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Protective Effect of Glutathione Administration on Ovarian Function in Female Rats with Cyclophosphamide-Induced Ovarian Damage

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Keywords
Glutathione · Cyclophosphamide · Primary ovarian insufficiency · Anti-Mullerian hormone · Antioxidants

Abstract

Objectives: We investigated the potential of glutathione to protect ovarian function in rats exposed to cyclophosphamide by measuring serum anti-Mullerian hormone (AMH) levels, follicle counts, and related parameters. ***Design:*** Forty-two adult female Sprague-Dawley rats were randomly divided into six groups and treated with various combinations of cyclophosphamide, glutathione, and sodium chloride. On day 21, the rats were anesthetized, and their ovaries were removed for examination. ***Participants/Materials, Setting, Methods:*** Histopathological examination, serum AMH concentrations, follicle counts, AMH-positive staining of follicle percentages were analyzed. Statistical analysis was performed using a one-way analysis of variance and Tukey's test, with significance set at $p < 0.05$. Secondary measures encompassed histopathological examination and percentages of AMH-positive staining of follicles. ***Results:*** Significant differences were observed in follicle counts, AMH-positive follicle parameters, and serum AMH concentrations among the six groups. Group 2 (treated with cyclophosphamide) had the lowest primordial, primary, secondary, and antral follicle counts and the highest atretic count. Group 6, treated with cyclophosphamide and 200 mg/kg glutathione, showed improved follicle counts compared to those in group 2. Reducing the glutathione dose to 100 mg/kg was ineffective. ***Limitations:*** This was an experimental animal investigation with a comparatively modest sample size. Experimental studies should be conducted to determine the optimal dosage and duration of glutathione therapy. Information gathered from an experimental animal model may not yield precisely similar outcomes in humans; therefore, additional investigations are necessary to examine the impact of glutathione on women experiencing POI. ***Conclusions:*** The anti-oxidative protective effect of directly administered glutathione was demonstrated for the first time. Low-dose glutathione was ineffective, whereas a high dose yielded significant ovarian protection against cyclophosphamide. Our findings provide valuable insights for supplementing clinical trials on the protective effects of glutathione against ovarian damage.

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Introduction

Most cases of primary ovarian insufficiency (POI) are idiopathic, and iatrogenic cases are common. Many cancer survivors experience POI as a frequent side effect of chemotherapy. In fact, the incidence of iatrogenic POI due to chemotherapy and radiotherapy increases as survival rates increase [1]. Therefore, preserving fertility has become a major concern, especially for young patients with cancer. Gonadotoxicity varies among different chemotherapeutics [2]. Alkylating agents such as cyclophosphamide (Cy) and ifosfamide are considered high risk. Table 1 summarizes chemotherapeutics regarding the gonadotoxicity risk they pose. They decrease ovarian reserves and induce POI [3]. In this study, Cy was used in an animal model to induce ovarian failure.

Follicular development involves several stages, starting with the formation of primordial follicles (PMFs) during embryonic development. Starting with menarche, these PMFs activate, leading to oocyte growth and the transformation of pregranulosa cells into granulosa cells, forming primary follicles. These evolve into secondary follicles as theca cells develop around them. In the antral phase, antral follicles appear, with one becoming the dominant Graafian follicle, while others become atretic. This phase depends on gonadotropins. Luteinizing hormone triggers ovulation in the dominant follicle, leading to oocyte release and corpus luteum formation. PMFs are selectively activated throughout reproductive years, balancing dormancy and activation. This pool's depletion, hastened by gonadotoxic agents, leads to menopause [4]. Cy was the first chemotherapeutic agent, which was accused for being associated with POI [5]. The primary target of Cy is proliferating cells. It promotes apoptosis and inhibits cell growth via intracellular DNA cross-linking [6]. The apoptosis of granulosa cells and follicular atresia significantly decreases serum anti-Müllerian hormone (AMH) concentrations; therefore, the recruitment of PMFs accelerates. This leads to a depletion of the ovarian reserve and a poor reproductive prognosis [7]. Cy also directly damages the follicle population [8].

The first reference to glutathione (GSH) in the literature was in 1888, when Rey-Pailhade described a molecule whose spontaneous reaction with sulfur produces hydrogen sulfide [9]. Rey-Pailhade named this molecule the "philothion". Later, it was established that philothion was GSH, and its structure was a tripeptide composed of glycine, cysteine, and glutamic acid [10]. Estrela et al. [11] described the effects of GSH on cell biology as a reducing agent and an antioxidant. It has many functions, such as being a free radical scavenger, a reservoir of cysteine, and a modulator of lymphatic functions and immune responses, and it contributes to mitochondrial processes that open the permeability transition pore complex and activate cell death. This multifunctional molecule is associated with resistance to drug-induced cytotoxicity and ionizing radiation [11–13]. Increased levels of GSH within tumor cells are implicated in conferring resistance to chemotherapy drugs, including cisplatin [14]. Administering precursors of GSH could potentially elevate GSH concentrations in healthy cells, while simultaneously reducing them in tumor cells. This approach may protect healthy tissues and increase the vulnerability of tumor cells to chemotherapeutic agents [15]. Following the discovery of these features, the potential of GSH in preserving ovarian function has become a popular research topic. GSH acts as an armor for oocytes against the oxidative stress produced during folliculogenesis [16]. Oocytes with higher intracellular GSH concentrations produce healthier and stronger embryos [17]. Gonadotoxic treatments, such as cisplatin, increase reactive oxygen species (ROS) and decrease ovarian antioxidant capacity [18], thus decreasing the activities of intracellular antioxidants such as superoxide dismutase [19] and GSH [20, 21]. This study aimed to investigate the protective effects of GSH against iatrogenic ovarian damage on ovarian function in female rats exposed to Cy by measuring serum AMH levels, follicle counts, and related parameters.

Methods

Animals

Forty-two adult female Sprague-Dawley rats (weight: 200–250 g; age: 65–75 days) were used in this study. All rats were provided by the Bezmialem University Animal Reproduction Centre and housed in the Animal Laboratory of Bezmialem University. The rats were maintained under standard housing conditions with a 12-h light-dark cycle and ad libitum access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Bezmialem University (Decision no: 2021/306). All procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (1996).

Experimental Design

Rats were randomly divided into six groups (7 rats per group) (Fig. 1). Group 1 (control, $n = 7$) was intraperitoneally administered sodium chloride 0.9% (1 mL/kg, single dose) on days 1, 7, and 14. Group 2 (Cy, $n = 7$) was intraperitoneally administered Cy (75 mg/kg, single dose) on the 1st day and sodium chloride 0.9% (1 mL/kg, single dose) on the 7th and 14th days. Drug doses were chosen based on our previous study (Özcan et al. [22], 2020).

Table 1. Gonadotoxicity of commonly used chemotherapy agents

Chemotherapeutic agent	Risk of gonadotoxicity	Mechanism of action
Alkylating agents Cyclophosphamide Ifosfamide	High	Induces single-stranded DNA breaks, targets PMFs and resting oocytes
Platinums Cisplatin Carboplatin	Intermediate	Induces chromosomal damage and DNA cross-links
Taxanes Paclitaxel	Intermediate	Inhibits microtubule formation and spindle function
Anthracyclines Doxorubicin	Intermediate	Inhibits DNA replication and transcription
Antimetabolites Gemcitabine 5-Fluorouracil	Low	Acts primarily on cells synthesizing DNA

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Group 3 (GSH group, $n = 7$) was intraperitoneally administered GSH (100 mg/kg, single dose) on days 1, 7, and 14. Group 4 (GSH group, $n = 7$) was intraperitoneally administered GSH (200 mg/kg, single dose) on days 1, 7, and 14. Group 5 (Cy plus GSH-100, $n = 7$) was intraperitoneally administered Cy (75 mg/kg, single dose) on the 1st day and GSH (100 mg/kg, single dose) on the 1st, 7th, and 14th days. Group 6 (Cy plus GSH-200, $n = 7$) was intraperitoneally administered Cy (75 mg/kg, single dose) on the first day and GSH (200 mg/kg, single dose) on days 1, 7, and 14.

Sample Collection, Histological Analysis, and Ovarian Follicle Count

On day 21, all rats were anesthetized by intramuscular administration of 50 mg/kg ketamine hydrochloric acid (Ketalar; Eczacibasi Warner-Lambert Ilac Sanayi, Levent, Istanbul, Turkey) and 7 mg/kg xylazine hydrochloric acid (Rompun; Bayer Sisli, Istanbul, Turkey). They were then immobilized on a standard rat surgical board. An aseptic technique was used to create a ventral midline incision to expose the reproductive organs. The ovaries were then removed. The ovaries were fixed in a 10% neutral buffered formaldehyde solution for 72 h. After fixation, the ovaries were washed with water and gradually dehydrated using increasing concentrations of ethanol (70%, 90%, 96%, and 100%), followed by clearing in xylene. Subsequently, the samples were immersed in paraffin overnight at 60°C and embedded in paraffin blocks. From these paraffin blocks, 5-µm-thick sections were prepared and placed on slides. Hematoxylin and eosin staining was performed on the paraffin sections for histomorphometric analysis, and they were examined under a photomicroscope (ZEISS Axio Zoom.V16, Germany). The follicles were categorized into five stages: primordial, primary, secondary, antral, and atretic follicles. The number of follicles was evaluated using the histomorphometric techniques described by Tilly [23]. Histopathological examinations were performed by a histologist blinded to the treatment allocation.

Immunohistochemistry of AMH

The paraffin sections of the ovaries were incubated overnight at 37°C. Following deparaffinization with xylene and rehydration in descending grades of ethanol, the sections were treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous enzyme activity. After rinsing with tap and distilled water, antigen retrieval was performed by microwaving the sections at 200 W with a citrate buffer (pH 6.1) for 20 min. Subsequently, the sections were washed with phosphate-buffered saline, incubated in blocking solution for 10 min, and then exposed to a mouse AMH antibody (1:20, GeneTex, Cat: GTX42794) at 4°C overnight. For secondary antibody staining, the Histostain®-Plus 3rd Gen IHC Detection Kit (Cat: 85-9073, Invitrogen, CA, USA) was used according to the manufacturer's instructions. After washing, the sections were incubated with streptavidin-peroxidase (ready-to-use) for 10 min at room temperature, followed by incubation with 3,3′-diaminobenzidine for 5 min. Finally, the slides were counterstained with Mayer's hematoxylin and covered with mounting medium.

If all or some of the granulosa cells forming the follicle express AMH, the follicle is considered as an AMH-positive follicle. In each section, the AMH-positive follicles were counted, and the staining intensity was evaluated semi-quantitatively on a scale of 0–3 (0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining). This evaluation was made by classifying the follicles as pre-antral (primary and secondary) and antral follicles [22].

ELISA for AMH

Intra-cardiac blood samples were collected in centrifuge tubes and centrifuged at 3,000 rpm for 20 min. Serum was separated and then quickly stored at −80°C for biochemical analyses. AMH levels were determined by ELISA kit (Cat No.: CEA228RA, Wuhan USCN Business Co., Ltd., Wuhan, China) according to the manufacturer's instructions. The absorbance of individual wells at 450 nm was recorded and quantified against a standard curve.

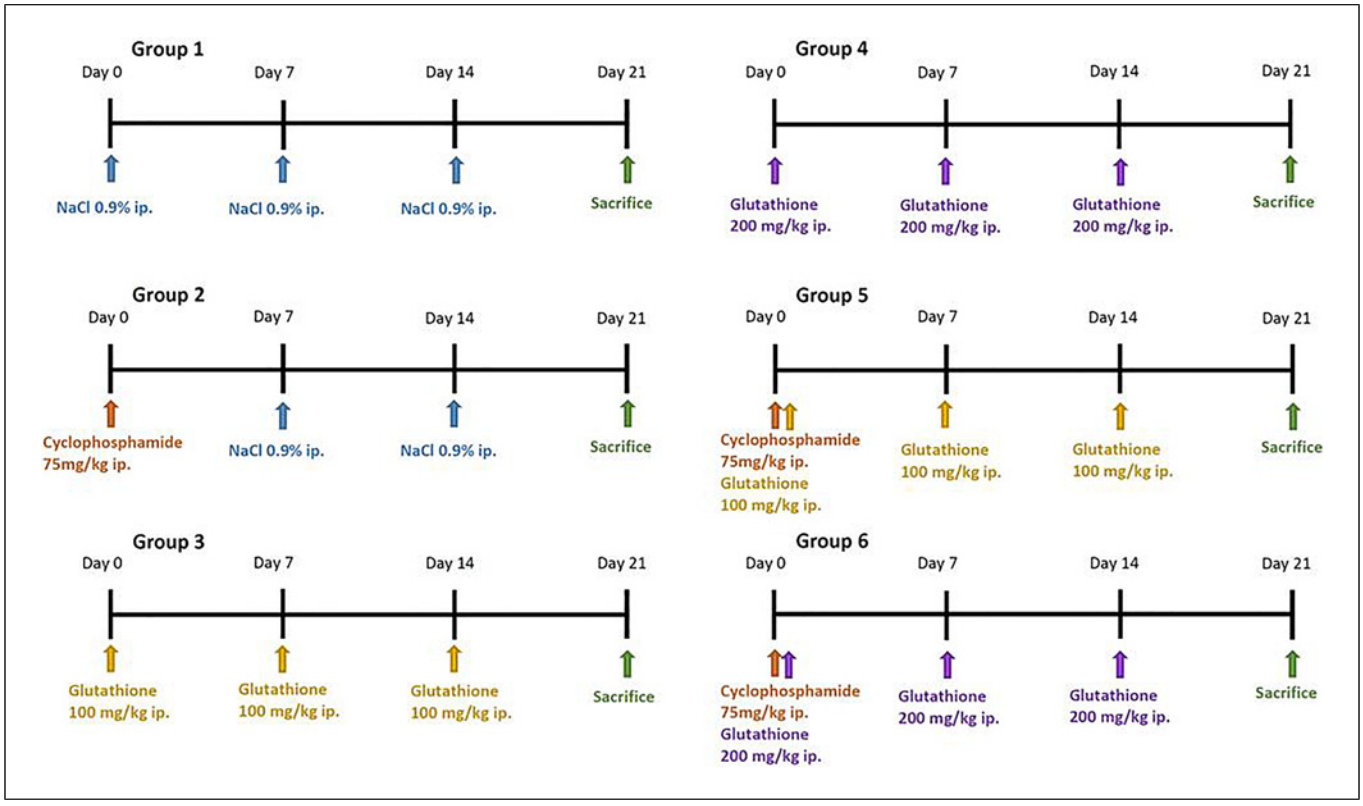

Fig. 1. Timeline of the experiment (the rats were randomly divided into six groups; 7 rats per group).

Statistical Analysis

Sample size and power calculations determined that sufficient statistical power required 7 rats for each group (power = 0.80, type 1 error = 0.05, and type 2 error = 0.20). The power calculation was based on the PMF count reported by Özcan et al. [22], using the standard formula/program. Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) by one-way analysis of variance, followed by Tukey's test. Data were reported as mean ± standard deviation, number, or percentage. $p < 0.05$ was considered statistically significant for all tests.

Results

Serum AMH concentrations, follicle count, including primordial, primary, secondary, atretic and antral, and pre-antral and antral AMH-positive staining follicle number, percentage, and intensity scores in all groups are shown in Table 2 and Fig. 2. Statistically significant variances were observed across all parameters among the six groups when subjected to a collective comparative analysis ($p < 0.05$).

Subsequent to the collective comparative assessment of all groups, individual group evaluations were conducted, followed by pairwise and triadic analyses. No significant differences were observed in the primordial, primary, secondary, atretic, and antral follicle counts between groups 1, 3, and 4. Statistically significant differences were found in all parameters between groups 1 and 2. Group 2 had the lowest primordial, primary, secondary, and antral follicle counts and the highest atretic follicle count. There was a significant difference in all these parameters between groups 2 and 6, while statistically significant differences were not observed between groups 2 and 5. Primordial, primary, and antral follicle counts were significantly higher, and atretic follicle count was significantly lower in group 6 than in group 2. No significant differences were observed in primary, secondary, atretic, and antral follicle counts between groups 1 and 6. The details of this analysis are summarized in the online supplementary Table (for all online suppl. material, see https://doi.org/10.1159/000536055) provided.

The pre-antral and antral AMH-positive follicle counts, percentages, and intensity scores in all groups are shown in Table 2. Immunohistochemical analysis of AMH-positive follicles in all groups is shown in Fig. 3. No significant differences were observed in any of the

Table 2. Serum AMH concentrations, follicle count, including primordial, primary, secondary, atretic and antral, and pre-antral and antral AMH-positive staining follicle number, percentage, and intensity scores in all groups

Variables	Group I (n = 7)	Group II (n = 7)	Group III (n = 7)	Group IV (n = 7)	Group V (n = 7)	Group VI (n = 7)	p value
Serum AMH concentrations	2.38±0.35	0.76±0.28	1.61±0.41	2.03±0.24	1.05±0.27	2.21±0.27	0.0001*
Pre-antral AMH-positive staining follicle counts	34.33±9.81	8.2±5.4	21.2±11.52	17.2±13.22	9±2.68	26.5±9.73	0.002*
Antral AMH-positive staining follicle counts	2.6±0.89	0.4±0.54	1.6±1.14	1.8±0.83	1.16±0.75	2.33±1.36	0.014*
Pre-antral AMH-positive staining follicle percent	86.62±12.49	58.9±14.94	69.88±9.55	77.14±18.64	57.62±10.01	88.48±7.1	0.0013*
Antral AMH-positive staining follicle percent	75±20.41	14.66±14.44	48.34±38.37	55.34±14.09	43.05±35.13	68.45±8.51	0.024*
Pre-antral AMH-positive staining follicle intensity score	1.94±0.49	0.53±0.03	1.08±0.31	1.73±0.9	1.49±0.76	2.26±0.4	0.003*
Antral AMH-positive staining follicle intensity score	1.3±0.57	0.17±0.23	0.65±0.54	0.96±0.29	0.47±0.34	1.02±0.3	0.003*
PMF counts	183.8±17.99	91±12.55	146.6±13.3	156.6±22.61	107.7±11.88	122.7±15.11	<0.0001*
Primary follicle counts	78.5±12.45	48±14.18	73.2±10.89	74.8±14.84	62.17±12.22	70.67±5.53	0.005*
Secondary follicle counts	24.75±5.85	14.4±5.32	24±3.67	25.2±5.45	19±2.6	22.83±3.81	0.004*
Antral follicle counts	18.75±4.11	10.8±3.03	16.4±2.96	17.4±2.6	12±2.75	17.5±2.16	0.0006*
Atretic follicle counts	4.25±1.7	8.6±2.3	3.6±0.89	4±1.58	7.5±1.37	4.66±1.36	<0.0001*

All values are expressed as mean ± standard deviation (SD). *$p < 0.05$ significant difference, comparison of all groups.

parameters between groups 1, 3, and 4. A statistically significant difference was found in all parameters between groups 1 and 2 (p = 0.008 for pre-antral and p = 0.01 for antral AMH-positive staining follicle counts; p = 0.03, pre-antral and 0.02; antral percentage, p = 0.03; pre-antral intensity score, p = 0.004 for antral intensity scores). Pre-antral and antral AMH-positive staining follicle counts, percentages, and intensity scores were significantly lower in group 2. No significant difference was found between groups 1 and 6 regarding pre-antral and antral AMH-positive follicle counts, percentages, and intensity scores. There was a significant difference in these parameters between groups 2 and 6. No significant difference was found between groups 2 and 5 regarding pre-antral and antral AMH-positive follicle counts, percentages, and intensity scores. There was a significant difference in pre-antral AMH-positive staining follicle counts, percentages, and intensity scores between groups 1 and 5, whereas no statistically significant differences were observed between groups 1 and 5 regarding antral AMH-positive staining follicle counts, percentages, and intensity scores.

The mean serum AMH concentrations were highest in the control group (group 1) and lowest in the Cy group (group 2) (Table 2) (Fig. 4). The online supplementary Table presents the analysis for the remaining pairwise group comparisons.

Discussion

Iatrogenic POI (chemotherapy-induced POI) is a long-term morbidity associated with cancer treatment. Researchers have uncovered various mechanisms that cause chemotherapy-induced ovarian damage [24]; however, many more are yet to be elucidated. Cy was used in our animal model for inducing ovarian damage. Cy functions by increasing ROS. Since the production rate of free radicals surpasses the rate of neutralization by antioxidants, oxidative stress is inevitable [25, 26]. Cy lacks specificity in targeting the cell cycle, resulting in its toxicity to cells not actively undergoing division [27]. Cy also triggers inflammation, and the increased expression of cytokines facilitates programmed cell death by activating

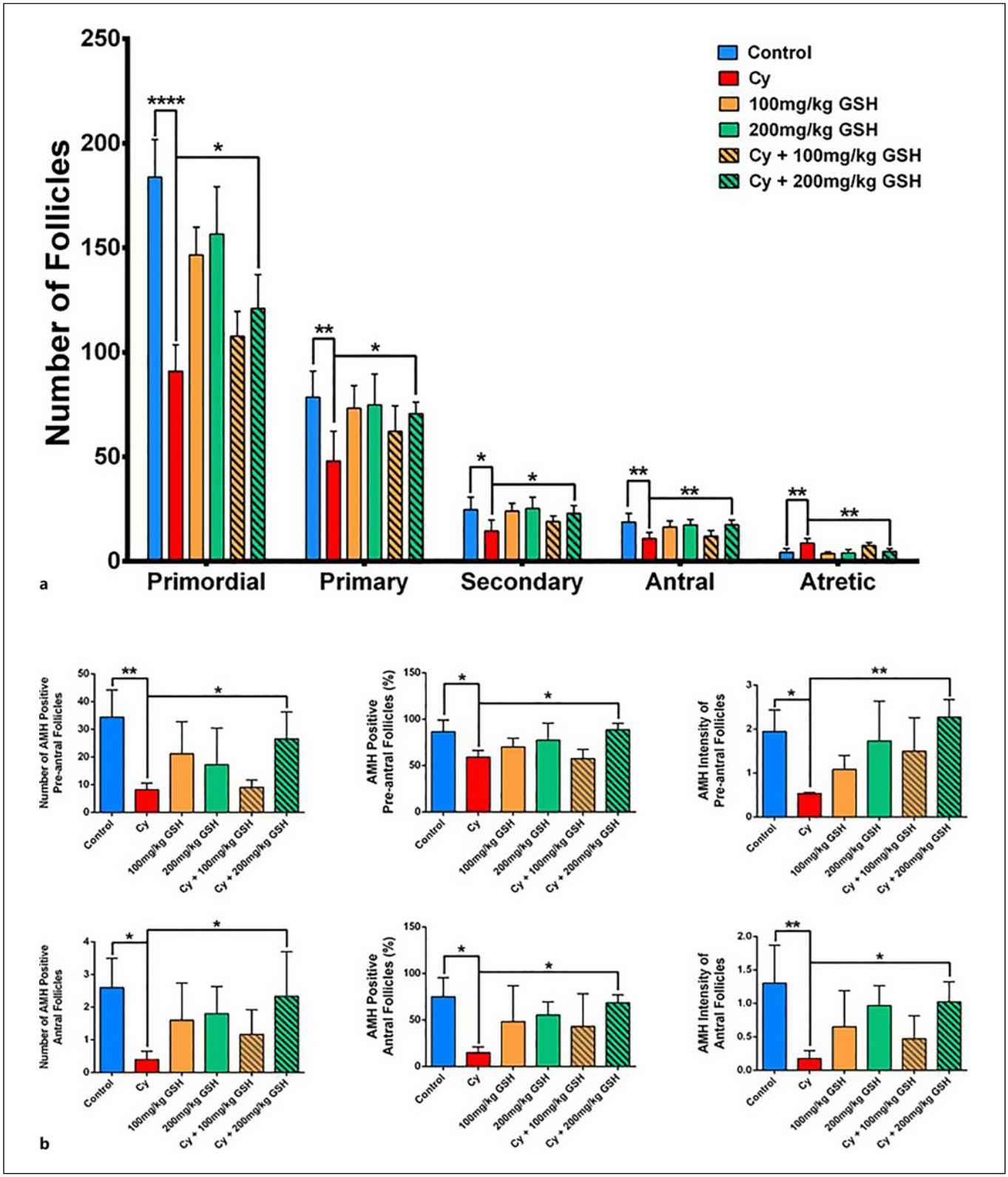

Fig. 2. a Follicle count, including primordial, primary, secondary, atretic, and antral. **b** AMH-positive staining follicle counts and pre-antral and antral AMH-positive staining follicle number, percentage, and intensity scores in all groups. AMH, anti-Müllerian hormone.

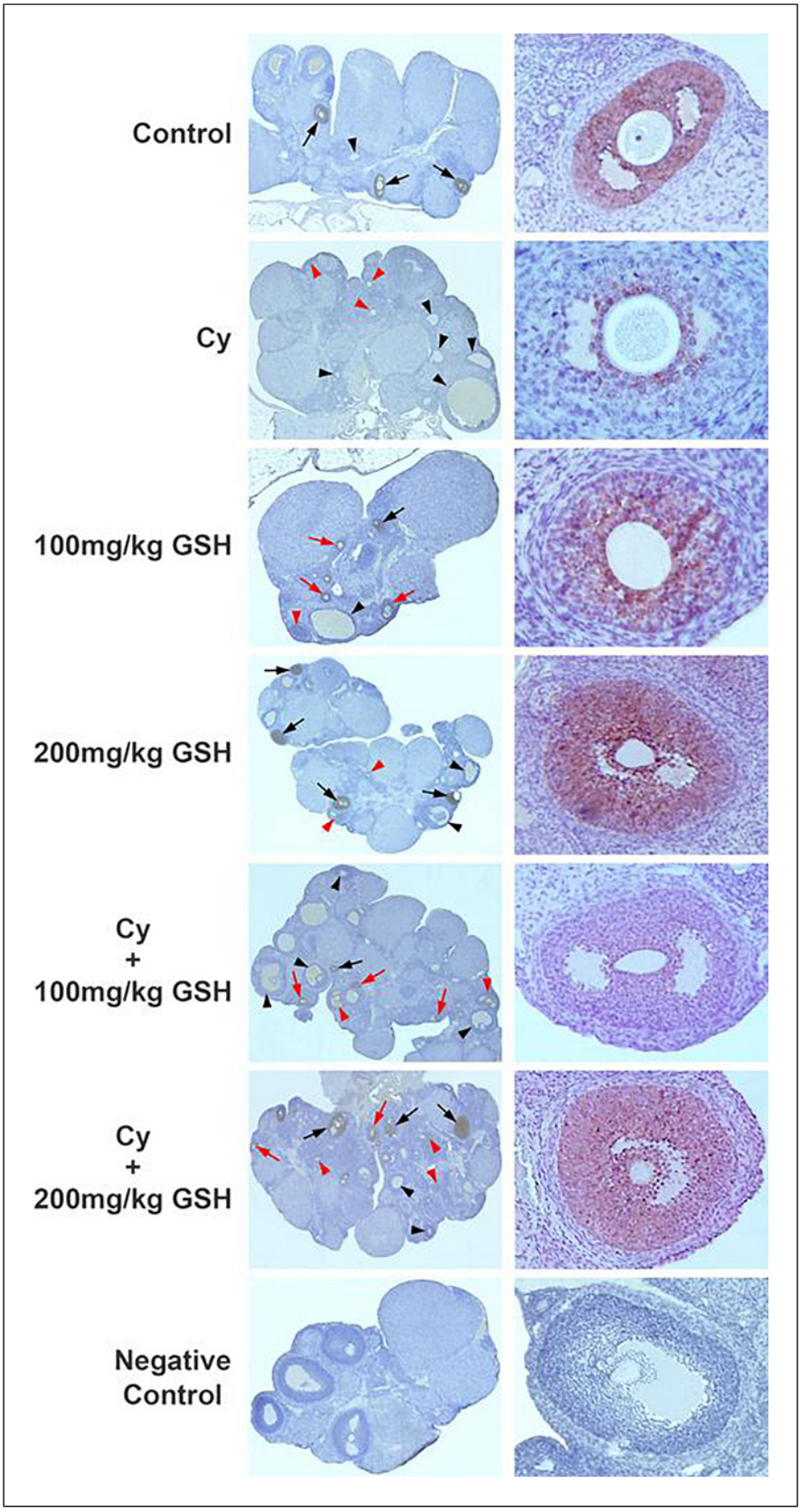

Fig. 3. Immunohistochemistry of AMH in the ovary. AMH, anti-Müllerian hormone; strong staining, black arrow; moderate, red arrow; weak, red arrowhead; non-staining, black arrowhead.

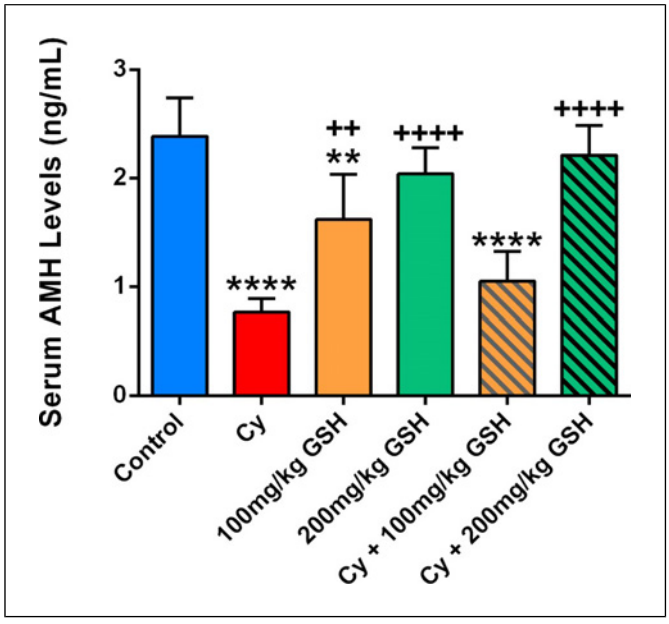

Fig. 4. AMH ELISA Results. *Compared with control group. +Compared with Cy group.

interferon alpha and gamma [28]. CYP2B and CYP3A activate Cy to produce 4-hydroxycyclophosphamide, which is eventually metabolized to phosphoramide mustard (PM) [29]. PM has been demonstrated to provoke the activation of ataxia telangiectasia mutated, an essential component in regulating and stimulating double-strand break repair, initiating cell cycle checkpoints, and signaling for apoptosis induction [30].

Like many chemotherapeutic agents, Cy induces the apoptosis of PMFs through different molecular pathways. It triggers the apoptosis of PMFs and granulosa cells in growing follicles by causing DNA cross-linking with its gonadotoxic effect. TUNEL-positive signals are especially evident in the granulosa cells of secondary follicles [25]. In another study, it has been demonstrated that Cy induces apoptosis in granulosa cells surrounding growing follicles through signaling pathways involving DNAPK/(ATM), CHK2, p53, and TAp63α [31]. Liu et al. [32] found a significant atrophy in ovarian tissues, accompanied by increased empty spaces in the ovarian stroma and necrosis in granulosa cells. Indirectly, Cy influences the normal development of follicles by adversely affecting the ovarian stroma and the vasculature [33]. It is observed that Cy causes a serious decrease in the number of follicles by causing apoptosis in PMFs and granulosa cells of growing follicles, with both direct and indirect effects. Without evaluating the apoptotic process, we found that Cy caused decreased serum anti-Müllerian hormone (AMH) levels, loss of PMFs, primary follicles, and secondary follicles, increased atretic follicles, and decreased ovarian reserve function in rats.

Antioxidants are widely used by patients with cancer worldwide to alleviate the side effects of toxic chemotherapies [34], and GSH is known for its antioxidant properties. PM and acrolein, the active metabolites of Cy, are detoxified by conjugation with GSH [27]. COV434 granulosa cells were exposed to 4-hydroperoxycyclophosphamide, a derivative of Cy, and a rapid reduction in intracellular GSH was observed. This was followed by an increase in ROS levels and the induction of apoptosis. GSH supplementation or co-treatment with antioxidants significantly suppressed this effect [35]. Raeeszadeh et al. [28] studied N-acetylcysteine, a precursor of GSH and vitamin E, on Cy-induced ovarian damage and demonstrated a synergistic protective effect. Other studies investigating the protective effects of 2-mercaptoethane sulfonate [20] and mirtazapine [36] against gonadotoxic medications have also revealed that preservation of antioxidant substances contributes to the preservation of ovarian function, like reduction of GSH depletion. Coenzyme Q10 and resveratrol are also widely studied antioxidants. Our previous studies demonstrated the protective antioxidant properties of coenzyme Q10 and resveratrol on ovarian function in the same animal models [18, 37]. Directly dispensing GSH to investigate its antioxidant properties in tissues has been studied by several researchers. GSH has been shown to improve cardiovascular function in old rats [38]. In another recent study, a GSH-enriched formulation improved glucose tolerance in rats [39]. To our knowledge, this is the first study to explore the role of directly administered GSH in counteracting gonadotoxicity in vivo. Our findings revealed that GSH administration plays a role in protecting ovarian function against Cy-induced gonadotoxicity. The observed protective effect appears dose-dependent, an intriguing and significant finding.

In this study, we focused mainly on the potential protective effects of two different doses of GSH on ovarian function against Cy-induced ovarian damage. Our data demonstrate the catastrophic damage caused by Cy in the ovaries. However, the hormonal, histopathological, and immunohistochemical methods used in our study demonstrated the potential protective effects of GSH against ovarian damage induced by Cy. After administering 200 mg/kg GSH with Cy, serum AMH concentrations and follicle count, including primordial, primary, secondary, antral, pre-antral, and antral AMH-positive staining, follicle number, percentage, and intensity scores significantly increased, whereas the atretic follicle count significantly decreased. In contrast, 100 mg/kg GSH appeared ineffective in reversing Cy toxicity. A dose-dependent effect was also observed in another study that investigated mirtazapine. In a

study by Altuner et al. [36], both mirtazapine doses were effective; however, the effect correlated with the dose administered. In our study, the lower dose exhibited a minimal protective effect, suggesting that Cy generates substantial oxidative stress and severely depletes GSH levels.

Our findings agree with past studies on using antioxidants to reduce gonadotoxic damage from Cy. Research shows that superoxide dismutase and GSH together help protect against this damage by removing ROS and preserving antioxidants [40]. However, GSH cannot be considered a purely beneficial substance. Elevated GSH levels in tumor cells may protect them by causing resistance to various chemotherapeutics [41, 42]. Inhibition of GSH S-transferase is studied to overcome cisplatin resistance [43]. Antioxidants also promote metastasis [44]. Thus, this dose-dependent benefit of GSH in preserving ovarian function may be unfavorable from an oncological perspective. Nonetheless, exploring antioxidant mechanisms as a strategy to mitigate the irreversible adverse effects of chemotherapeutic agents remains a viable approach. Antioxidant treatments could selectively shield normal cells while diminishing antioxidant levels in cancerous cells [15, 45]. Research examining a range of chemotherapeutic drugs and tissue types is crucial to determine whether antioxidants can safely shield healthy tissues without contributing to resistance in cancerous tissues.

Our study had several limitations. First, this was an experimental animal investigation with a comparatively modest sample size, a common restriction encountered in animal research. However, we argue that this study provides valuable insights for supplementing large-scale clinical trials. Second, experimental studies should be conducted to determine the optimal dosage and duration of GSH therapy. Furthermore, information gathered from an experimental animal model may not yield precisely similar outcomes in humans; therefore, additional investigations are necessary to examine the impact of GSH on women experiencing POI.

Regarding the strengths of this research, to our knowledge, this is the first study to explore the protective effects of GSH on ovarian tissue upon direct administration. Further, we demonstrated a dose-dependent effect of GSH, achieved by administering low and high doses of the compound. There is a necessity to explore the protective impact of GSH against Cy's apoptotic influence on follicle development.

In conclusion, this is the first study to examine the protective effects of GSH against ovarian damage associated with oxidative stress in a chemotherapy-induced experimental model. Our findings suggest that preserving antioxidant activity offers significant protection against ovarian damage associated with oxidative stress. The intense oxidative stress caused by Cy was highlighted by the fact that increasing the GSH dose resulted in remarkably improved effectiveness. GSH plays antioxidant roles by significantly improving serum AMH concentrations; primordial, primary, secondary, and antral follicles; antral AMH-positive staining follicle number, percentage, and intensity scores; and significantly decreasing atretic follicle count. We believe our findings will shed light on future clinical studies on the protective effects of GSH against ovarian damage, including detailed analyses of the dose and method of use.

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Statement of Ethics

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Bezmialem University (Decision no: 2021/306). All procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (1996).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Ebru Tansu Yurttancikmaz performed the study, contributed to data entry, and conducted literature review. Pinar Ozcan developed the study idea and design, interpreted the data, wrote the draft, and edited, reviewed, and finalized the manuscript. Fatma Basak Tanoglu and Caglar Cetin contributed to data entry and conducted literature review. Olgu Enis Tok performed the immunohistochemical staining of tissue samples and contributed to statistical analysis and interpretation of the data. Hikmet Tunc Timur finalized the manuscript and conducted literature review.

Data Availability Statement

All data generated or analyzed during this study are included in this manuscript. Further inquiries can be directed to the corresponding author.

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