Histological assessment for investigation of dose-dependent ovarian toxicity of cyclophosphamide in the rat

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Short Report

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Abstract

Background

Cyclophosphamide (CPA) have significant effects on ovarian follicles which lead to ovarian toxicity and impair the normal female reproductive function. This study aimed to evaluate the dose-dependent effects of CPA on rat folliclenumbers.

Methods

The experimental groups consisted of rats administered a single intraperitoneal injection of CPA at doses of either 50, 75,150, or 200 mg/kg followed by daily doses of 8 mg/kg for 14 days and control group given no treatment. After the treatment period, the histological evaluation was done.

Results

Primordial and primary follicles were affected by all doses of CPA, but differential follicle counts revealed that graaf and preantral follicles were most sensitive to CPA, followed by primary and primordial follicles. The greatest reduction in all type of studied follicles caused by CPA doses of 50 mg/kg.

Conclusion

Differential follicle counts revealed that CPA-induced ovarian toxicity is exhibited in structural feature of the ovary, particularly in destruction of graaf and preantral follicles in a dose-dependent manner so that the highest decrease in all type of studied follicles caused by 50 mg/kg of CPA and is suggested as the best concentration for ovotoxicity induction. These findings give insight into ovarian response to structural disruption of folliculogenesis.

1. Introduction

Different degrees of impaired ovarian function are ordinary among women treated with chemotherapy drugs such as cyclophosphamide (CPA). Cyclophosphamide, an alkylating chemotherapeutic agent (1), belongs to the oxazaphosporines group. It has been known for many years that CPA has been exploited widely for treatment of cancer and autoimmune or immune-mediated diseases (2). The clinical study illustrated that women suffering from cancer which treated with CPA can experience infertility due to various degrees of ovarian toxicity (3). The American Society of Clinical Oncology Guideline demonstrates that CPA therapy leads to ovarian failure or impaired infertility in the cancerous patients due to the apoptotic alternation in granulosa and theca cells followed by follicles demolition (4). It has been known that CPA could induce severe ovary damage and may cause premature ovarian failure (POF) due to ovarian toxicity by applying various mechanism including oxidative stress, inflammation, and apoptosis (5). The researches indicated that although CPA has been broadly applied in numerous researches to induce ovarian toxicity or even POF in female animal models (6), optimization of the administered dose is essential due its adverse effects on the other organs such as heart, kidney, brain,

liver, and bone marrow (4, 7, 8, 9), and more importantly, its twofold mechanism (10, 11). CPA is capable of activating the quiescent follicles which caused the proliferation and growth of them in specific dose by its metabolites such as 4-hydroperoxycyclophosphamide and phosphoramide mustard which seem to enhance the human primordial follicle activation to developing follicles (10, 11)[13,14]. In this regard, we tried to investigate the optimum dose for CPA- induced POF model in female rat based on its structural effects on ovarian follicle destruction. Thus, this study aimed to evaluate the dose-dependent effects of CPA on follicle numbers.

2. Materials and methods

2.1. Ethical statements

The application of animals was confirmed with the guide for the care and use of laboratory animals published by the ethic committee according to the institutional guidelines and national animal welfare with the principles of Medical University of Fasa (Fars, Iran).

2.2. Experimental animals

Adult female Sprague–Dawley rats (250-300 g, 10—12 weeks old) were housed in sterilized polypropylene cages in the experimental animal care center at Fasa University of Medical Sciences under standard conditions, dark-light cycle 12/12 h, temperature 22 -24 and free access to food and tap water.

2.3. The chemotherapy-induced POF rat model

Cyclophosphamide was used to induce the experimental model of ovarian toxicity in rats. In order to determine the dose-response of follicle destruction and morphometric changes, rats were administered a single intraperitoneal injection of cyclophosphamide (Baxter Oncology GmbH, Germany) at doses of either 50, 75,150, or 200 mg/kg body weight followed by daily doses of 8 mg/kg for 14 days. Each group contained five animals and control group given no treatment. At the end of the induction period, rats were euthanized using an increasing anesthesia dose of thiopental (PANPHAMA, France) by IP injection and the ovaries were removed.

2.4. Histological preparations

2.4.1. Tissue processing

The ovaries were fixed in 10% formalin for 24-72 hours. Due to the presence of a large amount of water in the tissue, ovaries dehydrated with increasing concentrations of ethyl alcohol (5 min each in 70, 80, 90, and 100% [v/v]). The tissue specimens cleared using xylene (Sigma-1330-20-7) twice for 50 min, embedded with paraffin, and sectioned (5.0 um).

2.4.2. Hematoxylin and eosin (H&E) staining

The slides were placed in a Vinteb oven at a temperature of 90C° for 20 min to paraffin melting. Deparaffinization was performed using two changes of xylene each for 15 min**.** The hydration was done with decreasing concentrations of ethanol (5 min each in 100, 90, 80, and 70% [v/v]) until it reaches the water. The slides were placed in hematoxylin dye (Sigma-H9627) for 7 seconds, and washed in distilled water for 1 min. The samples were placed in lithium carbonate (Sigma-1.05680) for 2 seconds and placed in eosin dye (Sigma-HT110116) for 3 min. Dehydration of tissues were performed by alcohol as follows: ethanol 90% (4 seconds), and two changes of 100% ethanol for 4 min per change. The slides were placed in two changes of xylene for 15 min to clarification. After mounting with entellan (Sigma-1.07961), photographs were taken with an optical microscope (LABOMED) (12).

2.5. Differential follicle counts

Differential follicle categorizations were made using the standard definition of follicle classifications. The follicles were grouped as primordial, primary, preantral and graaf follicles. Total follicle numbers were calculated using image J software.

2.6. Statistical analysis

The results were analyzed by GraphPad Prism 6 software. After normal distribution verification, analyses of variance (ANOVA) and Tukey post hoc test was performed to distinguish the statistical differences between groups at a significant level of $P \leq 0.05$. Data reported as mean ± standard deviation (SD).

3. Results

3.1. Dose-response of primordial follicles destruction by cyclophosphamide

The mammalian ovarian reserve is reflected by the primordial follicle pool. These smallest ovarian follicles were observed in a large number in the control group (Figure 1). The mean number of the primordial follicles of all CPA-administrated groups (20.33 ± 1.52, 24.67 ±3.05, 22.33 ± 1.52, and 27.33 ± 1.52 in 50, 75, 150 and 200 mg/kg, respectively) had a significant decrease relative to control group (34.67 ± 3.51). At dose of 50 mg/kg, a significant decrease (41.36% of control) was observed with primordial follicles of rats administrated 200 mg/kg CPA (21.17% of control) and control groups ($P \leq 0.05$, Figure 2, A). Increased percent of 75 (28.84% of control) and 150 (35.59% of control) mg/ kg CPA-administrated groups was not significant relative to 50 mg/ kg CPA-administrated group ($P > 0.05$, Figure 2, A).

3.2. Dose-response of primary follicles destruction by cyclophosphamide treatment with CPA doses

In the control group, the means of the primary follicles was 26.67 ± 1.52. In 50 (8.33 ±1.52), 75 (14.33 ± 2.08), 150 (14.67 ±2.08) and 200 (21.33 ± 1.52) mg/kg CPA-administrated groups, primary follicle numbers were significantly reduced to 68.76%, 42.26%, 44.99%, and 20.02% of control, respectively. The greatest and the least reduction in primary follicles caused by CPA doses of 50 and 200 mg/kg, respectively. These groups had significant difference with all other groups.

In 75 and 150 mg/kg CPA-administrated groups, significant increase relative to 50 mg/kg and significant decrease compared to 200 mg/kg CPA-administrated and control groups was observed ($P \leq 0.05$ 0.05, Figure 2, B).

3.3. Dose-response of preantral follicles destruction by cyclophosphamide

Preantral follicles originate from primary follicles and contain several layers of granulosa and theca cells (Figure 1). The mean number of preantral follicles in 50 (3 ± 1), 75 (7.33 ± 1.52), and 150 (7.66 ± 1.52) mg/kg CPA-administrated groups statistically differed from 200 mg/kg CPA dose (12.33 ± 1.52) and control (14.67 ± 1.52) groups. The reduced percent of these follicles in 50 mg/kg CPA-treated group was statistically differ from other administrated doses and control group. Also, significant increase in 75 (70.69% of control) and 150 (48.19% of control) mg/kg CPA-administrated groups relative to 50 mg/kg (79.55% of control) and significant decrease in these groups compared to 200 (15.95% of control) mg/kg CPA-administrated and control groups was observed ($P \leq 0.05$, Figure 2, C).

3.4. Dose-response of graaf follicles destruction by cyclophosphamide

The mean number of graaf follicles in 50 (1.33 ± 0.57), 75 (3 ± 1), and 150 (4.33 ± 0.57) mg/kg CPA-administrated groups statistically differed from control (8.66 ± 1.52) group. The least reduction in graaf follicle counts caused by CPA doses of 50 mg/kg. This group had significant difference with control, 150, and 200 (7 ± 1) mg/kg CPA-administrated groups. The reduced percent of these follicles in 75 (65.11% of control) and 150 (50% of control) mg/kg CPA-treated groups was statistically differed from 200 (18.6% of control) and 50 (84.88% of control) mg/kg administrated doses, respectively. Among the four CPA-administrated groups, the number of graaf follicles at 200 mg/kg had a significant increase relative to 50 and 75 mg/kg groups ($P \leq 0.05$, Figure 2, D).

3.5. The most and least sensitivity to CPA

Histological assay showed that primordial and primary follicles were affected by all concentrations of CPA. Meanwhile graaf and preantral follicles were the most sensitive ones to CPA, followed by primary and primordial follicles. This reduction in graaf, preantral, primary and primordial follicles was to 54.64%, 53.59%, 45%, and 31.74% of controls, separately ($P \leq 0.05$, Figure 2). Results of differential follicle counts also showed that the greatest and the least reduction in all type of studied follicles caused by CPA doses of 50 and 200 mg/kg, respectively ($P \leq 0.05$, Figure 2).

4. Discussion

Our results showed that the number of all types of follicles (primordial, primary, preantral, and graaf) was normal in control group which did not receive any treatment. Also, CPA administration led to a noticeable decrease in the ovarian reserve, which was associated with increased histological damage. Our histological assay showed that primordial and primary follicles were affected by all doses of CPA. Effects of chemotherapeutic agents on ovarian reservation range from partial damage resulting in reduced

fertility, until the complete injury with total loss of primordial follicles, ovarian atrophy and subsequent complete ovarian failure (13). Impact of chemotherapy on ovarian reserve is one of the mechanisms of chemotherapy-induced ovarian damage which can be assessed by histological analysis (14). Previous histological studies in human ovaries have shown that chemotherapy treatments can lead to loss of primordial follicles and ovarian atrophy (15, 16). Several studies utilizing rodents did show presence of apoptosis in oocytes of primordial follicles after chemotherapy (17, 18). Cyclophosphamide, the common drug in chemotherapy, has been known to give rise to ovotoxicity. It has direct association with impaired ovarian function which may lead to premature ovarian failure and consequently infertility. It seems that there is a strong relation between cumulative doses of CPA and ovarian toxicity (13, 14). Furthermore, CPA has been applied to induce the experimental model of premature ovarian failure in rats. It has been illustrated in several investigations that the depletion of follicles caused by direct or indirect side effects of CPA which led to induction of cell death in oocytes and granulosa cells, respectively (19, 20, 21). *Oktem et al*. have previously characterized the *in vivo* impact of CPA in human ovarian xenograft model. They showed that a single dose of 200 mg/kg CPA resulted in significant primordial follicle death by apoptosis. They reported that earlier at 12 hours after the injection, the injury to the primordial follicles was initiated almost immediately upon administration of CPA (22). Our differential follicle counts revealed that graaf and preantral follicles were the most sensitive ones to CPA, followed by primary and primordial follicles. The follicle atresia occurs in response to unfavorable changes in many factors, such as follicles response to gonadotropins, autocrine and paracrine factors. The effects of antineoplastic agents on the ovaries are clinically inferred from a variety of surrogate markers, including antral follicle count (13). The antral follicle count records the total number of antral follicles on ovaries which are observed during histological evaluation. Our findings showed the reduction of primordial follicles in all doses of CPA in one side and the most sensitivity to CPA in graaf follicles on the other hand. In this line, *Hendriks et al.* reported that the number of antral follicles correlated with the number of remaining primordial follicles (23). Also, *Frattarelli e al.* showed in their studies that as the supply of primordial follicles decreases, the number of antral follicles observable on ultrasound also declines (24). *Bedoschi et al.* demonstrated that the primordial and preantral follicles are more susceptible to atresia compared to the antral and graafian follicles (13). *Bahmanpour* and her coworkers explained that primordial and preantral follicles were more sensitive to one single dose of 150 mg/kg CPA administration in their animal model (19). Previous reports have shown that the preantral follicles, as the sensitive ones, may be affected by chemotherapy drugs easily (19, 25, 26) that is in consistent with our results. The investigation of ovarian morphological for different doses showed that CPA dose of 50 mg/kg had the most remarkable decrease in the number of primordial, primary, preantral and graafian follicles. *Zheng et al.* applied 50 mg/kg CPA to design their POF model. It was shown that administration of this dose led to reduction of all types of follicles in the experiment. The primordial, primary, secondary, and early antral follicles were 43.24, 40.76, 57.14, and 80.34% of control (21). The greatest reduced follicles in *Zheng* study and our current research are early antral and graaf follicles followed by preantral, primary, and primordial follicles. Furthermore, 50 mg/kg as initial dose followed by 8 mg/kg/day to 15 days has been applied in *Li et al.* study as the optimum dose for induction of POF in animal models (27). It was demonstrated that primary and antral follicles had more reduction compared to the other types of ovarian

follicles.*Pascuali et al.* explained that a single intraperitoneal injection of CPA (75 mg/kg) led to noticeable decrease in number of primordial, primary and preantral follicles of CPA treated mice compared to control group in POF mice model (28). Interestingly, the least reduction in all type of follicles number was related to CPA dose of 200 mg/kg. *Song et al.* exploited the intraperitoneally injection of 200 mg/kg of CPA on the first day and then 8 mg/kg/day for the 15 consecutive days to design the POF model in rat. Their results determined that the secondary follicles were more sensitive to CPA injection. It was reported that the mean number of primordial, primary, and early antral follicles had no significant difference with that of control group (29). Although in some studies, CPA dose of 200 mg/kg followed by 8 mg/kg/day has been introduced for the POF induction in animal models (1, 30, 31, 32), our findings showed that the least decrease in graaf (18.6%), preantral (15.95%), primary (20.02%) and primordial (21.17%) follicles relative to controls caused by CPA doses of 200 mg/kg. Overall, our results suggest that CPA dose of 50 mg/kg is more suitable for ovarian toxicity induction relative to other concentrations. The primordial follicles constitute the ovarian reserve and are continuously recruited throughout life (33). On the other hand, scientific evidence indicates that the number of primordial follicles constituting ovarian reserve is finite (13). A role of CPA in indirect damage induction to primordial follicles and increased follicle activation have been reported. *Kalich-Philosoph* has proposed a new hypothesis to the chemotherapy-induced ovarian damage. It was suggested that chemotherapy leads to an increase in follicular recruitment, causing decrease of ovarian reservation and subsequently the ovarian failure (10). Damages to the growing follicles reduce their inhibitory effects on primordial follicles recruitment, so resulting in activation of the primordial follicles in an effort to replace the cohort of damaged antral follicles (34). These findings suggested that CPA acted by a twofold mechanism. As *Kalich-Philosoph et al.* and *Lande et al.* demonstrated in their works (10, 11), CPA is toxic for the dividing cells and may lead to death of the growing ovarian follicles as described in our current study in various doses of 50-150 mg/kg CPA. At the same time, CPA is capable of activating the quiescent follicles which caused the proliferation and growth of them in specific dose such as 200 mg/kg CPA. *Lande et al.* explained that CPA metabolites such as 4-hydroperoxycyclophosphamide and phosphoramide mustard seem to enhance the human primordial follicle activation to developing follicles *in vitro*. In general, the present study determined that CPA dose of 50 mg/kg had the most remarkable decrease in the number of all types of follicles and on the other hand had the least decrease effects on primordial follicle (41.36% of control) relative to primary (68.76% of control), preantral (79.55% of control) and graaf (84.88% of control) follicles causing the less activating the quiescent follicles. So, with regard to the evidences that has shown the twofold mechanism of CPA and according to the results of the present study, the 50 mg/kg CPA is suggested as the best concentration for ovotoxicity induction. This study was limited to rat data. So, further clinical studies are needed to reveal the effects of CPA on ovarian reserve and infertility.

5. Conclusion

The present study evaluated the effect of various doses of CPA on different ovarian follicles by histological assessment in the rat model. Our findings suggested that the primordial and primary follicles were affected by all doses of CPA, while the graaf and preantral follicles were the most sensitive follicles. In addition, the greatest reduction in the number of all follicle types were observed in dose of 50 mg/kg CPA. On the other hand, the least decrease in graaf, preantral, primary, and primordial follicles was related to CPA doses of 200 mg/kg. So, it seems that 50 mg/kg CPA is the best concentration for ovotoxicity induction. This study gives the researches brilliant insight into the dose-dependent ovarian toxicity of CPA and its twofold mechanism for further research.

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of Fasa, Iran. The protocol was approved by the Fasa University of Medical Sciences Institutional Animal Care.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

N.E. wrote the main manuscript text and M. A. and J.A. reviewed the literature. Z.M. designed the study, and analyzed the data. All authors contributed to the drafting of the manuscript and approved the final version.

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A statement

All experimental protocols were approved by Fasa University of Medical Sciences committee and all methods were carried out in accordance with relevant guidelines and regulations.

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Figures

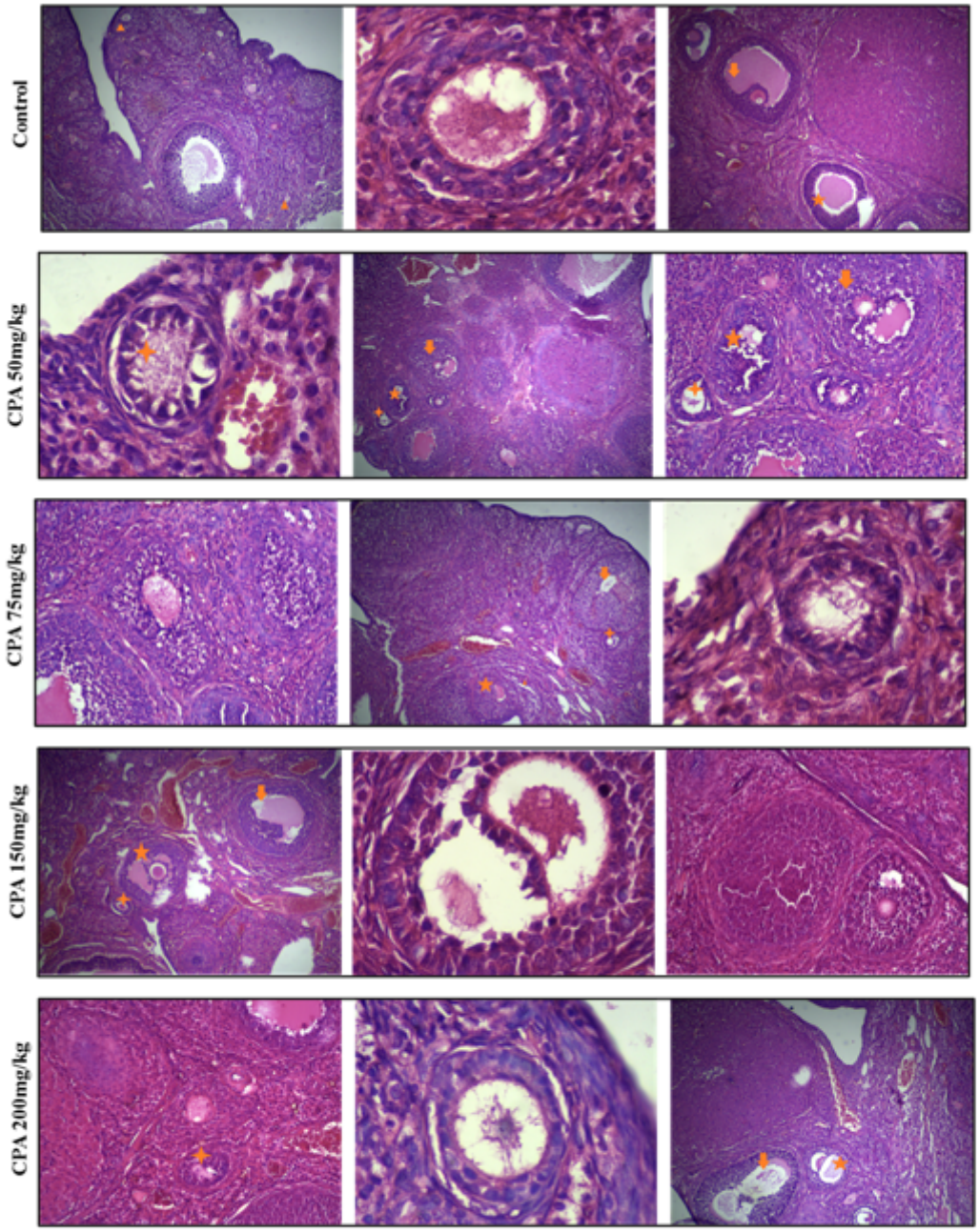

Figure 1. Dose-response of primordial (▲), primary (+), preantral (★) and graaf (⬇) follicles destruction by cyclophosphamide (CPA; 50, 75, 150 and 200 mg/kg followed by 8 mg /kg/day for 14 days).

Figure 1

See image above for figure legend.

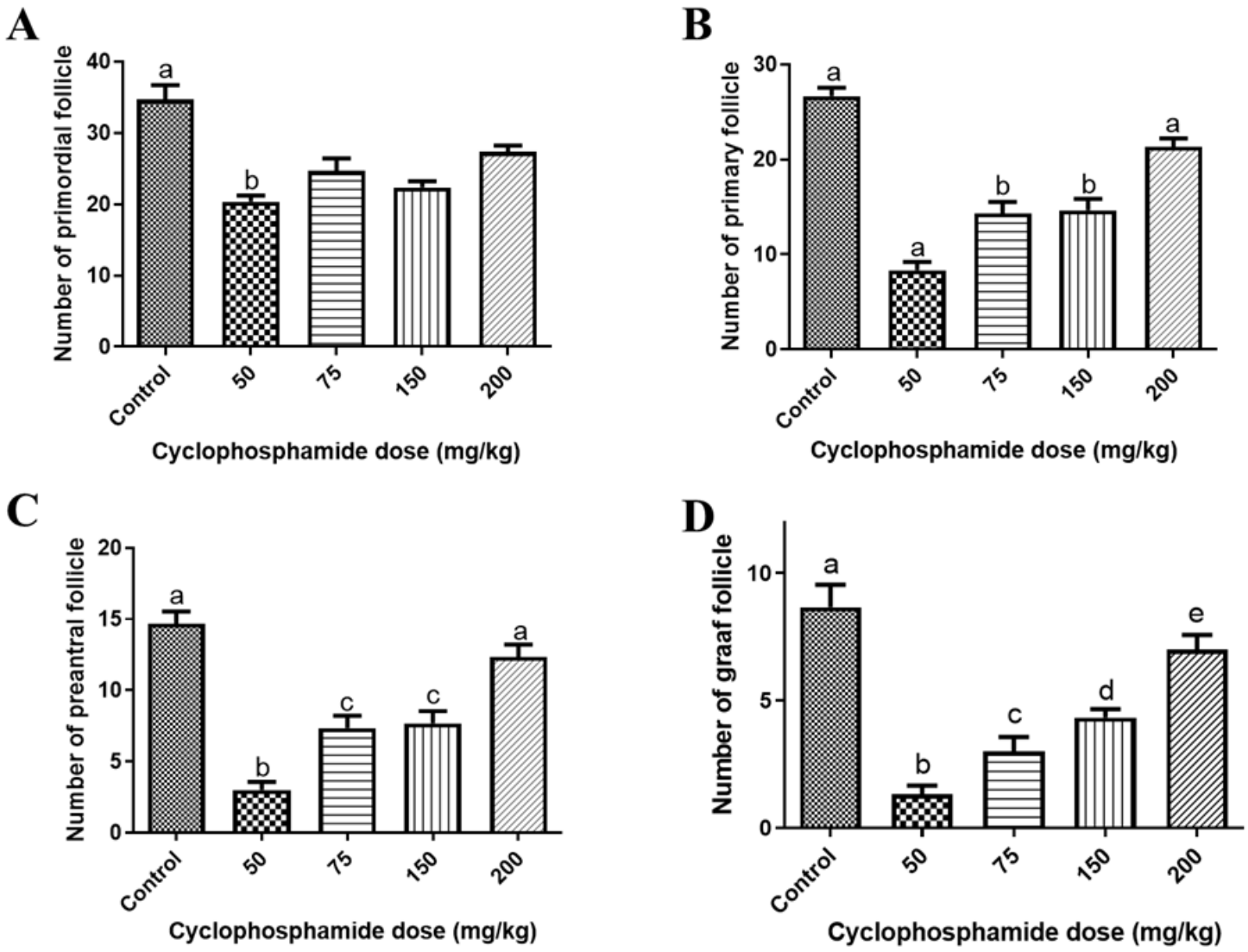

Figure 2

Differential follicle counts after treatment with cyclophosphamide (50, 75, 150 and 200 mg/kg followed by 8 mg /kg/day for 14 days). Each point shows the mean number of follicles of 5 rats ± SD. In figure A, “a” showed significant differences to other groups and “b” showed significant differences with control and 200 mg/kg CPA-administrated groups. In figure B, “a” showed significant differences to other groups and “b” showed significant differences with control, 50 and 200 mg/kg CPA-administrated groups. In figure C, “a” showed significant differences with 50, 75 and 150 mg/kg CPA-administrated groups, “b” showed significant differences to other groups, and “c” showed significant differences with control, 50 and 200 mg/kg CPA-administrated groups. In figure D, “a” showed significant differences with 50, 75 and 150 mg/kg CPA, “b” showed significant differences with control, 150 and 200 mg/kg CPA, “c” showed significant differences with control and 200 mg/kg CPA, “d” showed significant differences with control and 50 mg/kg CPA, and “e” showed significant differences with 50 and 75 mg/kg CPA-administrated groups.