [3].

Ethephon is one of the most widely used OPs that controls crop growth and development, including fruit ripening, leaf senescence, and seed germination [4]. It is commonly used for a variety of crops, including fruit, vegetables, cereals, and oilseed crops. In recent times, for the better yield of crops, overuse of ethephon has led to the accumulation of pesticide residues, which can enter the human body through the food chain and may impact health [5]. Ethephon, a type of OP, inhibits acetylcholinesterase activity(AchE) and leads to nervous system damage [6]. The clinical signs of ethephon poisoning include nausea, headache, dermal irritation, and dyspnoea [7]. Ethephon has been reported to cause inflammatory response and fibrosis in the liver and colon [8], increase mammary gland ectasia [7], induce nephrotoxicity and kidney dysfunction [9], suppress both humoral and cell-mediated immunity [10], decrease RNA and DNA content, and induce structural chromosomal aberrations [11].

There is increasing concern regarding the effects of environmental pollutants on the reproductive system. The secretion of sex hormones affects the development of the reproductive system. Abd Eldaim et al. reported that exposure to ethephon in male rats decreased serum levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), and increased immotile sperm and sperm abnormalities [12]. Yan et al. reported that ethephon exposure in male Sprague-Dawley rats decreased serum levels of testosterone and oestradiol (E_2), increased the spermatogenic cell apoptosis index, and decreased the spermatogenic capability [13]. In

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contrast, another study found that sperm parameters or relative organ weight did not change significantly in male rats exposed to ethephon [14].

The toxicity resulting from ethephon exposure is well known; however, its effects on the reproductive system of mammalian females have not been thoroughly studied. Therefore, this study investigated the effects of exposure to ethephon on serum levels of reproductive hormones, apoptosis, cell cycle in ovarian cells, and the mutual influence between them, in female mice. These results are useful for assessing the risk of oral exposure and exploring the role of ethephon in reproductive endocrine disruption.

Material and methods

Animal care, diets, and ethephon exposure

This research protocol was approved by the Animal Experiment Ethics Committee of Jilin University (2020 Joint Trial No. 2020-10-02). Forty clean-grade mature female C57/BL6 mice, weighing 18–22 g, were provided by Liaoning Changsheng Biological Technology Co., Ltd (Changchun, China). The laboratory temperature was 18–24°C. The relative humidity was 50–60%, and the light/dark cycle was 12/12. All mice were provided with food and water ad libitum. The mice were divided randomly into 8 groups (5 mice per group) and were acclimatized to laboratory conditions for 3 days before beginning the experiment. The mice were treated with 0, 107.25, 214.5, and 429 mg/kg body weight (bw)/day of ethephon (Shanghai Yuanye Bio-Technology Co. Ltd, minimum purity 85%) in pure water for 20 days and 40 days by gavage at a dose of 0.1 mL/10 g bw. All experimental animals were treated humanely with the alleviation of suffering.

Weight measurement and sample collection

After the collection of blood samples, the female mice were weighed and killed by cervical dislocation between 8:00 A.M. and 10:00 A.M. The ovaries were removed and weighed to calculate organ coefficients. Blood was collected by bleeding of the orbital sinus and placed in a cooled room (4°C) for 2 h. After centrifugation, the serum was saved at –20°C until further use. The serum levels of FSH, LH, progesterone (P), and E2 were measured by enzyme-linked immunoassay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The interplate and intraplate coefficients of variation for all the ELISA kits were less than 15%. The sensitivities for P, E_2, FSH, and LH were 0.1 μmol/L, 1.0 pmol/L, 1.0 mIU/mL, and 1.0 pg/mL, respectively. Each standard and each serum sample was analysed in duplicate, and the mean value was used.

Cell cycle and apoptosis analysis

Flow cytometry using propidium iodide (PI) nuclear staining was performed for apoptosis and cell cycle analysis in ovarian tissue [15]. The mouse ovaries were triturated on a glass slide and transferred to 0.1 mL of phosphate buffered saline (PBS). The cells were centrifuged at 1000 rpm for 5 min. Following treatment, the cells were fixed in 1 mL of cold 70% ethanol and stored at –20°C overnight. The cells were centrifuged again, washed in 1 mL of PBS and resuspended in 1 mL of PBS. The samples were centrifuged, and the supernatant was discarded. PI (Meilunbio, CHN) was added to the cell suspension, gently mixed, and placed at 4°C for 30 min in the dark. Cell apoptosis and cell cycle distribution were analysed using flow cytometry. (BD, USA).

Statistical analysis

Each experiment was repeated a minimum of 3 times. The results are expressed as x ± SD, where x is the mean value and s is the standard deviation. SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, N.Y., USA) was used for statistical analyses. One-way analysis of variance was used to determine differences among groups, and pairwise comparisons were made using the least significant difference method. In this study, $p < 0.05$, (2-tailed tests) was considered statistically significant.

Results

Effect of ethephon exposure on body weight and ovarian organ coefficient

In this study, the bodyweight of female mice treated with 429 mg/kg ethephon was significantly lower than that of mice in the control group after 20 days of exposure ($p < 0.05$) (Fig. 1A).

As shown in Figure 1B, after 20 days of exposure, compared with mice in the control group, the ovarian organ coefficient of mice was significantly higher in the 107.3 and 214.5 mg/kg ethephon groups ($p < 0.05$). After 40 days of exposure, the ovarian organ coefficient of female mice was significantly lower in each dose group than in the control group ($p < 0.05$).

Effect of ethephon exposure on the serum level of sex hormones in female C57 mice

In this study, after 20 and 40 days of exposure, the serum level of P was significantly lower in each dose group than in the control group ($p < 0.05$) (Fig. 2A).

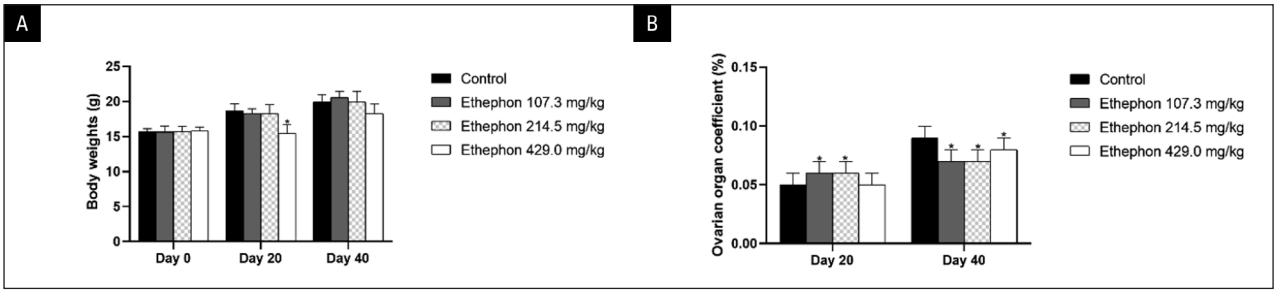

Figure 1. *The effect of ethephon on the body weight and ovarian organ coefficient of female mice. (***A***) Bodyweight of mice (***B***) ovarian organ coefficient of mice. Data are represented as the mean ± standard deviation (SD) (n = 5 per group). *p < 0.05 compared to the control group*

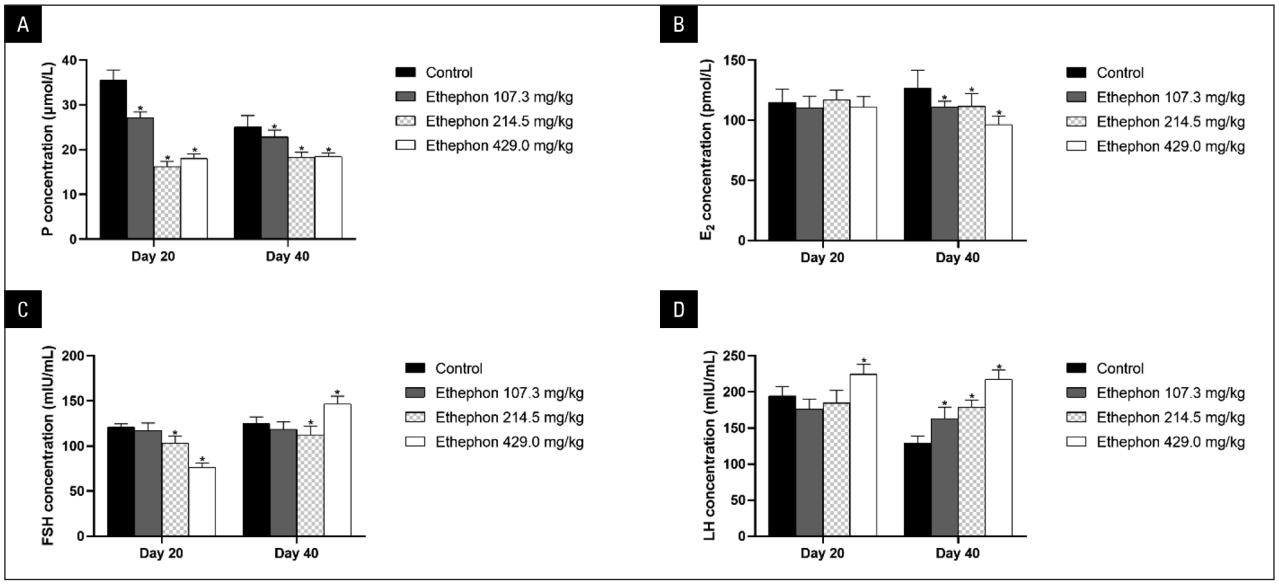

Figure 2. *The effect of ethephon exposure on the serum levels of sex hormones in female C57 mice.* **A**. *Progesterone (P);* **B**. *Oestradiol (E_2);* **C**. *Follicle-stimulating hormone (FSH);* **D**. *Luteinizing hormone (LH). Data are represented as the mean ± standard deviation (SD) (n = 5 per group). *p < 0.05 compared to the control group*

As shown in Figure 2B, we found no significant changes in E_2 levels in mouse serum after 20 days of exposure. The serum level of E_2 was significantly lowered in each dose group compared to that in the control group after 40 days of exposure ($p < 0.05$).

As shown in Figure 2C, the serum level of FSH was significantly decreased in the 214.5 and 429.0 mg/kg ethephon groups after 20 days of exposure compared to the control group ($p < 0.05$). After 40 days of exposure, the serum level of FSH was significantly decreased in the 214.5 mg/kg ethephon group ($p < 0.05$), but the serum level of FSH was significantly increased in the 429.0 mg/kg ethephon group compared to the control group ($p < 0.05$). As the treatment time increased, the serum level of FSH first decreased and then increased.

As shown in Figure 2D, the serum level of LH was significantly increased in the 429.0 mg/kg ethephon group compared to the control group after 20 days of exposure ($p < 0.05$). After 40 days of exposure, the serum level of L4 was significantly increased in each dose group compared to that in the control group ($p < 0.05$). These results indicated that ethephon caused a disorder in the serum levels of sex hormones.

Effect of ethephon exposure on cell apoptosis and cell cycle of ovarian cells in female C57 mice

The apoptosis ratio of ovarian cells in each group is shown in Figure 3A–H. As shown in Figure 3I, after 20 days of exposure the apoptosis of ovarian cells was significantly decreased in the 214.5 and 429.0 mg/kg ethephon groups ($p < 0.05$) compared to the control group. After 40 days of exposure, the apoptosis of ovarian cells was significantly decreased in the 429.0 mg/kg ethephon group ($p < 0.05$). These results indicate that ethephon inhibited the apoptosis rate in ovarian cells.

In this study, the cell cycle distribution of ovarian cells in each group is shown in Table 1 and Figure 4. After 20 days of exposure, compared to the control group, G0/G1 phase cells in the 107.3 mg/kg ethephon groups were significantly increased ($p < 0.05$), S phase cells in each dose group were significantly decreased ($p < 0.05$), and G2/M phase cells in the 214.5 and 429.0 mg/kg ethephon groups were significantly increased ($p < 0.05$).

After 40 days of exposure, compared to the control group, G0/G1 phase cells in each dose group were significantly increased ($p < 0.05$), S phase cells in each dose group were significantly decreased ($p < 0.05$), and G2/M phase cells in the 214.5 and 429.0 mg/kg ethephon groups were significantly increased ($p < 0.05$). These results indicate that ethephon induced cell cycle arrest in the G0/G1 and G2/M phase of ovarian cells.

Discussion

In China, infertility affects an estimated 15% of women of reproductive age [16]. Ethephon has been shown to induce reproductive dysfunction in male animals. However, there is a dearth of studies on the effects of ethephon on the female reproductive system. We designed the experiment, which involved exposure of female C57 mice to ethephon for 20 and 40 days, respectively, to demonstrate changes in serum sex hormone levels, apoptosis, and ovarian cell cycle in mice.

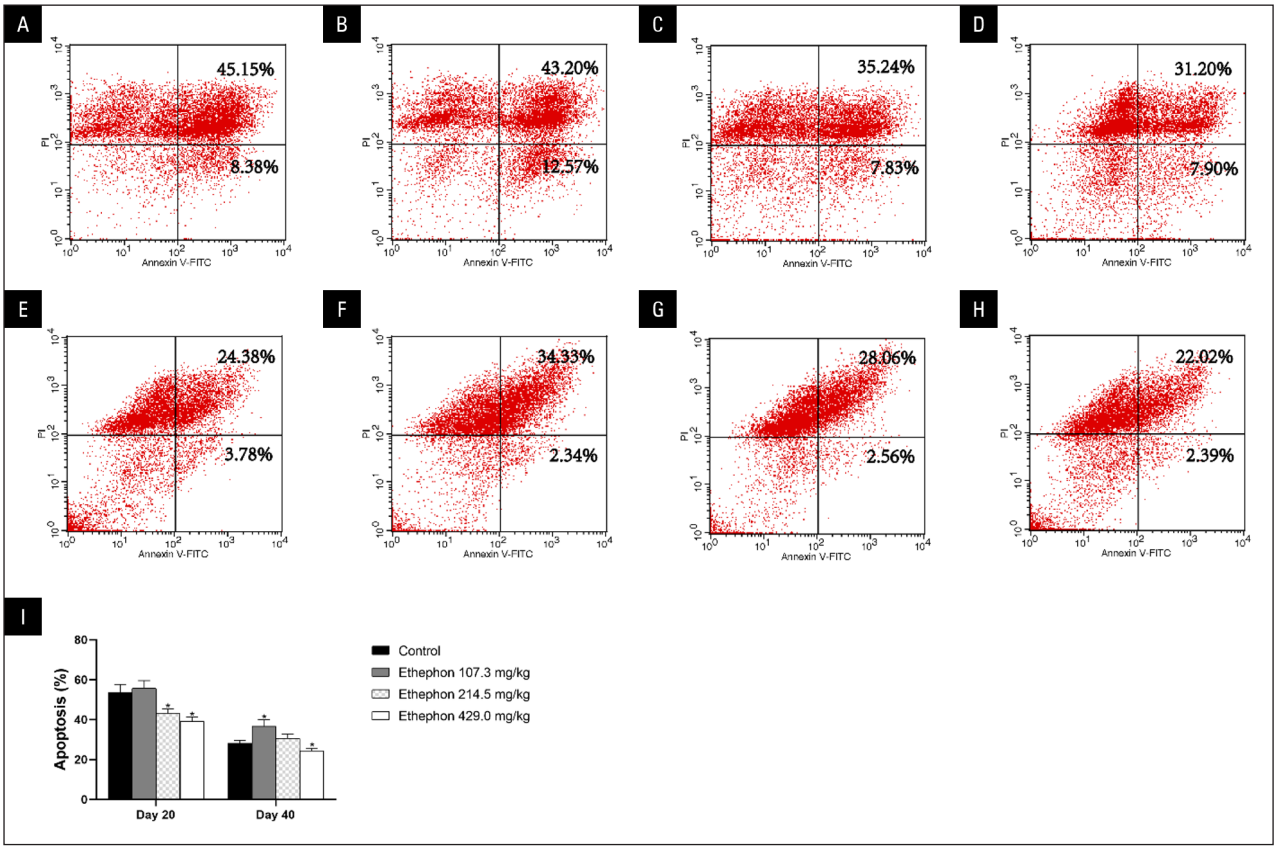

Figure 3. *The effect of ethephon exposure on the apoptosis of ovarian cells in female C57 mice.* **A**. *The apoptosis ratio of the control group after 20 days;* **B.** *The apoptosis ratio in the 107.3 mg/kg group after 20 days;* **C.** *The apoptosis ratio in the 214.5 mg/kg group after 20 days;* **D**. *The apoptosis ratio in the 429.0 mg/kg group after 20 days;* **E**. *The apoptosis ratio of the control group after 40 days;* **F.** *The apoptosis ratio in the 107.3 mg/kg group after 40 days;* **G.** *The apoptosis ratio in the 214.5 mg/kg group after 40 days;* **H.** *The apoptosis ratio in the 429.0 mg/kg group after 40 days. Data are represented as the mean ± standard deviation (SD) (n = 5 per group). *p < 0.05 compared to the control group*

Table 1. ***Effects of ethephon exposure on the cell cycle of ovarian cells in female C57 mice***

Treatment	20 Days			40 Days		
	G0/G1 (%)	S (%)	G2/M (%)	G0/G1 (%)	S (%)	G2/M (%)
Control	80.01 ± 1.15	11.32 ± 0.94	8.67 ± 0.36	51.97 ± 2.00	39.10 ± 2.48	8.93 ± 0.64
Ethephon [mg/kg]						
107.3	83.69 ± 0.60*	7.48 ± 0.50*	8.83 ± 0.62	70.73 ± 1.59*	10.42 ± 0.97*	18.85 ± 1.25*
214.5	79.55 ± 1.09	10.22 ± 0.68*	10.23 ± 0.97*	68.34 ± 1.49*	11.59 ± 1.04*	20.08 ± 0.95*
429.0	80.182 ± 0.93	9.76 ± 6.65*	10.05 ± 0.46*	72.52 ± 2.15*	11.27 ± 0.99*	16.21 ± 1.44*

The data are presented as mean ± standard deviation (SD); n = 5 for each group. *significantly different from control ($p < 0.05$).

Bodyweight is often used as a common indicator to evaluate health. In this study, the body weight in the 429.0 mg/kg ethephon group was significantly decreased after 20 and 40 days of exposure, suggesting that ethephon exerts a toxic effect on mice. Simultaneously, a previous study found that ethephon decreased body weight after 14 days of exposure in adolescent rats [4]. The organ coefficient of variation indicates that the organ may be susceptible to toxicity [17]. In this study, the increase or decrease in ovarian organ coefficient indicated that ethephon may have toxic effects on the reproductive system.

This study indicated that ethephon has a dual effect on the pituitary-ovarian axis. We found that serum levels of P decreased significantly in each dose group after 20 and 40 days of exposure, while levels of E_2 decreased remarkably in each dose group after 40 days. This finding was in line with that of Huang et al., who reported

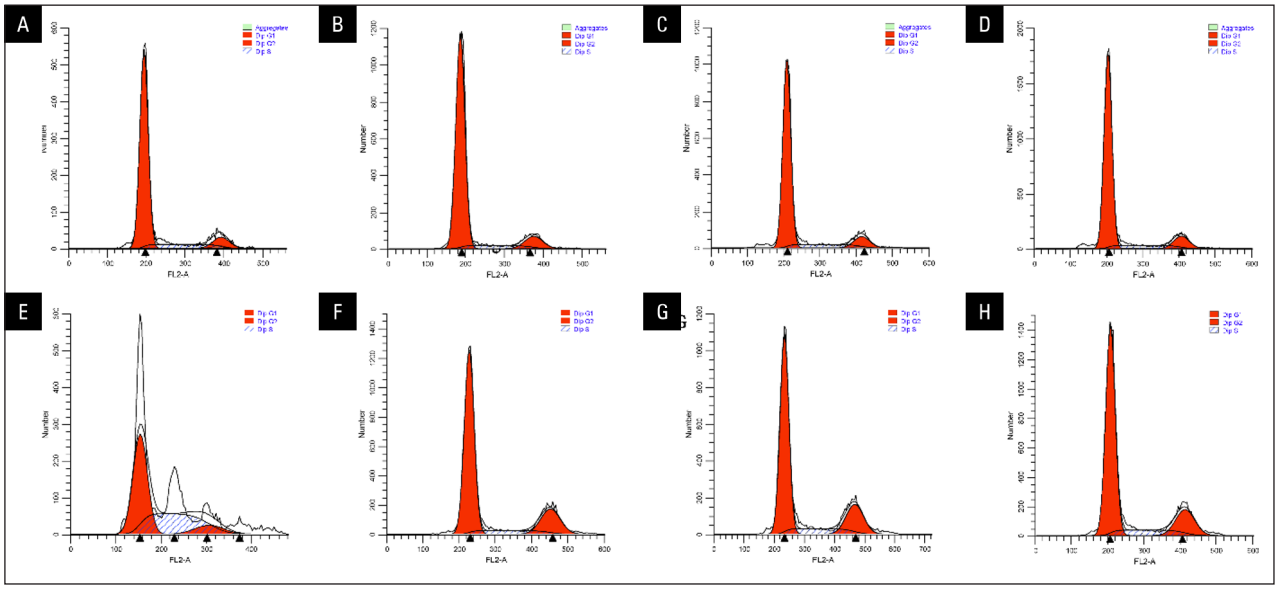

Figure 4. *The effect of ethephon exposure on the cell cycle distribution of ovarian cells in female C57 mice.* **A.** *The cell cycle distribution of the control group after 20 days;* **B**. *The cell cycle distribution in the 107.3 mg/kg group after 20 days;* **C**. *The cell cycle distribution in the 214.5 mg/kg group after 20 days;* **D.** *The cell cycle distribution in the 429.0 mg/kg group after 20 days;* **E.** *The cell cycle distribution of the control group after 40 days;* **F.** *The cell cycle distribution in the 107.3 mg/kg group after 40 days;* **G.** *The cell cycle distribution in the 214.5 mg/kg group after 40 days;* **H.** *The cell cycle distribution in the 429.0 mg/kg group after 40 days. Data are represented as the mean ± standard deviation (SD) (n = 5 per group). *p < 0.05 compared to the control group*

that intragastric administration of 285 mg/kg ethephon for 8 days reduced the serum levels of E_2 and P in pregnant mice [5]. Evidence has shown that changes of gonadotropin- releasing hormone (GnRH) pulse frequency can influence the secretion of gonadotropins (Gn). Higher and lower pulse frequencies are associated with selective secretion of LH and FSH, respectively [18]. It is well known that ovarian hormones have a strong inhibitory effect on the pituitary secretion of FSH and LH [19]. Oestradiol binds to its α receptor, which inhibits LH secretion [20]. In this study, we found that levels of FSH in the 429.0 mg/kg dose group decreased significantly after 20 days, but a significant increase was recorded after 40 days. After 40 days, LH levels increased significantly in each dose group, and FSH levels increased significantly in the 429.0 mg/kg dose group. We speculate that ethephon increases the GnRH pulse frequency, which leads to inhibition of FSH secretion and increased LH secretion after 20 days. With the extension of exposure dose and time, after exposure for 40 days, the falling serum E_2 results in the removal of feedback inhibition of E_2, which promotes the secretion of LH and FSH. This finding was in accordance with Fattahi et al., who found that treatment of mice with diazinon, a type of OP, increased the serum levels of LH and FSH [21]. However, this finding was not in line with those of Abd Eldaim et al., who demonstrated that treatment of mice with ethephon at a dose of 200 mg/kg for 28 days decreased serum levels of LH and FSH [12]. In addition, Alaa-Eldin et al. found that treatment of rats with chlorpyrifos, a type of OP, decreased serum levels of LH and FSH [22]. A previous study indicated that under the feedback of the pituitary-ovary axis, E_2 has negative feedback regulation for FSH secretion [23]. The rising level of serum FSH was only observed in the 429.0 mg/kg dose groups, not in the 107.3 and 214.5 mg/kg dose groups. This may be because the suppression of E_2 secretion was better in the high-dose group than in the lower-dose group. Further research is required to evaluate this effect.

This study indicated that ethephon inhibited the apoptosis rate and induced cell cycle arrest in the ovary, which may result in the suppression of cell proliferation. Apoptosis is an important mechanism of tumour elimination [24]. Most follicles undergo atresia via cell apoptosis [25]. Only a few follicles develop fully and undergo ovulation, and gonadotropin is an important factor for developing follicles to escape atresia [26]. Elevated gonadotropin levels have been reported to be associated with ovarian tumourigenesis [27]. In this study, after 20 and 40 days of exposure, the apoptosis of ovarian cells decreased in the 429.0 mg/kg dose group. We speculated that ethephon increased LH secretion, which allowed some follicles to escape apoptotic demise; thus, the rate of ovarian cell apoptosis decreased, which may cause ovarian tumourigenesis. This finding was in accordance with that of Xia et al., who found that treatment of ovarian cancer cells with LH (40 U/L)

inhibited apoptosis in ovarian cancer cells [28]. However, after 40 days of exposure, the apoptosis of ovarian cells was increased in the 107.3 mg/kg dose group. We surmised that this was associated with the interference of low-dose ethephon. Further research is required to evaluate this.

P53 protein is a critical factor involved in cell cycle arrest. It has 2 types: wild-type (wtp53) and mutant (mtp53). Wtp53 is a tumour suppressor that suppresses tumour cell proliferation; Mtp53 leads to tumour cell proliferation [29]. A previous study showed that mtp53 in breast cancer cells may promote tumour formation by default of p53-mediated cell cycle arrest [30]. In this study, after 20 days, ovarian cells were arrested in the G0/G1 phase in the low-dose group. Ovarian cells were arrested in the G2/M phase in the middle- and high-dose groups. After 40 days, ovarian cells were arrested in the G0/G1 and G2/M phases, respectively. We speculate that ethephon caused overexpression of p53 protein in ovarian tissue. Research by Abd Eldaim et al. agrees with our conjecture [12]. They found that adult male rats exposed to 200 mg/kg ethephon for 28 days induced the expression of P53 protein.

Conclusion

We found that prolonged exposure to ethephon had a dual effect on the pituitary–ovarian axis: it decreased serum P and E2 levels and increased secretion of FSH and LH hormones. The increased rate of apoptosis caused ovarian tumourigenesis. The ovarian cells were arrested in the G0/G1 and G2 phases, which may be attributed to overexpression of the p53 protein. However, further experiments are required to confirm this hypothesis.

Authors' contribution

H.H. and S.Z. conceived and designed the experiments; H.H., X.Z., X.S., Q.T., R.Z., Q.C., M.Y. and R.M. performed the experiments; H.H. analysed the data; Z.S. contributed reagents, materials, and analysis tools; L.Y. revised the manuscript.

Conflict of interest

The authors have no conflicts of interest.

Ethical approval

This research protocol was approved by the Animal Experiment Ethics Committee of Jilin University (2020 joint trial no. 2020-10-02).

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