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Research article

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

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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 follicle numbers.
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 common among women treated with chemotherapy drugs such as cyclophosphamide (CPA). Cyclophosphamide, an alkylating chemotherapeutic agent, belongs to the oxazaphosporines group [1]. CPA has long been used extensively to treat cancer, and autoimmune/immune-mediated diseases. It is effective against numerous cancers and has immunosuppressive properties, making it valuable in treating certain autoimmune diseases and preventing organ transplant rejection [2]. Clinical researches have revealed that women with cancer who are treated with CPA can experience infertility due to various degrees of ovarian toxicity [3]. Understanding the dose-dependent effects of cyclophosphamide on follicle numbers helps clinicians

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assess the potential impact on fertility and develop strategies to preserve reproductive function [4].

The American Society of Clinical Oncology Guideline demonstrates that CPA therapy leads to ovarian failure or impaired infertility in cancer patients due to apoptotic changes in granulosa and theca cells, followed by follicle destruction [5]. 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 mechanisms, including oxidative stress, inflammation, and apoptosis [4,6]. Adjusting the dose of cyclophosphamide based on individual patient needs ensures the desired level of immunosuppression for cancer treatment while minimizing the impact on ovarian function.

The researches indicated that although CPA has been broadly applied in numerous researches to induce ovarian toxicity or even POF in female animal models [4–10], optimization of the administered dose is essential due its adverse effects on the other organs such as heart, kidney, brain, liver, and bone marrow [5,11–13], and more importantly, its twofold mechanism [14,15]. Cardiac toxicity associated with this chemotherapeutic agent can be a lethal complication [16]. It was reported that a total cyclophosphamide dose exceeding 150 mg/kg predicts an increased risk of acute heart failure, with an estimated incidence between 7 % and 33 %. The exact mechanism of cyclophosphamide-induced cardiac toxicity remains unclear [17]. Furthermore, exposure to cyclophosphamide causes various toxic effects in mice, including: inhibition of body weight increase, decreased leukocyte density and spleen coefficient, disruption of oxidation-antioxidation balance in the liver, impaired kidney function, and disturbance of amino acid metabolism in liver and kidney [18]. Cyclophosphamide treatment in male Wistar rats induced multi-organ toxicity, including in the testes, liver, lungs, spleen, and kidneys, compared with the control group [19]. On the other hand, 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 [14,15]. In our recent review paper, we noted that varying doses of CPA (50 mg/kg to 200 mg/kg) have been used in different studies to induce a POF model in animal models. However, due to CPA's diverse side effects on different organs, which can affect morbidity and mortality, we aimed to determine the optimal dose for a CPA-induced POF model in female rats based on its structural effects on ovarian follicle destruction. Thus, this study evaluated 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 ethics committee (IR.FUMS.AEC.1401.008), in accordance with the institutional guidelines and national animal welfare principles of the 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. Vaginal smears were collected daily to identify the phases of the estrous cycle. The four stages were defined as follows: proestrus (marked by a complete presence of intact, living epithelial cells), estrus (characterized by 100 % cornified epithelial cells), metestrus (showed a blend of roughly 50 % cornified epithelial cells and 50 % white blood cells), and diestrus (predominantly comprised of 80–100 % white blood cells). The rat estrous cycle typically spans approximately 4 days and encompasses the proestrus, estrus, metestrus, and diestrus phases. Only animals exhibiting at least two consecutive normal 4-day vaginal estrous cycles were included in the research.

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 [20–22], 75 [23,24], 150 [25,26], or 200 [27,28] 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, the animals were weighed and euthanized using an increasing anesthesia dose of thiopental (PANPHAMA, France) by IP injection and the ovaries were removed.

2.4. Histological preparations

For histological analysis, the ovaries were fixed in 10 % formalin (Sigma-Aldrich) for 24–72 h. The fixed ovaries were dehydrated, encased in paraffin, and sliced into 5 μm-thick sections. These sections were then mounted onto glass slides. Standard hematoxylin and eosin staining was applied for microscopic examination under a light microscope [29]. Using an Olympus BX-51 microscope, ovarian tissue sections were analyzed. A count of follicles was performed following established methods [30].

2.5. Differential follicle counts

Follicles were categorized according to standard definitions, grouping them into primordial, primary, preantral, and Graafian follicles. To count the total follicles per ovary, every fifth section of the ovary was examined. Only follicles containing an oocyte with a visible nucleus were counted to avoid duplication [22]. The following criteria were used for follicle classification [31]:

- Primordial follicles: An oocyte enclosed by a single layer of flattened follicular cells.
- Primary follicles: An oocyte surrounded by a single layer of cuboidal granulosa cells.
- Preantral follicles: Oocytes with two or more layers of granulosa cells but lacking an antral cavity.
- Graaf follicles: Oocytes with a distinct nucleus surrounded by multiple layers of granulosa cells and an antral cavity.

Total follicle numbers were calculated using image J software.

2.6. Statistical analysis

The results were analyzed using GraphPad Prism 6 software. After verifying normal distribution, analysis of variance (ANOVA) and Tukey post hoc test were used to distinguish the statistical differences between groups at a significance level of $P \leq 0.05$. Data are reported as mean ± standard deviation (SD).

3. Results

3.1. Assays in experimental animals

We examined various concentrations of the chemotherapy drug CPA to determine the optimal dose for a CPA-induced POF model in female rats based on its structural effects on ovarian follicle destruction. Table 1 revealed a significant reduction in follicle counts across all developmental stages. Furthermore, while control rats exhibited normal 4-day estrous cycles, all CPA-treated groups displayed irregular cycles. On day 5 of treatment, the treated rats exhibited decreased appetite and reduced activity (data not shown). All

Table 1
Mean number of ovarian follicles after treatment with cyclophosphamide (50, 75, 150 and 200 mg/kg followed by 8 mg/kg/day for 14 days).

	Control	CPA 50 mg/kg	CPA 75 mg/kg	CPA 150 mg/kg	CPA 200 mg/kg
Primordial follicles	34.67[a]	20.33[a]	24.67[a]	22.33[a]	27.33[a]
Primary follicles	26.67 [b]	8.33 [b]	14.33 [b]	14.67 [b]	21.33 [b]
Preantral follicles	14.67[c]	3.00[c]	7.33[c]	7.67[c]	12.33
Graaf follicles	8.67 [d]	1.33 [d]	3.00 [d]	4.33 [d]	7.00

The mean numbers of follicles were counted at different stages of maturation in the ovary section of experimental groups. The mean numbers of primordial, primary, preantral, and graaf follicles in the CPA groups decreased significantly compared with the control group ($p < 0.05$). “a” showed significant differences in primordial follicles between control and CPA groups. “b” showed significant differences in primary follicles between control and CPA groups. “c” showed significant differences in preantral follicles between control and CPA 50, 75, and 150 mg/kg groups. “d” showed significant differences in graaf follicles between control and CPA 50, 75, and 150 mg/kg groups.

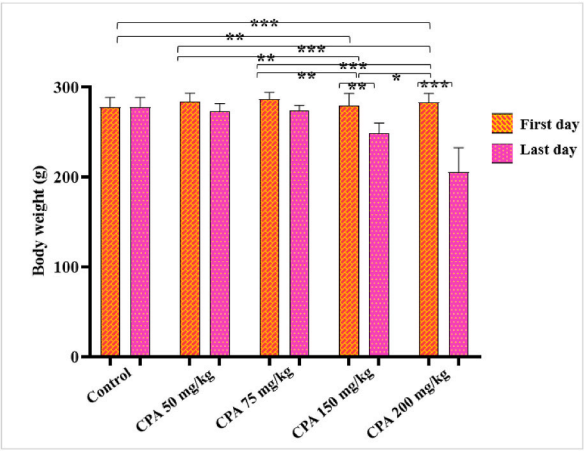

Fig. 1. The body weight of treated rats was obviously decreased by increasing the CPA dose during first and last days. *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$.

animals were weighed before and after treatment, and a notable decrease in body weight was observed in the treated animals compared to the control group (Fig. 1). Our results indicated significant differences in body weights between the CPA 200 mg/kg and CPA 150 mg/kg groups between the first and last day of the experiment. While our data showed significant differences in body weights between the control group and the CPA 150 mg/kg group ($p < 0.01$) and the CPA 200 mg/kg group ($p < 0.001$), no significant differences were observed between the control group and the CPA 50 mg/kg or 75 mg/kg groups, or between the CPA 50 mg/kg and 75 mg/kg groups on the first and last day of the experiment. Furthermore, there were no apparent differences in animal weights between the CPA 50 mg/kg and 75 mg/kg groups. However, significant differences were observed between the animal groups of CPA 50 mg/kg versus 200 mg/kg ($p < 0.001$), CPA 50 mg/kg versus 150 mg/kg ($p < 0.01$), CPA 75 mg/kg versus CPA 150 mg/kg ($p < 0.01$), CPA 75 mg/kg versus CPA 200 mg/kg ($p < 0.001$), and CPA 200 mg/kg versus CPA 150 mg/kg ($p < 0.05$).

3.2. Assessment of differential follicles count

3.2.1. Dose-response of primordial follicles destruction by cyclophosphamide

The mammalian ovarian reserve is reflected by the primordial follicle pool. Fig. 2 shows the primordial follicles after H & E staining in various CPA-administrated and control groups. These smallest ovarian follicles were observed in a large number in the control group as it is shown in Fig. 2. The statistical analysis showed that the mean number of primordial follicles in all CPA-administrated groups had a significant decrease relative to the control group. At a dose of 50 mg/kg, a significant decrease (41.36 % of control) was observed in the primordial follicles of rats administered 200 mg/kg CPA (21.17 % of control) and the control group ($P \leq 0.05$, Fig. 2). The increased percentage of primordial follicles in the 75 (28.84 % of control) and 150 (35.59 % of control) mg/kg CPA-administrated groups was not significant relative to the 50 mg/kg CPA-administrated group ($P > 0.05$).

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

The primary follicles are indicated in Fig. 2 in all groups of this study. The primary follicle numbers in doses of 50, 75, 150, and 200 mg/kg were significantly reduced to 68.76 %, 42.26 %, 44.99 %, and 20.02 % of the control group, respectively. The greatest reduction in primary follicles was caused by a CPA dose of 50 mg/kg, and the least reduction was caused by a CPA dose of 200 mg/kg. These groups had significant differences with all other groups. In the 75 and 150 mg/kg CPA-administrated groups, a significant increase relative to the 50 mg/kg group and a significant decrease compared to the 200 mg/kg CPA-administrated and control groups was observed ($P \leq 0.05$, Fig. 3).

3.2.3. Dose-response of preantral follicles destruction by cyclophosphamide

Preantral follicles originate from primary follicles and contain several layers of granulosa and theca cells (Fig. 2). The reduced percentage of these follicles in the 50 mg/kg CPA-treated group was statistically different from the other administered doses and the control group. Also, a significant increase in the 75 (70.69 % of control) and 150 (48.19 % of control) mg/kg CPA-administrated groups relative to the 50 mg/kg (79.55 % of control) group and a significant decrease in these groups compared to the 200 (15.95 % of control) mg/kg CPA-administrated and control groups was observed ($P \leq 0.05$, Fig. 3).

3.2.4. Dose-response of graaf follicles destruction by cyclophosphamide

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 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 ($P \leq 0.05$, Fig. 3).

3.2.5. 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 ($P \leq 0.05$). 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 ($P \leq 0.05$, Fig. 3).

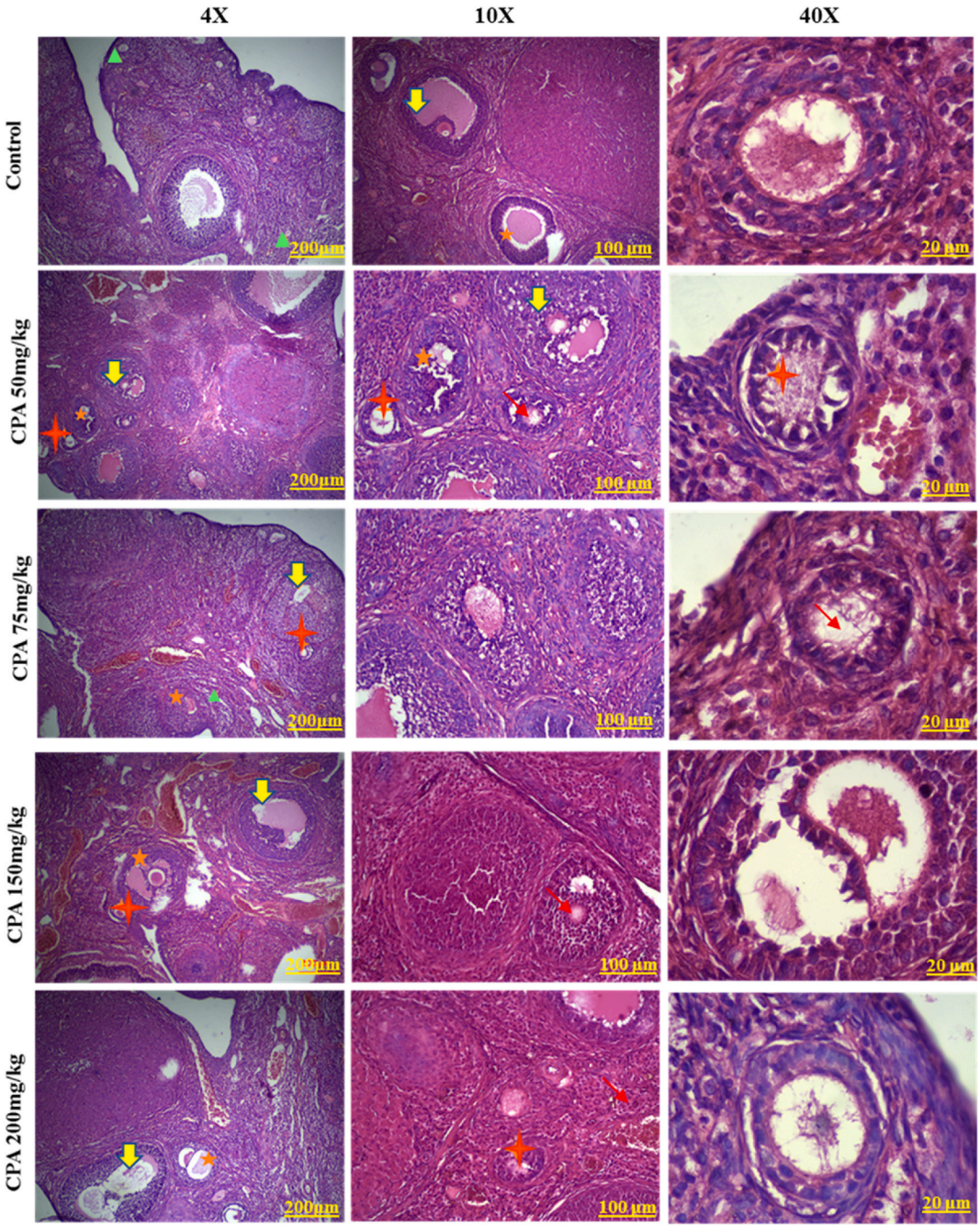

Fig. 2. Dose-response of ovarian follicles destruction by cyclophosphamide (CPA; 50, 75, 150 and 200 mg/kg followed by 8 mg/kg/day for 14 days) with different magnification 4× = 40 magnification, 10× = 100 magnification, and 40× = 400 magnification); POF (Premature Ovarian Failure) (↘), Primordial follicles (▲): An oocyte enclosed by a single layer of flattened follicular cells. Primary follicles (+): An oocyte surrounded by a single layer of cuboidal granulosa cells. Preantral follicles (★): Oocytes with two or more layers of granulosa cells but lacking an antral cavity. Graaf follicles (⇩): Oocytes with a distinct nucleus surrounded by multiple layers of granulosa cells and an antral cavity.

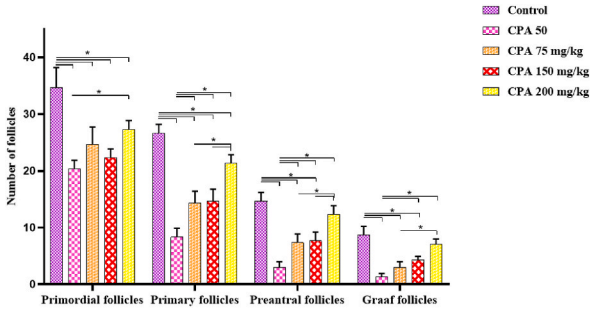

Fig. 3. Comparative follicle counts following cyclophosphamide administration (50, 75, 150, and 200 mg/kg followed by 8 mg/kg/day for 14 days). Ovarian tissue sections stained with hematoxylin and eosin (H&E) were obtained from both the control group and the group treated with CPA. The follicles in these sections were counted and classified. Each point shows the mean number of different stages follicles (primordial, primary, preantral, and graaf follicles of POF group versus control group) of 5 rats ± SD. * indicates $p < 0.05$. All data were performed by utilization of one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

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 [32]. Impact of chemotherapy on ovarian reserve is one of the mechanisms of chemotherapy-induced ovarian damage which can be assessed by histological analysis [33]. Previous histological studies in human ovaries have shown that chemotherapy treatments can lead to loss of primordial follicles and ovarian atrophy [34,35]. Several studies utilizing rodents did show presence of apoptosis in oocytes of primordial follicles after chemotherapy [36,37]. 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 [32,33]. 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 [20,38,39]. *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 h after the injection, the injury to the primordial follicles was initiated almost immediately upon administration of CPA [40]. 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 [32]. 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 [41]. 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 [42]. *Bedoschi* et al. demonstrated that the primordial and preantral follicles are more susceptible to atresia compared to the antral and graafian follicles [32]. *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 [38]. Previous reports have shown that the preantral follicles, as the sensitive ones, may be affected by chemotherapy drugs easily [9,38,43] 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 [20]. 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 [44]. 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 [45]. 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 (those follicles with oocytes surrounded by two to four complete GC layers which aligned with our definition for preantral follicles; or preantral 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 [28]. 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 [1,10,46,47], 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 [48]. On the other hand, scientific evidence indicates that the number of primordial follicles constituting ovarian reserve is finite [32]. 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 [15]. 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 [49]. These findings suggested that CPA acted by a twofold mechanism. As *Kalich-Philosoph* et al. and *Lande* et al. demonstrated in their works [14,15], 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

This study aimed to determine the optimal dose of cyclophosphamide for inducing premature ovarian failure in a rat model. Our findings demonstrate that a 50 mg/kg dose of CPA significantly reduced ovarian follicle numbers, particularly affecting graafian and preantral follicles, making it the most effective dose for inducing POF in this model. This dose-dependent effect of CPA on follicle depletion suggests a threshold effect, with higher doses (200 mg/kg) showing a lesser reduction in follicle numbers. These findings provide valuable insights for researchers developing preclinical models of POF and for clinicians treating cancer patients with CPA, emphasizing the importance of considering fertility preservation strategies and optimizing treatment plans to minimize the impact on ovarian function.

6. Limitations

The study's findings demonstrated a dose-dependent effect of CPA on ovarian toxicity, with 50 mg/kg being the most effective concentration for inducing ovotoxicity. Further investigation including hormonal and biochemical assays, is necessary to elucidate the precise mechanism of CPA's effects on ovarian function. Additionally, examining the dose-response relationship of various drugs on follicle differentiation is crucial for developing accurate animal models for POF research. This would involve investigating a range of doses to identify the optimal concentration for inducing desired changes in follicle differentiation.

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 (IR.FUMS.AEC.1401.008). The protocol was approved by the Fasa University of Medical Sciences Institutional Animal Care.

Consent for publication

Not applicable.

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Data availability statement

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

CRediT authorship contribution statement

Narges Elahi: Writing – original draft, Project administration, Investigation, Formal analysis. **Mohammad Ebrahim Astaneh:** Writing – review & editing, Visualization. **Jafar Ai:** Writing – review & editing, Visualization. **Zohreh Makoolati:** Writing – review & editing, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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