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Resveratrol May Reduce Apoptosis due to Doxorubicin-Induced Hepatotoxicity by Regulating the Bax/BcL2 Ratio in Male Rats

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Objective: Doxorubicin (DX) is an antibiotic of the anthracycline group with chemotherapeutic effects, which is widely used in cancer treatment. However, its clinical use is limited due to liver toxicity. The main causes of DX hepatotoxicity are generally considered to be oxidative stress and apoptosis. In this study, it was aimed to investigate the antiapoptotic effect of Resveratrol (RSV), a powerful antioxidant, at different doses on DX-induced hepatotoxicity.

Materials and Methods: 42 Sprague-Dawley male rats were randomly divided into six groups (n=7). While the control group (n=7) was not administered, the DX group (n=7) was administered DX intraperitoneally (i.p) at a dose of 15 mg/kg once at the beginning of the experiment. DX+RSV I (n=7) and DX+RSV II (n=7) groups were administered DX ip at a dose of 15 mg/kg once at the beginning of the experiment followed by 1 and 5 mg/kg/day RSV ip, respectively. RSV I (n=7) and RSV II (n=7) groups were administered 1 and 5 mg/kg/day RSV i.p, respectively. The experiment was terminated on the 15th day.

Results: It was observed that DX administration caused biochemical and histopathological changes in the liver. It was observed that these changes were reduced/mitigated with RSV (5 mg/kg/day) treatment. It was determined that DX administration in liver tissue increased Bax and Casp3 immunoreactivity while decreasing BcL2 immunoreactivity.

Conclusion: RSV treatment was observed to regulate DX-induced altered apoptotic protein immunoreactivities. RSV, a dietary polyphenol, treatment may exert an antiapoptotic effect in DX-induced hepatotoxicity by regulating the Bax/BcL2 ratio.

Key Words: *Doxorubicin, resveratrol, hepatotoxicity, antiapoptotic*

Resveratrol, Erkek Sıçanlarda Bax/BcL2 Oranını Düzenleyerek Doksorubisin Kaynaklı Hepatotoksisiteye Bağlı Apoptozu Azaltabilir

Amaç: Doksorubisin (DX), kanser tedavisinde yaygın olarak kullanılan kemoterapötik etkileri olan antrasiklin grubu bir antibiyotiktir. Ancak karaciğer toksisitesi nedeniyle klinik kullanımı sınırlıdır. DX hepatotoksisitesinin ana nedenleri genellikle oksidatif stres ve apoptoz olarak kabul edilir. Bu çalışmada, güçlü bir antioksidan olan Resveratrol'ün (RSV) farklı dozlarda, DX kaynaklı hepatotoksisite üzerine antiapoptotik etkisinin araştırılması amaçlandı.

Gereç ve Yöntem: 42 Sprague-Dawley erkek sıçan rastgele altı gruba ayrıldı (n=7). Kontrol grubuna (n=7) uygulama yapılmazken, DX grubuna (n=7) deney başlangıcında 15 mg/kg dozunda intraperitoneal (i.p) DX uygulandı. DX+RSV I (n=7) ve DX+RSV II (n=7) gruplarına, deneyin başında bir kez 15 mg/kg dozunda DX ip uygulamanın ardından sırasıyla 1 ve 5 mg/kg/gün RSV ip uygulandı. RSV I (n=7) ve RSV II (n=7) gruplarına sırasıyla 1 ve 5 mg/kg/gün RSV i.p uygulandı. Deney 15. günde sonlandırıldı.

Bulgular: DX uygulamasının karaciğerde biyokimyasal ve histopatolojik değişikliklere neden olduğu gözlemlendi. Bu değişikliklerin RSV (5 mg/kg/gün) tedavisi ile azaldığı/hafiflediği gözlemlendi. Karaciğer dokusunda DX uygulamasının Bax ve Casp3 immünoreaktivitesini artırırken BcL2 immünoreaktivitesini azalttığı belirlendi.

Sonuç: RSV tedavisinin, DX kaynaklı değişen apoptotik protein immünoreaktivitelerini düzenlediği gözlemlendi. Bir diyet polifenol olan RSV ile tedavi, Bax/BcL2 oranını düzenleyerek DX'in neden olduğu hepatotoksisiteye karşı antiapoptotik etki gösterebilir.

Anahtar Kelimeler: *Doksorubisin, resveratrol, hepatotoksisite, antiapoptotik*

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Introduction

Doxorubicin (DX) or Adriamicin, a widely used chemotherapeutic agent, has serious side effects, including hepatotoxicity (1-3). Hepatotoxicity caused by DX is due to oxygen radical damage to hepatic cell membrane lipids. It has been reported to membrane lipid oxidation cause functional and structural changes that, hepatocyte necrosis or apoptosis and increase liver enzymes in the blood (4). Apoptosis plays an important role in many defense mechanisms, such as triggered immune response when a disease or harmful agents damage cells. There are two main apoptotic pathways, the extrinsic pathway, also known as the death receptor pathway, and the intrinsic pathway, also known as the mitochondrial pathway. Intrinsic and extrinsic paths can intersect

each other. These pathways initiate the activation of Caspase 3 (Casp3), which is the initiator of apoptosis and is characterized by degeneration of nuclear proteins and cytoskeletal, DNA fragmentation, apoptotic body formation, and antigen production for phagocytic cell receptors (5, 6). Studies have reported that DX administration can trigger apoptosis by inducing inflammation and oxidative stress in liver tissue (3, 7). Therefore, the use of antioxidants together with DX therapy may be an appropriate approach to minimize the toxic effects on healthy normal cells (4).

According to the studies published in the last few decades, the use of herbal and natural products or their derivatives has received a lot of interest to reduce side effects caused by chemotherapy or to increase the sensitivity of cancer cells to chemotherapy drugs (8). The anticancer effect of Resveratrol (RSV), a polyphenolic compound; It shows by activating or inhibiting various cellular targets that induce apoptosis, inhibit cell growth, prevent metastasis or block angiogenesis (9). Studies have shown that RSV can induce apoptosis in different cancer cells (10, 11). In addition to its apoptotic activities, this herbal agent can protect normal cells/tissues by showing antiapoptotic effects. Many studies have shown that the combined treatment of RSV and DX reduces the apoptosis level of heart cells compared to the groups treated with DX alone (12, 13).

Therefore, in this current study, we targeted to examine the protective activity of RSV against liver damage caused by DX, based on its protective effects and various biological properties. With the aim of determining the hepatoprotective effect of RSV in DX-induced hepatotoxicity, biochemical parameters, histopathological changes and immunohistochemically apoptotic protein immunoreactivities were investigated.

Materials and Methods

Research and Publication Ethics: This study was carried out taking into account the national and international guidelines on the use of experimental animals. The study was confirmed by Fırat University Animal Experiments Local Ethics Committee (05.07.2021-2832).

Experimental Design: RSV (Sigma-Aldrich, USA) and DX (Saba Farma, Turkey) were purchased from commercial companies. Lyophilized DX was reconstituted with sterile water for injection to acquire a final concentration of 2 mg/ml. Optimal conditions (12 hours light/dark, 22-25 °C, add-libitum water and feed) were provided for the experimental animals. 42 Sprague-Dawley male rats (10 weeks, 220±20 g weight) obtained from XXXX Experimental Research Unit were randomly divided into six groups (n=7). Only add-libitum feed and water was given to the control group (n=7). In the DX group (n=7), 15 mg/kg DX was applied intraperitoneally (i.p) once at the beginning of the experiment (14). DX+RSV I (n=7) and DX+RSV II (n=7) groups were administered DX i.p at a dose of 15 mg/kg once at the beginning of the experiment, after that 1 and 5 mg/kg RSV (15) i.p was administered daily for 14 days respectively. In the RSV I (n=7) and RSV II (n=7) groups, 1 and 5 mg/kg RSV i.p were administered daily for 14 days, respectively. On the 15th day, the rats were decapitated under anesthesia (xylazine and ketamine), and the experiment was terminated. Blood serum samples taken for biochemical analyzes were tucked away at -80 °C until the study day and were dissolved only once. Rapidly removed liver tissues were placed in 10% formalin solution for histopathological and immunohistochemical evaluations, and fixation was performed.

Histopathological Assessment: Liver tissues fixed in formalin solution (10%) were subjected to routine histological follow-up series. Sections of 5 µm thickness were taken from the tissues embedded in paraffin blocks. After the sections were paraffinized with xylene, they were passed through serial alcohol series. Hematoxylin Eosin staining procedure was applied for general histopathological evaluations. Preparations were examined and photographed with a light microscope (DM2500 LED, Leica, Germany). Histopathological changes in the liver tissue were evaluated and scored. Histopathological score criteria; sinusoidal dilatation, vacuolization, inflammatory cell focus, mononuclear cells increase and degenerated hepatocyte cords. Histopathological histoscore table was created by giving a value between 0 and 4 (0; none, 1; 0-25%, 2; 26-45%, 3; 46-75% and 4; 76-100%) according to the presence of the criteria (6).

Immunohistochemical Evaluation: Bax (sc–7480, Santa Cruz, USA), B-cell lymphoma 2 (BcL2) (201r.5304, SunRed, China) and Casp3 (bs0081R, Bioss Inc, China) immunoreactivities in liver tissues, Avidin-Biotin-Peroxidase Complex method applied according to the procedure described previously (16). All tissues were counter-stained with Mayer's Hematoxylin. Preparations were examined and photographed under a light microscope (DM-2500, Leica, Germany). Immunohistochemical evaluation of the prevalence of immunoreactivity (0.1: <25%, 0.4: 26-50%, 0.6:51-75%, 0.9:76-100%) and severity (0:none, 0.5:very little, 1: little, 2: moderate, 3: severe). Histoscore was formed according to immunoreactivity=prevalencexseverity (16). The ratio of Bax/BcL2=Bax immunoreactivity/BcL2 immunoreactivity was calculated.

Biochemical Analyzes: Serum Alanine transaminase (ALT), Albumin and Aspartate transaminase (AST) levels were measured using automatic biochemical analyzer (ADVIA 2400 Siemens) and kits.

Statistical Analysis: The obtained data were analyzed using the Statistical Package for Social Sciences (SPSS) 22.0 package program. Data were presented as mean±standard error. Krusskall Wallis H followed by post-hoc Dunn multiple comparison test was used for statistical analysis of the results. A value of $p<0.05$ was considered statistically significant. GraphPad Prism 9.3.1 software was used for graph drawings.

Results

In the histopathological examination of liver tissues, it was observed that the control group and RSV (I and II) groups had normal histological structures (Figure 1; a, e, f). Vacuolization, inflammatory cell foci, increased mononuclear cells in the central and preportal areas, sinusoidal dilatation, vascular congestion, hemorrhagic areas and hepatocyte degenerations were detected in the DX group (Figure 1; b). The histopathological changes induced by DX were partially reduced in the RSV I group (Figure 1; c). On the other hand, in the group receiving 5 mg/kg/day RSV treatment (RSV II), it was observed that almost all histopathological findings improved (Table 1, Figure 1; d).

Bax immunoreactivity was similar in the control, RSV I and RSV II groups (Figure 2A; a, e, f). It was observed that Bax immunoreactivity was significantly increased in liver tissues with DX administration (Figure 2A; b). 5 mg/kg/day RSV treatment relatively decreased Bax immunoreactivity, which was increased by DX (DX+RSV II group compared to DX group, p=0.066, Figure 2A; d).

BcL2 immunoreactivity in the RSV I and RSV II groups was similar to the control group (Figure 2B; a, e, f). BcL2 immunoreactivity was found to be significantly decreased in the DX group (p<0.001) (Figure 2B; b). The DX+RSV I group showed similar BcL2 immunoreactivity as the DX group. A significant increase in BcL2 immunoreactivity was observed in the DX+RSV II group compared to the DX group (p<0.001) (Table 1, Figure 2B; d).

The Bax/BcL2 ratio was similar in the control, RSV I and RSV II groups. It was observed that the Bax/BcL2 ratio increased significantly in the DX group compared to the control group (p<0.001) (Table 1). Although this rate decreased partially in the DX+RSV I group compared to the DX group, this was not statistically significant (p>0.05) (Table 1). In the DX+RSV II group, the Bax/BcL2 ratio was found to be significantly decreased compared to the DX group (p=0.045) (Table 1).

Table 1 Effect of DX and/or RSV applications on liver tissue histopathological changes, Bax, BcL2 and Casp3 immunoreactivities and Bax/BcL2 ratio

	Control	DX	DX+RSV I	DX+RSV II	RSV I	RSV II	P
Histopathological histoscore	0.14±0.14	3.28±0.18[a]	2.57±0.2[a]	1.42±0.20[a,b]	0.28±0.18[b]	0.28±0.18[b]	<.001
Bax	0.12±0.18	1.62±0.11[a]	1.31±0.13[a]	0.74±0.03[a,b]	0.15 ±0.04[b]	0.11±0.02[b]	<.001
BcL2	1.27±0,14	0.54±0.03[a]	0.62±0.06[a]	1.24±0.15[b]	1.04±0.07[b]	1.21±0.16[b]	<.001
Bax/BcL2	0.10±0.01	3.07±0.27[a]	2.19±0.24[a]	0.87±0.17[a,b]	0.16±0.05[b]	0.25±0.15[b]	<.001
Casp3	0.21±0.05	2.44±0.16[a]	1.80±0.25[a]	0.58±0.06[a,b]	0.22±0.06[b]	0.24±0.05[b]	<.001

Values are presented as mean ± standard error. [a]: Compared with the Control group, [b]: Compared with the DX group (p<0.05). DX; Doxorubicin, RSV; Resveratrol, Casp3; Caspase 3.

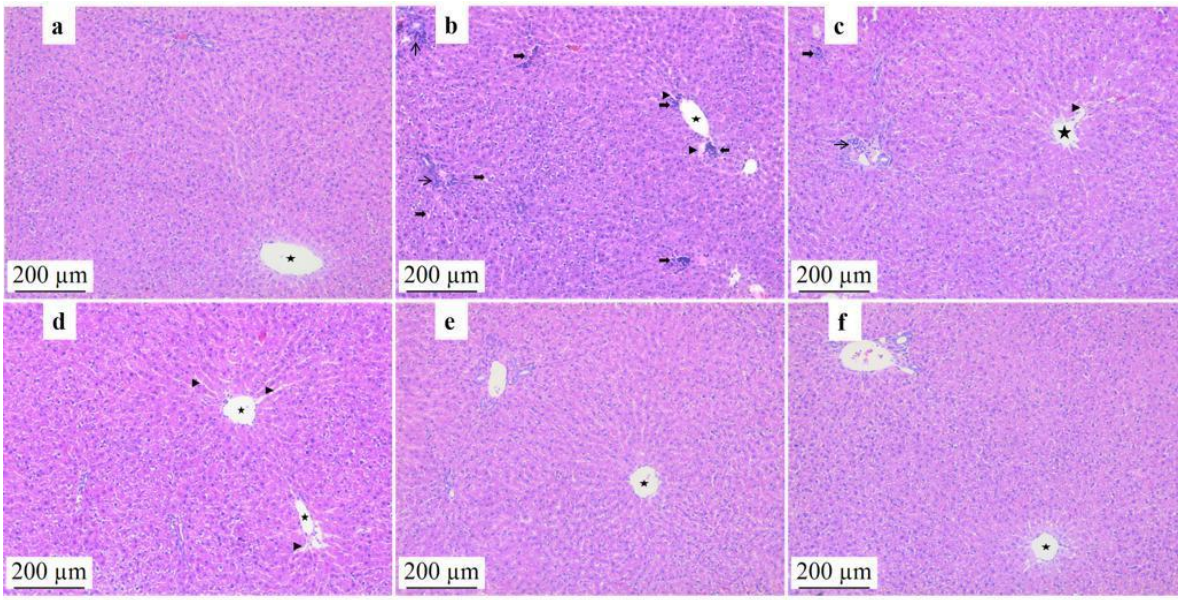


Figure 1. Effects of RSV administrations on histopathological changes in DX-induced liver tissue. Control (a), RSV I (e) and RSV II (f) liver tissues had normal histological appearance. DX group (b); mononuclear cells increasing in the preportal area (thin arrow), sinusoidal dilatation (triangle), vacuolization (notched arrow), inflammatory cell focus (thick arrow). DX-induced histopathological changes were alleviated by dose-dependent RSV applications (DX+RSV I (c), DX+RSV II (d)). Central vein (star), Hematoxylin Eosin, scale bar: 200µm, x100. DX; Doxorubicin, RSV; Resveratrol

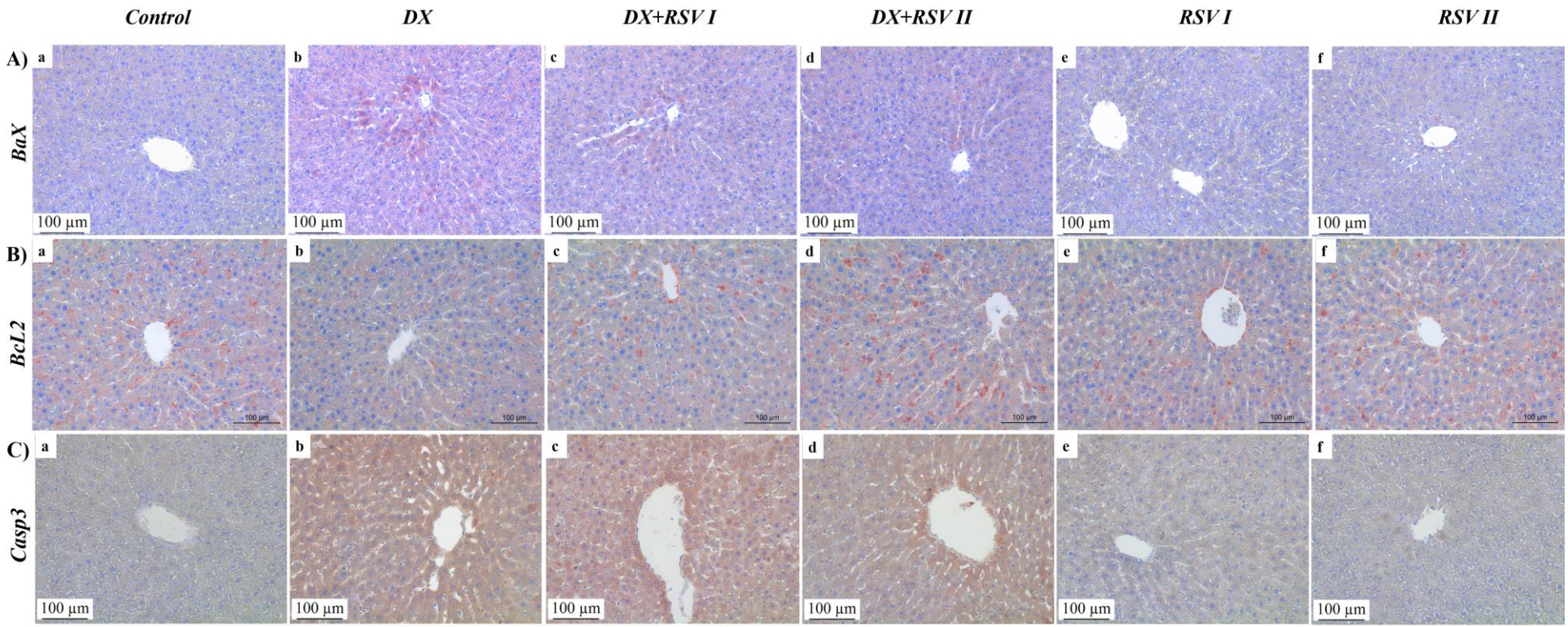


Figure 2 Effects of DX and/or RSV applications on the Bax, BcL2 and Casp3 immunoreactivities of the liver tissue. Liver tissues in the Control, RSV I and RSV II groups were observed to have similar Bax, BcL2 and Casp3 immunoreactivities ($p>0.05$) (A, B, C; a, e, f). DX group (A; b) Bax immunoreactivity was observed to be significantly increased ($p<0.05$) compared to the Control group. It was observed that Bax immunoreactivity in the DX+RSV II group (A; d) was relatively decreased compared to the DX group ($p>0.05$). In the DX group, compared to the control group, BcL2 immunoreactivity was decreased in liver tissue ($p<0.05$) (B; b). In the DX+RSV II group, compared to the DX group, BcL2 immunoreactivity was increased in liver tissue ($p<0.05$) (B; d). DX group (C; b) Casp3 immunoreactivity was observed to be significantly increased ($p<0.05$) compared to the Control group. Casp3 immunoreactivity of the DX+RSV II group (C; d) was observed to decrease at a statistically significant level ($p<0.05$) compared to the DX group. A) Bax immunohistochemical images, B) BcL2 immunohistochemical images, C) Casp3 immunohistochemical images, scale bar: 100 µm, ×200. Casp3; Caspase 3, DX; Doxorubicin, RSV; Resveratrol

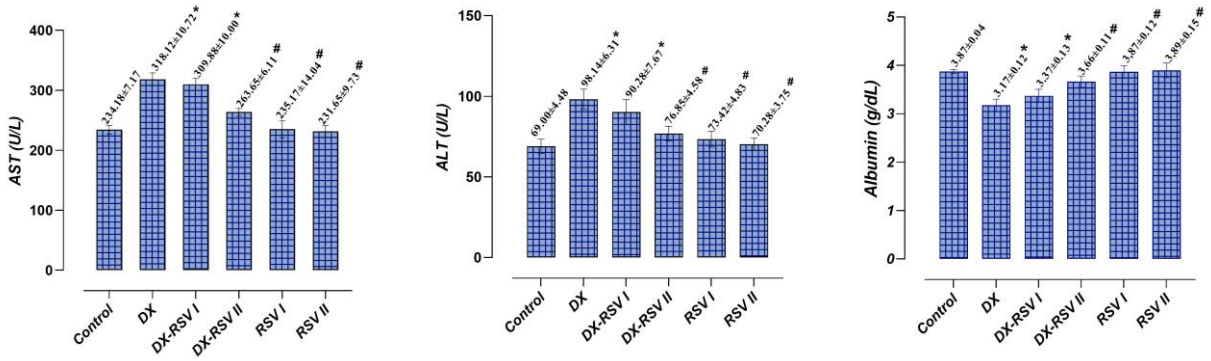


Figure 3 Effects of DX and/or RSV applications on the ALT, AST and Albumin serum levels. AST, ALT and Albumin serum levels were similar in the control and RSV-only groups. A significant increase in AST and ALT levels and a decrease in Albumin levels were detected in the DX group compared to the control group ($p<0.05$) (b). DX administration together with 5 mg/kg/day RSV administration arranged the biochemical parameters (d). AST; Aspartate transaminase, ALT; Alanine transaminase, DX; Doxorubicin, RSV; Resveratrol

Casp3 immunoreactivity was similar in the control, RSV I and RSV II groups (Figure 2C; a, e, f). Casp3 immunoreactivity was observed to be significantly increased in DX-treated rats compared to the control group ($p<0.001$) (Fig. 2C;b). The DX+RSV I group showed similar Casp3 immunoreactivity as the DX group (Figure 2C; c). In addition, DX-induced increased Casp3 immunoreactivity was observed to decrease significantly with 5 mg/kg/day RSV treatment (DX+RSV II group compared with DX group, $p=0.036$) (Table1, Figure 2C; d).

ALT and AST serum levels were similar in the control and RSV-only groups. There was a significant increase in ALT and AST levels in the DX-treated group compared to the control group (respectively; $p=0.002$, $p=0.001$). Although ALT and AST levels decreased relatively in the DX+RSV I group compared to the DX group, this was not statistically significant ($p>0.05$). However, it was observed that ALT and AST levels were significantly decreased in the DX+RSV II group, which received 5 mg/kg/day RSV, compared to the DX group (respectively; $p=0.039$, $p=0.035$, Figure 3). In addition, it was observed that serum Albumin level decreased with DX application compared to the control group ($p=0.002$). However, it was observed that albumin level increased significantly in the DX+RSV II group compared to the DX group ($p=0.039$, Figure 3).

Discussion

DX is a chemotherapeutic antibiotic that is frequently used in cancer treatment (17). Hepatotoxicity is a serious complication of DX therapy (18). A study reported increased AST and ALT levels as an indicator of liver damage from DX administration (19). The results obtained in this current study showed that serum AST and ALT levels increased and Albumin levels decreased due to DX administration. These data are consistent with other recent studies (20, 21). In addition, a study reported that DX administration caused histopathological changes in the liver tissue (22). In this study, in the liver tissue of DX application; It has been observed that it causes sinusoidal dilatation, vacuolization, mononuclear cell infiltration, degeneration of hepatocytes, hemorrhagic areas and vascular congestion. Some studies have similarly reported that DX chemotherapy may cause histopathological changes in liver tissue (2, 3, 20). However, in this study, it was determined that these biochemical and histopathological changes caused by DX were significantly reduced/mitigated with RSV (5mg/kg/day) treatment.

Apoptosis plays an important role in DX induced toxicity (23). Apoptosis is an organized process involving cellular proteins and signaling pathways. Apoptotic proteins are divided into two groups according to their roles as anti-apoptotic and pro-apoptotic. BcL2 family proteins, localized in the outer membrane of mitochondria, function as both anti-apoptotic (BcL2, etc.) and proapoptotic (Bax, etc.) (24). A recent study reported that DX administration caused an increase in Bax expression and a 60.6% decrease in BcL2 mRNA expression (1). Another study reported that in the liver, in contrast to the control group, the fluorescence intensity of BcL2 decreased significantly in the DX group, whereas the fluorescence intensity of Bax increased clearly in the liver. However, the same study revealed that the ratio of Bax/BcL2 protein analyzed by Western blot technique in the DX group was greatly increased (21). In this current study, it was observed that DX administration caused an increase in Bax immunoreactivity in liver tissue, while BcL2 immunoreactivity was significantly decreased. Consistent with the studies, it was determined that the Bax/BcL2 ratio increased in DX-induced hepatotoxicity. It was observed that these DX-induced apaptotic protein immunoreactivities changes were significantly alleviated by RSV (5mg/kg/day) treatment. In addition, immunohistochemistry analyzes of Casp3 immunoreactivity in studies revealed that the percentages of Casp3 positive areas increased significantly with DX application (21). Similarly, this current study showed that DX administration significantly

increased Casp3 immunoreactivity in liver tissue compared to the control group. In line with these data, a study showed that Casp3 immunoreactivity and the number of TUNEL-positive cells in liver tissue were significantly increased in the DX group compared to the control group (6). In addition, in this current study, it was determined that RSV (5mg/kg/day) treatment significantly reduced the DX-induced increased Casp3 immunoreactivity.

In this current study, it was determined that 1 mg/kg RSV administration was not as effective as 5 mg/kg RSV in improving DX-induced hepatotoxicity. These results showed that RSV treatment may have a dose-dependent hepatoprotective effect. Similarly, a recent study reported that low doses of RSV are beneficial in chronic treatments, while high doses of RSV treatment are needed for acute treatment (25). This suggests that the failure to achieve the desired result with 1 mg/kg RSV treatment may be about the duration of the study. In addition, in a study conducted on diabetic rats, the antidiabetic effects of 1, 5 and 10 mg/kg RSV treatment were compared, and it was reported that the best results were obtained with 5 mg/kg RSV treatment (15). Consistent with these data, it was observed that 5 mg/kg/day RSV treatment reduced/attenuated the DX-induced biochemical and histopathological changes in liver tissue. However, RSV (5 mg/kg/day) decreased Bax/BcL2 ratio and Casp3 immunoreactivity to inhibit cell apoptosis against DX-induced hepatotoxicity. Therefore, the data obtained in this study indicate that 5 mg/kg/day RSV has an important role in preventing apoptosis through Bax/BcL2 modulation.

In conclusion, in this study, liver tissue damage was determined biochemically, histopathologically and immunohistochemically in rats exposed to DX, a chemotherapeutic drug widely used in the clinic. On the other hand, the protective effect of RSV treatment was determined by liver function tests, apoptotic proteins immunoreactivities and histopathological examination of tissue degeneration. The data obtained from this study reveal that 5 mg/kg/day RSV treatment may provide an antiapoptotic effect against DX-induced liver injury.

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