ORIGINAL RESEARCH

Magnesium Sulfate Has Potential Scavenging Effects on Cyclophosphamide-Induced Ovarian Damage in A Rat Model

İlkin YERAL[a],
Cemile DAYANGAN SAYAN[a]

[a]Department of Gynecology and Obstetrics,
Kırıkkale University Faculty of Medicine,
Kırıkkale, TURKEY

Correspondence:
İlkin YERAL
Kırıkkale University Faculty of Medicine,
Department of Gynecology and Obstetrics,
Kırıkkale, TURKEY
ilkinyeraldr@hotmail.com

ABSTRACT Objective: This study proposed to investigate whether magnesium sulfate ($MgSO_4$) could reduce the ovarian damage induced by cyclophosphamide (Cyc). **Material and Methods:** Thirty female rats were used for this study: Control group (n=10)- Only laparotomy; Cyc group n=10)-75 mg/kg Cyc intraperitoneally; and Cyc+$MgSO_4$ group (n=10)-75 mg/kg Cyc on day 0 and 200 mg/kg $MgSO_4$ on days 1-7 (both intraperitoneally). The extent of histopathological damage and the number of ovarian follicles were determined. The levels of anti-Mullerian hormone (AMH) were measured in blood samples. **Results:** Statistically significant differences in the AMH values were observed in the control group versus Cyc group and Cyc group versus Cyc+$MgSO_4$ group (p<0.05). The levels of AMH were the least in the Cyc group. The total tissue damage in the Cyc group was significantly higher than that in the control, as well as in the Cyc+$MgSO_4$ group (p<0.05). The follicle counts were the least in the Cyc group. **Conclusion:** Cyc caused ovarian damage and reduced the ovarian reserves. The ovarian reserves in the $MgSO_4$-treated group were better than those in the other groups, and there was least ovarian damage in the $MgSO_4$-treated group.

Keywords: Cyclophosphamide; anti-Mullerian hormone; magnesium sulfate

Chemotherapeutic agents, which are presently widely used in the treatment of cancer, are highly effective but have a high incidence of side effects.[1] Various chemotherapeutic agents may be selected based on the type of cancer. Among chemotherapeutics, cyclophosphamide (Cyc) is one of the most effective alkylating agents but is also one of the most toxic to the ovaries.[1] Cyc affects the ability of ovarian primordial follicular cells to regenerate, and damage to these cells can cause ovarian dysfunction and premature ovarian failure.[2] Thus, the use of Cyc in reproductive women puts them at high risk of premature menopause and infertility.[3]

Anti-Mullerian hormone (AMH) is a dimeric glycoprotein produced from granulosa cells in preantral (primary) follicles, particularly antral follicles. Very large antral follicles (larger than 5 mm in size) lose their ability to produce AMH, thus inhibiting adjacent primordial follicles by exerting a negative paracrine effect.[4] Serum levels of AMH can provide information about ovarian reserves.[5,6] AMH is a better marker of ovarian reserves than estradiol, inhibin, or follicle-stimulating hormone.[7]

Previous research has demonstrated the strong anti-inflammatory, antioxidant, and anti-apoptotic effects of magnesium sulfate ($MgSO_4$), which inhibits the endotoxin-dependent inflammatory molecules.[8] Previous studies have also demonstrated the protective effects of $MgSO_4$ in lung, kidney, and brain.[8-10] However, none of these studies investigated the protective effect of $MgSO_4$ on the ovaries. The aim of this study was to evaluate the potential ability of $MgSO_4$ to reduce ovarian damage induced by Cyc.

MATERIAL AND METHODS

The study was approved by the Kırıkkale University's Animal Experiments Local Ethics Committee on 09.11.2017 (meeting number 17/7 and decision number 17/39) and was performed according to the criteria of the National Health and Medical Research Council. The study was carried out in accordance with the principles of the Declaration of Helsinki.

ANIMALS

Thirty female Wistar-Albino rats aged 10-12 weeks (weight 200-250 g) were used. The animals were fed standard rat chow and had unrestricted access to tap water. They were housed in cages at room temperature (22-25°C) under a 12-h light/12-h dark cycle.

STUDY GROUPS

Three groups were established, with 10 rats in each group: 1) Control group: Only a laparotomy was performed on day 7; 2) Cyc group: A single dose of 75 mg/kg of cyclophosphamide (Eczacibasi; Baxter Chemical Co., Istanbul, Turkey) was administered intraperitoneally on day 0; and 3) Cyc + $MgSO_4$ group: A single dose of 75 mg/kg of Cyc was administered on day 0, and 200 mg/kg of $MgSO_4$ (both intraperitoneal) was administered on days 1-7.

ANESTHESIA

Anesthesia was induced by the intramuscular administration of 5 mg/kg of 2% xylazine (Rompun® vet, Bayer AG, Leverkusen, Germany) and 50 mg/kg of 10% ketamine (Ketaminol® vet, Farmaceutici Gellini SpA, Aprilia, Italy).

SURGICAL METHODS

At the end of one week, the animals from all the groups were surface-sterilized in the supine position and a 2-cm midline vertical incision was made. Bilateral oophorectomy was performed and intracardiac blood samples (5 mL) were collected. All the operations were performed by the same researcher.

MEASUREMENT OF SERUM AMH LEVELS

The blood samples were centrifuged at 1000 rpm for 20 min and the obtained serum samples were stored at -80°C until further use. The serum was diluted 1:30 with distilled water and the serum AMH values were measured using an enzyme-linked immunosorbent assay kit (Cusabio, MD, USA).

HISTOPATHOLOGICAL ANALYSIS

Tissues from the right ovary were fixed in 10% formalin and then embedded in paraffin. Serial sections of 5 μm thickness were cut and stained with hematoxylin-eosin. All the specimens were examined by light microscopy (Olympus BX50F4; Olympus, Tokyo, Japan), wherein at least five fields per specimen were scanned at a minimum magnification of 10-fold. A single pathologist blinded to the specimen group performed the examination and scoring of the samples (Figure 1a).
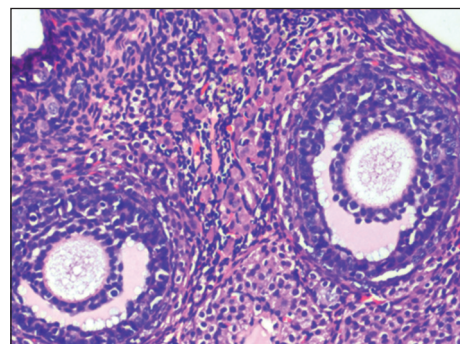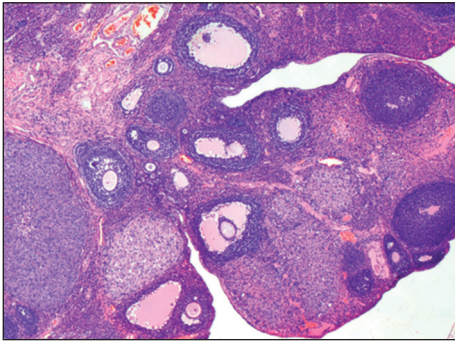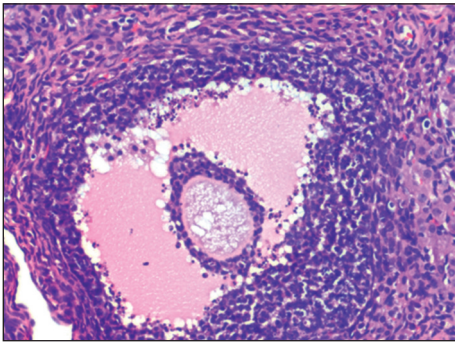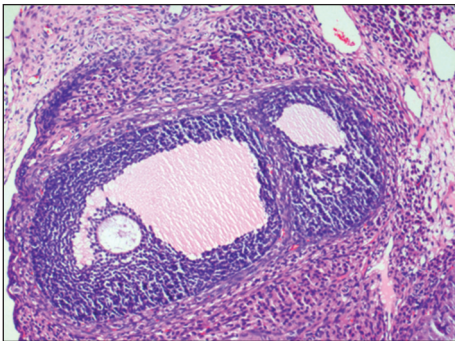

FIGURE 1a: Primordial follicle in the sham group (H-E; X200).

FIGURE 1b: Follicular degeneration in the Cyc group (H-E; X40).

FIGURE 1c: The degenerated follicle in the Cyc group (H-E; X200),

FIGURE 1d: The intact follicle in the Cyc+MgSO$_4$ group (H-E; X100).

The follicles in the ovaries were divided into four groups based on their size: primordial follicles (<20 μm), preantral follicles (20-220 μm), small antral follicles (221-310 μm), and large antral follicles (311-370 μm). For follicle counts, both the follicle size and total number were recorded.

The level of ovarian damage was evaluated using a histopathological damage-scoring system (Figure 1b, Figure 1c, Figure 1d). According to this system, follicular cell degeneration, vascular congestion, hemorrhage, and inflammation levels were scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) based on damage of less than 25%, between 25 and 50%, between 50 and 75%, and over 75%, respectively. The scores were added up to determine the total tissue damage.

STATISTICAL ANALYSIS

All the results were reported as the average standard deviation of mean values. The statistical analyses were performed using the software SPSS, version 16.0 (Chicago, IL). Due to the limited number of rats in each group, nonparametric methods were used for the statistical analysis. Differences among the three groups were evaluated with Kruskal-Wallis's variance analysis. Pair-wise comparisons were done with Mann-Whitney's U-test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

The serum AMH values and parameters of ovarian damage such as hemorrhage, congestion, inflammation, follicle degeneration, total tissue damage, and follicle number in the ovarian tissues were compared between different groups. The examined parameters in the control group and the Cyc+MgSO$_4$ group were found to be statistically similar ($p>0.05$).

The serum AMH values of the control and Cyc groups, and those of the Cyc and Cyc+MgSO$_4$ groups were significantly different ($p<0.05$). The Cyc group showed the least serum AMH values, while the control group showed the highest (Table 1).

In terms of tissue damage, a statistically significant difference in the follicular degeneration and total tissue damage was observed between the control and Cyc groups, and between the Cyc and Cyc+MgSO$_4$ groups ($p<0.05$). The Cyc group showed the highest follicular degeneration and total tissue damage (Table 2).

The number of primordial follicles, preantral follicles, small antral follicles, large antral folli-

TABLE 1: Biochemical results of the groups (mean±SD) and p values of the multiple comparison test results between the groups.

Groups	AMH (pg/ml)
Sham (n=10)	13.66±8.49
Cyc (n=10)	6.16±0.76
Cyc+MgSO$_4$ (n=10)	9.22±2.70
p-value*	
Sham vs Cyc	p<0.05
Sham vs Cyc+MgSO$_4$	p=0.280
Cyc vs Cyc+MgSO$_4$	p<0.05

*p<0.05 value was considered as statistically significant.
SD: Standard deviation, AMH: Anti-Mullerian hormone, Cyc: Cyclophosphamide, MgSO$_4$: Magnesium sulfate.

cles, and the total number of follicles was statistically different between the control and the Cyc group, and between the Cyc and the Cyc+MgSO$_4$ group (p<0.05). The Cyc group showed the least number of primordial follicles, preantral follicles, small antral follicles, and large antral follicles, as well as the total number of follicles (Table 3).

DISCUSSION

To the best of our knowledge, the effect of MgSO$_4$ against Cyc-induced ovarian damage in rat models has not been investigated yet. The results of our study indicated that Cyc caused ovarian damage and reduced the ovarian reserve. The ovarian reserves in the MgSO$_4$-treated group were better than those in the other groups and there was least ovarian damage in the MgSO$_4$-treated group.

Cyc shows cytotoxic effects, especially against rapidly dividing cells.[1] Hence, an important side-

TABLE 2: Histopathological results of the groups (mean±SD) and p values of the multiple comparison test results between the groups.

Group	Hemorrhage	Congestion	Inflammation	Follicular degeneration	Total damage
Sham (n=10)	0.00±0.00	1.00±0.00	0.50±0.52	0.30±0.48	1.80±0.78
Cyc (n=10)	0.00±0.00	1.00±0.00	0.9±0.31	1.00±0.00	2.90±0.31
Cyc+MgSO$_4$ (n=10)	0.00±0.00	1.00±0.00	0.4±0.51	0.40±0.51	1.60±0.84
p-value*					
Sham vs Cyc	p=1.00	p=1.00	p=0.143	p<0.05	p<0.05
Sham vs Cyc+MgSO$_4$	p=1.00	p=1.00	p=0.739	p: 0.739	p=0.579
Cyc vs Cyc+MgSO$_4$	p=1.00	p=1.00	p=0.63	p<0.05	p<0.05

*p<0.05 value was considered as statistically significant.
SD: Standard deviation, Cyc: Cyclophosphamide, MgSO$_4$: Magnesium sulfate.

TABLE 3: Follicle counts of the groups (mean±SD) and p values of the multiple comparison test results between the groups.

Group	Hemorrhage	Congestion	Inflammation	Follicular degeneration	Total damage
Group	Primordial	Preantral	Small antral	Large antral	Number of follicle
Sham (n=10)	3.20±1.81	1.70±0.82	2.50±1.64	2.00±1.33	9.40±3.62
Cyc (n=10)	1.40±1.17	0.70±0.82	0.90±1.28	0.60±0.69	3.60±2.63
Cyc+MgSO$_4$ (n=10)	4.50±2.32	2.70±2.31	2.90±1.79	2.90±2.23	13.00±4.76
p-value*					
Sham vs Cyc	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05
Sham vs Cyc+MgSO$_4$	p=0.218	p=0.436	p=0.684	p=0.436	p=0.052
Cyc vs Cyc+MgSO$_4$	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05

*p<0.05 value was considered as statistically significant.
SD: Standard deviation, Cyc: Cyclophosphamide, MgSO$_4$: Magnesium sulfate.

effect of Cyc is ovarian insufficiency, which can occur as a result of ovarian follicular damage.[1] Several studies have examined ovarian toxicity induced by Cyc and other chemotherapeutic agents in various animal models.[11-13] As the toxicity by Cyc surpasses that of most of the other chemotherapeutic agents, the present study focused on the effects of Cyc. Based on the results of the serum and tissue analyses, the ovarian reserve in the Cyc group was the least.

In a previous study on Cyc-induced ovarian toxicity in rats, the number of follicles increased in a group treated with an antagonist of the gonadotropin-releasing hormone (GnRH).[11] In another study using rat models, a specific inhibitor of tyrosine-specific protein kinases called genistein provided a protective effect against ovarian toxicity induced by Cyc.[12] In other studies, antioxidants such as ascorbic acid, alpha-tocopherol, and selenium reduced Cyc-induced toxicity in rat ovaries.[13] Furthermore, few other studies have reported that dienogest and the blue-green alga spirulina, which has known antioxidant and antiapoptotic properties, provided protection against Cyc-induced toxicity in rat ovaries.[14,15]

A previous study demonstrated that treatment with $MgSO_4$ prevented the damage of oligodendrocytes after the induction of cerebral ischemia-reperfusion in rats.[10] In this study, the treatment with $MgSO_4$ appeared to reduce the ovarian damage induced by treatment with Cyc.

The glycoprotein AMH is a member of the transforming growth factor-B (TGF-B) family, and its structure is similar to that of inhibin and activin.[16] Previous research has demonstrated that serum AMH is a sensitive marker of decreased ovarian reserve and apoptosis caused by cisplatin.[17] Human studies support the role of serum AMH levels as a reliable marker of ovarian reserves.[18] Thus, we used serum AMH levels as an indicator of ovarian reserves in the present study and validated our findings using histopathological scores or follicle counts. The findings from the serum AMH levels and tissue histopathological

scores were consistent. A study comparing serum AMH values before and after exposure to cisplatin-induced ovarian toxicity reported decreased ovarian reserves following drug exposure.[19] In the present study, wherein serum AMH levels were compared between groups following Cyc-induced toxicity, the serum AMH values were lower in the rats exposed to Cyc, as compared to those in the other groups.

As noted earlier, chemotherapeutic agents are effective against rapidly dividing cells. Although this is beneficial while combating malignancy, it has negative consequences for rapidly dividing healthy cells, which are also killed by chemotherapeutic drugs. By reducing the number of follicles, these drugs result in a decrease in the ovarian reserve. As a result, ovarian failure and infertility may occur. The effects of chemotherapeutics on the ovaries are dose-dependent, and they mainly affect the number of primordial follicles. Chemotherapeutics exert primary (direct follicle damage) and secondary (decrease in the number of antral follicles) effects on the ovarian reserve. Thus, an actual decrease in the ovarian reserve is not apparent until a later period during which secondary effects occur. In a previous study, the numbers of primordial and preantral follicles were significantly lower in a cisplatin-treated group as compared with controls, whereas there was no significant difference in the number of antral follicles between the groups.[19] In the present study, although the decrease in follicle number was most apparent in the primordial follicles, there was a statistically significant decrease in all the follicle types in the Cyc-administered group compared to that in the control group. This difference may be attributed to the greater ovarian toxicity induced by Cyc compared to cisplatin. Atli et al. evaluated ovarian changes induced by cisplatin and resveratrol in rats that received a low dose of resveratrol and saline for 21 days.[20] On day 15, the animals in both groups received a single dose of cisplatin. In the resveratrol group, the numbers of primordial and preantral (primary) follicles were significantly higher than those in the saline group. In the resveratrol group, the numbers of primordial and preantral (primary)

follicles were significantly higher than those in the saline group, indicating that resveratrol reversed the toxic effects of cisplatin. Thus, resveratrol could also be a potential agent to reverse the cytotoxic effects of chemotherapeutics.[20]

CONCLUSION

In conclusion, our findings indicate that $MgSO_4$ appears to have a protective effect and may even be able to reverse the damage caused by Cyc to the ovaries. However, the results need to be supported further by more extensive studies on animal models as well as humans.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: İlkin Yeral, *Design:* İlkin Yeral, *Control/Supervision:* İlkin Yeral, *Data Collection and/or Processing:* İlkin Yeral, Cemile Dayangan Sayan, *Analysis and/or Interpretation:* İlkin Yeral, Cemile Dayangan Sayan, *Literature Review:* İlkin Yeral, *Writing the Article:* İlkin Yeral, *Critical Review:* İlkin Yeral, *References and Fundings:* İlkin Yeral, *Materials:* İlkin Yeral.

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