Rosmarinic Acid Alleviated Cyclophosphamide Induced Gonadal Toxicity in Adult Male Rats

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Abstract

Aim: This study aimed to investigate the potential protective effects of rosmarinic acid (RA) against cyclophosphamide (CP)-induced gonadal toxicity in male Wistar Albino rats. Specifically, the research focused on the modulation of apoptotic pathways, with an emphasis on Bax protein expression, and utilized bioinformatic analyses to elucidate the key molecular mechanisms and signaling pathways underlying the observed effects.

Methods: The experimental design consisted of four groups: Control (administered saline), RA (administered rosmarinic acid), CP (administered cyclophosphamide), and RA+CP (administered a combination of rosmarinic acid and cyclophosphamide). Following a 14-day treatment period, body weight, serum malondialdehyde (MDA) levels, and Bax protein expression in testicular tissue were evaluated. Additionally, a protein-protein interaction (PPI) network influenced by RA and CP was constructed using STITCH and subsequently analyzed in Cytoscape. Functional enrichment analysis was performed to identify key molecular pathways associated with Bax regulation, with an emphasis on clusters exhibiting significant associations ($p < 0.05$) for enhanced interpretability.

Results: In the CP group, a significant reduction in body weight was observed, alongside elevated serum malondialdehyde (MDA) levels, indicative of heightened oxidative stress, and increased Bax protein expression, reflecting enhanced apoptotic activity. In contrast, the RA+CP group exhibited preservation of body weight, reduced Bax expression, and lowered MDA levels, closely resembling the profiles of the control group. Bioinformatic analyses revealed that CP predominantly activated molecular pathways associated with oxidative stress, apoptosis, and lipid metabolism. In comparison, RA treatment modulated pathways involved in mitochondrial protection, endoplasmic reticulum (ER) stress response, and the regulation of cytochrome c release, highlighting its potential protective role.

Conclusions: This study demonstrates that the antioxidant and anti-inflammatory properties of rosmarinic acid (RA) significantly mitigate cyclophosphamide (CP)-induced gonadal toxicity in male rats. The protective effects of RA are evident in its ability to preserve body weight, reduce oxidative stress, and suppress Bax protein expression, a key marker of apoptosis. Furthermore, in-silico analyses confirm that RA exerts its protective effects by modulating critical apoptotic pathways, specifically through the inhibition of Bax expression and the reduction of oxidative stress. These findings underscore the potential of RA as a therapeutic agent to prevent CP-induced gonadal damage, offering promise for its future application in protecting against chemotherapy-related reproductive toxicity.

Keywords: *Bax expression; cyclophosphamide; gonadal toxicity; rosmarinic acid.*

1. Introduction

Cyclophosphamide (CP) stands out as a highly successful anticancer drug, continuing to be utilized even 50 years after its synthesis. Widely employed in chemotherapy, blood, and bone marrow transplantation procedures, CP exhibits a broad range of clinical applications[1]. Despite its efficacy, CP is associated with reproductive toxicity in both humans and experimental animals[2]. Adverse effects include reduced gonad weight, impaired spermatogenesis, azoospermia, oligospermia, and significant abnormalities in the repro-

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ductive system[3]. Histological changes, particularly in the seminiferous tubule epithelium, have been observed following CP exposure, which may lead to degeneration and cell losses in spermatogenesis[4]. The exact cause of CP-induced gonadal toxicity remains unclear, but studies indicate that severe oxidative and nitrative stress, inflammation, apoptosis, and genomic changes play pivotal roles. Long-term exposure to CP has been linked to male infertility, various reproductive dysfunctions, and oncogenic effects[5]. The therapeutic and toxic effects of the drug are primarily dependent on hepatic metabolism, where the cytochrome P450 mixed-function oxidase system generates active metabolites, including phosphoramide mustard and acrolein[6]. Elangovan et al. have reported that the administration of a high dose of cyclophosphamide to the testes may lead to permanent functional impairments[7]. Natural antioxidants, like Rosmarinic Acid (RA), found in plants of the Lamiaceae family, have demonstrated potential in reducing CP toxicity. RA acts as a free radical scavenger, exhibiting antiviral, antibacterial, and immunomodulatory properties[8,9]. RA is widely used in food preservation, cosmetics, and the medical field due to its antimicrobial and antioxidant activities. Experimental studies indicate RA's protective effects in conditions such as Alzheimer's, wound healing, and renal ischemia-reperfusion damage[10,11]. Additionally, RA has been shown to significantly increase serum testosterone levels in rats, emphasizing its potential impact on reproductive functions[12,13].

In conclusion, cyclophosphamide (CP)-induced gonadal toxicity remains a significant clinical concern, prompting the investigation of antioxidants, particularly rosmarinic acid (RA), as potential mitigators of these adverse effects. This study aims to provide a comprehensive evaluation of RA's protective role against CP-induced reproductive toxicity, with a particular emphasis on histological alterations and functional impairments in the male reproductive system. By exploring RA's potential to counteract CP-induced gonadal damage, this research seeks to contribute valuable insights that may guide the development of therapeutic strategies to alleviate the reproductive side effects associated with CP treatment.

2. Materials and Methods

2.1. Experimental design

The Animal Ethics Committee approval, designated as 2020/16, was secured from the local ethics committee of Dicle University. Male Wistar Albino rats, aged 15-16 weeks and weighing between 200-240 grams, were obtained from the Dicle University Health Sciences Research and Application Center for the study. The study divided into four groups: Control (n=7), Rosmarinic Acid (RA, n=7), Cyclophosphamide (CP, n=7), and Rosmarinic Acid + Cyclophosphamide (RA+CP, n=7). The rats were housed in stainless steel cages under controlled conditions, maintaining a 12-hour light/dark cycle at a temperature of 22±2°C. Throughout the study, the rats had unrestricted access to both water and food. The experimental procedure lasted for 14 days, involving daily intraperitoneal injections. Comprehensive assessments through immunohistochemical and Western blot examinations. Additionally, biochemical analyses were conducted following specific protocols, ensuring a rigorous and consistent methodology throughout the study. The experimental design and durations were adapted from Alami et al.[14] and Sabik et al.[8] for consistency and referencing within the scientific literature.

2.2. Malonaldehyde (MDA) level analysis

MDA, a byproduct of cellular polyunsaturated fatty acid peroxidation, serves as an oxidative stress indicator. Post-experiment, rat blood samples underwent centrifugation, and plasma was stored at -80°C for subsequent MDA analysis. Thawed plasma was mixed with TCA and TBA, heated, and spectrophotometrically read at 532 nm. MDA values, calculated using extinction coefficients and dilution factors, underwent statistical analysis in SPSS 24.0, employing Anova and Post-Hoc Tukey and Games-Howell tests ($p \leq 0.05$).

2.3 Tissue processing for immunohistochemical staining

After sacrifice, rat testicular tissues underwent fixation in 10% neutral buffered formaldehyde (Catalog no: HT501128, 4L, Sigma, Germany) for 6 hours, followed by an additional 18 hours in clean neutral formalin. Post-fixation, tissues were rinsed in tap water for 12 hours to eliminate excess formalin. Sequential dehydration occurred in 50%, 70%, 80%, 90%, and 96% ethanol baths for 8 hours, concluding with a final step of 2x30 minutes in absolute alcohol. To remove residual alcohol, tissues underwent 2x15 minutes of xylene treatment. For infiltration, tissues were incubated in molten paraffin at 58°C for 3x30 minutes in an oven. Paraffin-embedded tissue blocks were then embedded at room temperature, and 5 µm thick sections were obtained using a Leica R52265 rotary microtome, mounted on positively charged slides. Sections were incubated in xylene for 15 minutes in two consecutive series, followed by treatment with decreasing alcohol concentrations (100%, 90%, 80%, 70%) for 5 minutes each. After a 2x15 minute distilled water rinse, sections underwent a 3-minute antigen retrieval process in EDTA solution at 90°C using a microwave. Post-microwave, sections were incubated in Phosphate Buffer Saline (PBS) at room temperature for 20 minutes. Hydrogen peroxide block solution was applied for 20 minutes, followed by 2x5 minute PBS washes. Ultra V Block solution was then applied for 8 minutes. Subsequently, Bax primary antibody (Thermo fisher/PA5120029) was applied overnight at 4°C. The next day, sections were left at room temperature for 1 hour, followed by 2x5 minute washes. After washing, sections were incubated with a secondary antibody for 14 minutes. Following 2x5 minute PBS washes, sections underwent enzyme binding with Streptavidin peroxidase for 15 minutes. Further washes were performed, and sections were subjected to a DAB chromogen reaction. Specific reaction sections were collected in PBS, followed by counterstaining with Mayer's hematoxylin, dehydration, xylene treatment, and mounting with Entellan. Prepared slides were examined using a Zeiss Imager A2 light microscope and Zen 3.00 software.

2.4. Western blot protocol

Testicular tissue lysates, frozen at -80°C, were processed for protein analysis using the Smart BCA assay. In the Western blot laboratory, resolver and stacker gels were prepared with the TGX Stain-Free™ FastCast™ Acrylamide Solution. Protein samples were loaded onto these gels for electrophoresis. Following gel electrophoresis, proteins were transferred onto a PVDF membrane. Subsequent steps, including antibody incubation and imaging, were carried out using the Bio-Rad ChemiDOC MP system.

2.5. Statistical analysis and Bioinformatics Approaches

Rat weights were measured pre-experiment, and post-experiment weight checks were conducted for rats receiving the appropriate dose (mg/kg). Prior to the experiment, rat weights were measured. Prior to the experiment, rat weights were measured. Following administration of the appropriate dose (mg/kg), post-experiment weight assessments were conducted.

Statistical analysis was done using the IBM SPSS 25.0 software (IBM, Armonk, New York, US). The data were recorded as median (minimum-maximum). Normality of the data distribution was evaluated with the Shapiro-Wilk test. Group comparisons were done with the Kruskal Wallis and post hoc Mann- Whitney U test. Significance was considered for $p \leq 0.05$. The number of animals for each group was calculated by G Power analysis (version 3.1).

3. Results

3.1 Statistical Findings and Bioinformatics Findings in Evaluating Toxicity

In the pre-experiment phase, rat body weights were measured, and doses were administered. Post-application, body weights were re-measured. The control group showed no significant difference in weights before (237±11.02 g) and after (232±14.07 g) the experiment (p>0.05). The RA group also exhibited no significant difference in weights before (211±7.02 g) and after (204±10.03 g) the experiment (p>0.05). However, the CP group displayed a significant difference in weights before (207±3.02 g) and after (149±11.02 g) the experiment (p≤0.01), indicating a significant decrease. In contrast, the RA+CP group showed no significant difference in weights before (213±5.02 g) and after (199±13.09 g) the experiment (p>0.05). CP significantly decreased the animal's weight, but RA treatment mitigated this effect, helping to maintain the animal's wellbeing (**Fig. 1.**and **Table 1.** show the results).

Average animal weights before and after the experiment were shown in **Table 1**. A significant decrease in animal weight is observed following the administration of CP. However, in the RA+CP group, the RA treatment mitigates this weight loss, preventing significant reductions in body weight.

Figure 1

Graphical representation illustrates the variations in animal weights before and after the initiation of the experiment. The chart presents a comparative analysis of the average weights of rats within each group, both prior to and following the experimental intervention.

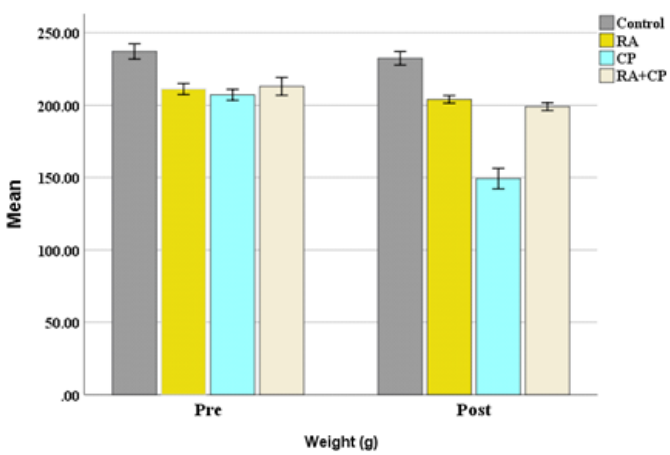


Table 1

Pre and post experiment animal weights

Groups	Pre-experiment	Post-Experiment	p
Control	237±11.02	232±14.07	0.067
RA	211±7.02	204±10.03	0.081
CP	207±3.02	149±11.02	0.001
RA+CP	213±5.02	199±19.09	0.002

3.2. MDA Analysis

Serum Malondialdehyde (MDA) concentrations in rats were measured as follows: 1.2 ± 0.03 nmol/ml for the control group, 1.3 ± 0.2 nmol/ml for the RA group, 2.5 ± 0.9 nmol/ml for the CP group, and 1.7 ± 0.03 nmol/ml for the CP+RA group. The difference in MDA levels between the control and RA groups was not statistically significant (p>0.05). However, a significant increase was observed in the CP group compared to the control group (p≤0.01). Additionally, there was a statistically significant reduction in MDA levels in the RA+CP group compared to the CP group (p<0.05). MDA levels increased after CP induction compared to the control group, but RA treatment significantly reduced MDA concentrations compared to the CP group (**Fig. 2.** and **Table 2**).

3.3 Immunohistochemical findings.

Examination of Bax immune stained testicular sections were show in in **Figure 4**. Control and RA group showed negative Bax expression in the seminiferous tubules, spermatogonia and spermatid cells (**Fig. 3a** and **3b**, respectively). In the CP group, positive Bax expression was observed in spermatogenic cells and in interstitial cells (**Fig. 3c**). In the RA+CP group, Bax expression was reduced in the seminiferous tubule structures. Bax expression was negative in Sertoli cells and in the interstitial connective tissue areas (**Fig. 3d**).

Figure 2

Graphical representation of Serum Malondialdehyde (MDA) concentrations by Group.

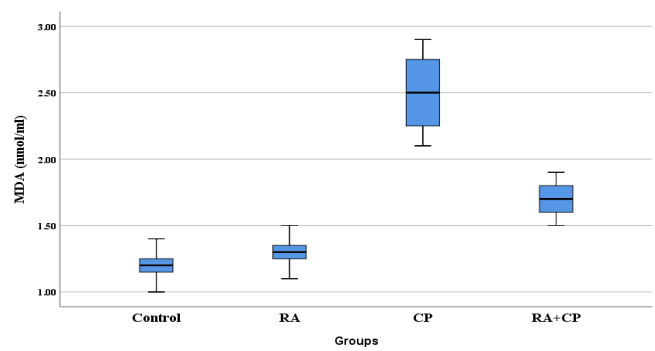


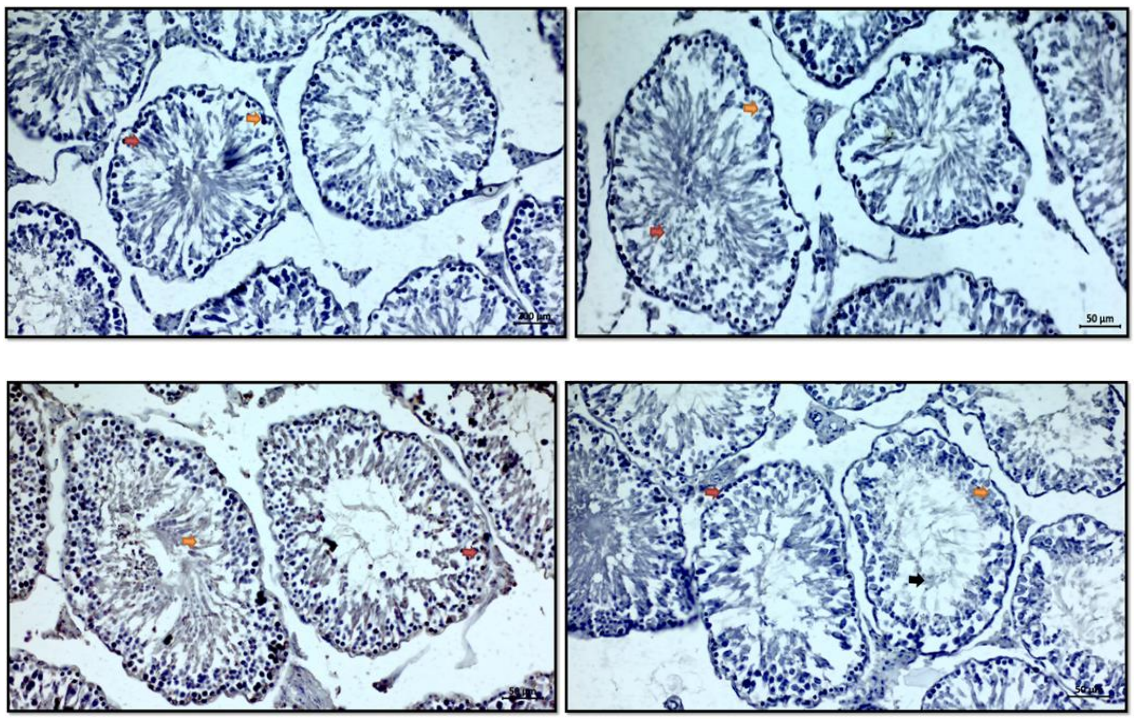
Table 2

The MDA values for each group. Following CP induction, MDA levels increased compared to the control group. However, RA treatment significantly reduced MDA content in comparison to the CP group (* Control vs CP, **CP vs RA+CP).

Groups	MDA (nmol/ml)	p
Control	1.2 ± 0.03 (1.0-1.4)	
RA	1.3 ± 0.2 (1.1-1.5)	
CP	2.5 ± 0.9 (2.1-2.9)	0.002*
RA+CP	1.7 ± 0.03 (1.5-1.9)	0.001**

Figure 3

Bax immunostained testicular sections.



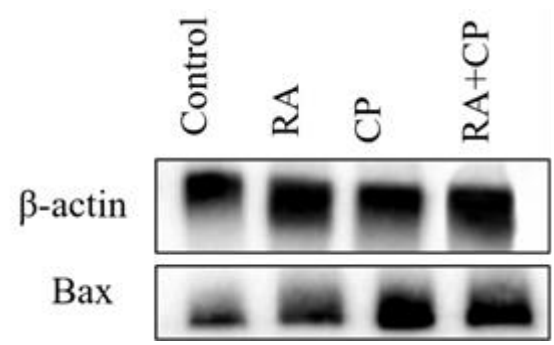
A) *Control group, negative Bax expression in spermatogonia cells (orange arrow) and spermatid cells (red arrow),*
B) *RA group, negative Bax expression in Sertoli cells (orange arrow) and spermatid cells (red arrow);*
C) *intense Bax expression in spermatogonia cells (red arrow) and spermatid cells (orange arrow);*
D) *CP+RA group, negative Bax expression in spermatogonia cells (red arrow) and spermatid cells (black arrow). Bax immunohistochemistry; Scale bar: 50 µm*

3.4. Western Blot Results

Protein bands of Bax in testicular tissues per group was visualized in **Figure 4**. β-Actin was used as a positive control. The CP group showed a significantly increased band thickness compared to the control group and RA group, indicating elevated Bax expression. In the comparison of Bax expression between the CP and RA+CP groups, the CP group demonstrated higher Bax levels, whereas Bax protein levels were notably down-regulated in the RA+CP group. These findings suggest that CP treatment leads to increased Bax expression, as evidenced by the thicker bands, while RA+CP treatment results in reduced Bax expression. (**Fig. 5.**). Bioinformatic analyses revealed that CP primarily activated pathways related to oxidative stress, apoptosis, and lipid metabolism, all critical to chemotherapy-induced damage. Specifically, pathways such as "oxidation by cytochrome P450," "apoptosis," and "response to oxidative stress" were significantly enriched upon CP-treatment (**Figure 5.-A**). Conversely, RA treatment predominantly influenced pathways related to mitochondrial protection, ER stress response, and regulation of cytochrome c release from mitochondria, including "regulation of release of cytochrome c," "response to hypoxia," and "ER stress response." (**Figure 5**).

Figure 4

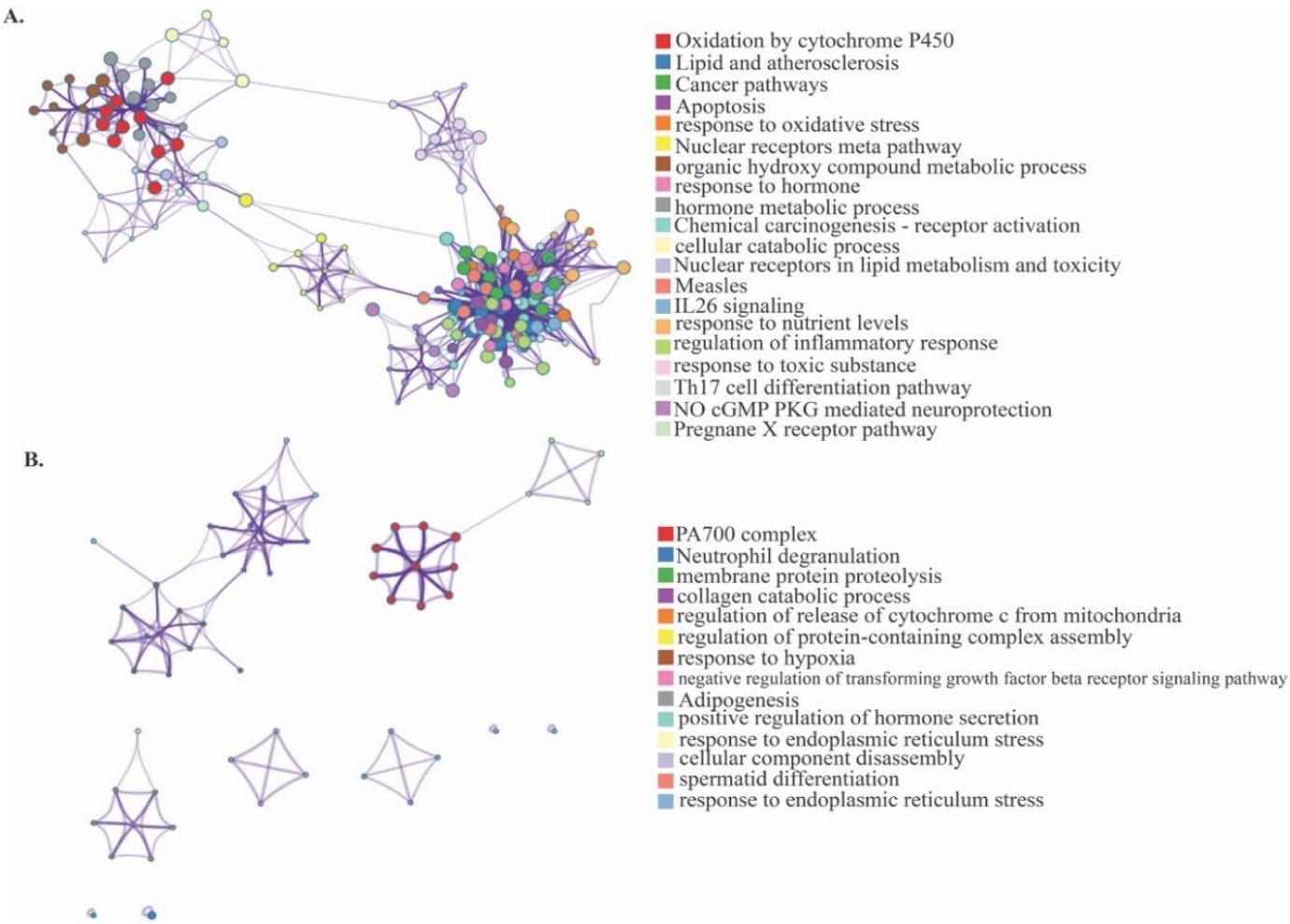
Bax Protein Levels Across Groups



In the CP group, there was a significant increase in Bax protein levels compared to the control group and RA group. RA treatment decreased Bax band intensity in the RA+CP group.

Figure 5

Protein-protein interaction (PPI) network and functional enrichment analysis of genes affected by Cyclophosphamide (CP) and Rosmarinic Acid (RA). A. PPI network of CP-affected genes and the top 10 statistically significant pathways influenced by CP. B. PPI network of RA-affected genes and the top 10 statistically significant pathways influenced by RA. Pathway annotations are listed from top to bottom in order of increasing p-values. Statistical significance was set at $p < 0.05$.



4. Discussion

The Cyclophosphamide (CP), a phosphoramide phosphoramide mustard derivative, is a widely used alkylating agent known for its antineoplastic and immunosuppressive properties. It is commonly administered alongside other chemotherapeutic agents in the treatment of various cancers such as malignant lymphomas, breast cancer, ovarian carcinoma, and myeloblastoma. Additionally, CP is employed in immunosuppressive therapy to prevent graft rejection and treat chronic autoimmune disorders, including rheumatoid arthritis and myasthenia gravis. CP acts by interfering with cell growth and differentiation, particularly affecting rapidly proliferating tissues like the gonads. While effective as a therapeutic agent, it also has notable toxic effects on various organs and tissues. At high doses (greater than 50 mg), over 65% of patients experience nausea and vomiting within approximately 12 hours of administration. Between 5% and 30% of patients undergoing CP treatment experience significant hair loss. Experimental studies in animal models have also confirmed CP's toxicity and teratogenicity, indicating its adverse effects on fetal development. In patients, CP can induce cystitis, with the incidence of this condition increasing with the dosage. CP, along with cumulative dosage and patient age, is also associated with an increased risk of early menopause in women and infertility in men. Pathological examinations reveal ovarian atrophy, fibrosis, and complete absence of follicular structures as key histological features in women. In men, prolonged CP treatment can result in irreversible azoospermia, with significant degeneration of seminiferous tubules and Sertoli cells, signaling extensive gonadal damage. In some cases, CP treatment in women leads to irreversible amenorrhea[15-17]. Trasler et al. evaluated the effects of cyclophosphamide (CP) on male Sprague-Dawley rats by administering low (5.1 mg/kg/day) and high doses (6.8 mg/kg/day) for up to 9 weeks. Sig-

nificant reproductive toxicity was observed, with oligospermia and azoospermia detected at both doses after 6 weeks. Histological and biochemical analyses confirmed substantial testicular damage, emphasizing the detrimental impact of CP on male fertility[18]. Hoorweg-Nijman et al. examined 23 male patients (ages 14.8–28.8) and found that cyclophosphamide (CP) treatment disrupted gonadotropin secretion, leading to decreased testosterone levels and testicular damage[19].

Aguilar-Mahecha et al. treated adult male rats with cyclophosphamide (70 mg/kg, i.p.) and compared them to a control group (saline solution, i.p.). Sixteen hours after injection, CP specifically disrupted the expression of stress response genes in germ cells during spermatogenesis[20]. Tımar et al. studied the effects of cyclophosphamide (CP) and saponin (SP) in 40 male mice divided into four groups. CP (15 mg/kg/week, i.p.) caused significant reductions in sperm viability, count, and normal morphology, alongside increased DNA fragmentation and malondialdehyde (MDA) levels. SP administration (2.5 mg/kg/day, i.p.) mitigated these effects, improving sperm parameters and enhancing antioxidant capacity[21]. Sabik et al. investigated the protective effects of vitamin E and ginger against cyclophosphamide (CP)-induced gonadal toxicity in 44 male rats. CP was administered at 20 mg/kg body weight for 14 days. Both the vitamin E + CP and ginger + CP groups had significantly higher testis weights compared to the CP group. The CP group showed the highest malondialdehyde (MDA) levels and the lowest testosterone levels. Testosterone levels were significantly higher in the vitamin E + CP and ginger + CP groups. Histopathological analysis revealed reduced spermatogonial cell death and apoptosis in these groups, demonstrating the protective effects of vitamin E and ginger[8].

Exposure to CP during chemotherapy, both before and after puberty, leads to abnormal sperm parameters. Higher CP doses are associated with an increased risk of infertility. In a study of 17 males, azoospermia was found in 58.8%, oligospermia in 29.4%, and normal sperm count in 11.8%[22]. High doses of CP can lead to azoospermia and hormonal disturbances. In a study of 31 male patients with Behçet's disease, CP treatment was found to increase the risk of infertility. These findings highlight the significant damage CP can cause to the male reproductive system[23]. Similar findings have been reported in studies involving other populations. These studies collectively emphasize the significant damage CP can cause to the male reproductive system[24,25]. Rocha et al. reported a 60% reduction in edema and inflammation in rats treated with 25 mg/kg RA[26]. Roland et al. demonstrated that RA provided protection against skin cancer[27]. Boonyarikpunchai et al. showed that RA, when administered at doses of 100 and 150 mg/kg, prevented both chronic and acute inflammation[28]. Our study aimed to explore the protective effects of RA against CP-induced testicular toxicity. CP is a commonly used alkylating agent known for its cancer-fighting and immune-suppressing properties. It is frequently used to treat various cancers, including lymphoma, breast cancer, ovarian cancer, and myeloblastoma, and to suppress the immune system in conditions such as rheumatoid arthritis and myasthenia gravis. Despite its therapeutic effectiveness, CP has been associated with several toxic effects, particularly on rapidly dividing cells, such as those in the gonads. These effects include nausea, vomiting, hair loss, and more serious reproductive issues, such as gonadal damage, which can ultimately lead to infertility. In light of this, our study aimed to determine whether RA, a compound known for its diverse biological activities, could mitigate the toxic effects of CP on the testes. To assess the protective potential of RA, we evaluated several parameters. To begin with, body weight changes were evaluated in the RA+CP group in comparison to the CP-only group. No significant difference in body weight was observed between pre- and post-experiment measurements within the RA+CP group ($p>0.05$), suggesting that RA may have prevented significant weight loss commonly associated with CP treatment (**Fig. 1**). Furthermore, to investigate the impact of RA on CP-induced oxidative damage, we measured malondialdehyde (MDA) levels, a biomarker of oxidative stress. The MDA levels were significantly lower in the RA+CP group (1.7±0.03 nmol/ml) compared to the CP group (2.5±0.9 nmol/ml), with a statistically significant difference ($p<0.05$) (**Fig. 2**). This reduction in MDA levels suggests that RA possesses antioxidative properties, potentially contributing to its protective role against CP-induced oxidative stress. Additionally, immunohistochemical analysis was performed to examine Bax expression, a pro-apoptotic marker, in the testes. In the RA+CP group, Bax expression was found to be absent in both seminiferous tubules and intertubular connective tissue (**Fig. 4**). This result indicates that RA treatment effectively inhibited Bax expression, suggesting a protective effect against CP-induced apoptosis. Finally, Western blot analysis was conducted to quantify Bax protein levels and further investigate the impact of RA on Bax expression. The results demonstrated a significant downregulation of Bax expression in the RA+CP group compared to the CP-only group (**Fig. 5**). This downregulation of Bax protein supports the hypothesis that RA attenuates CP-induced apoptosis in testicular tissues.

Our study demonstrates that rosmarinic acid (RA) provides significant protection against cyclophosphamide (CP)-induced gonadal toxicity in rats by modulating key apoptotic pathways. CP treatment led to a substantial increase in Bax protein expression, indicating an enhanced apoptotic response in testicular tissues. Bax, a pro-apoptotic protein, facilitates the release of cytochrome c from mitochondria, thereby initiating the intrinsic apoptotic pathway[29]. This upregulation of Bax is consistent with CP's well-established role in inducing oxidative stress and DNA damage, both of which activate apoptotic signaling cascades[30]. In contrast, RA treatment effectively counteracted the CP-induced upregulation of Bax, suggesting that RA protects against apoptosis by inhibiting Bax expression. This protective effect is likely due to RA's well-documented antioxidant and anti-inflammatory properties[31]. Further supporting this hypothesis, our bioinformatic analysis revealed that RA regulates several critical pathways related to the suppression of oxidative stress and endoplasmic reticulum (ER) stress—both of which are key contributors to Bax activation and apoptosis. Specifically, RA modulated pathways associated with cytochrome c release from mitochondria and ER stress, both of which are crucial players in apoptotic signaling. On the other hand, CP-induced toxicity was associated with the upregulation of oxidative stress and lipid peroxidation pathways, such as "cytochrome P450" and "response to oxidative stress." These findings align with the known role of CP in generating reactive oxygen species (ROS) through its metabolism, which leads to oxidative damage in gonadal tissues[32]. The observed increase in Bax expression is likely a direct consequence of CP-induced ROS production, as oxidative stress is a well-known trigger for the mitochondrial apoptotic pathway. Taken together, our findings suggest that RA mitigates CP-induced gonadal toxicity by modulating Bax expression and regulating key apoptotic pathways. By alleviating oxidative stress and maintaining mitochondrial integrity, RA emerges as a promising therapeutic strategy for protecting against chemotherapy-induced gonadal damage.

5. Conclusion

RA effectively mitigates CP- induced gonadal toxicity by modulating key apoptotic pathways, particularly through the inhibition of Bax protein expression and the reduction of oxidative stress. RA's antioxidant and anti-inflammatory properties contribute to preventing apoptosis in testicular tissues. These findings position RA as

a potential therapeutic agent for protecting against chemotherapy-induced gonadal damage.

Statement of ethics

Ethical approval was obtained from the Dicle University Animal Experiments Local Ethics Committee (ethical approval number: 2020/16).

Source of Finance

This study was supported Research Projects (DUBAP) with the by Dicle University Scientific project number TIP.21.003

Conflict of interest statement

The authors declare that they have no conflict of interest.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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