Original Article

DOI: 10.14235/bs.2018.2105

Resveratrol attenuates bleomycin–induced genotoxicity, pulmonary fibrosis and DNA damage in balb/c mice with ehrlich ascites carcinoma

Resveratrol, erhlich asit karsinomalı balb/c farelerde bleomisin kaynaklı genotoksisite, akciğer fibrozu ve DNA hasarını azaltır

İsmail KOYUNCU

Department of Medical Biochemistry, Harran University School of Medicine, Şanlıurfa, Turkey

Özet

Amaç: Oksidatif stres, bleomisin (BLM) kaynaklı akciğer fibrozis patogenezi ve genotoksisitesinde önemli rol oynamaktadır. Bu çalışmanın amacı, resveratrolün (RES) Ehrlich asit karsinoması (EAT) taşıyan farelerde BLM'ye bağlı gelişen genotoksisite, akciğer fibrozisi ve DNA hasarı üzerindeki koruyucu etkisini araştırmaktır.

Yöntemler: Çalışmada 60 adet Balb/c fare rastgele altı gruba ayrıldı. Sham grubu hariç diğer gruplardaki farelere $3x10^5$ canlı EAT hücreleri intraperitonal (ip) olarak enjekte edildikten 24 saat sonra, farelere altı gün boyunca; BLM (10 mg/kg, i.p) tek başına, RES (50 mg/kg, i.p) tek başına, BLM (10 mg/kg, i.p) +RES (25 mg/kg, i.p), BLM (10 mg/kg, i.p) +RES (50 mg/kg, i.p) uygulandı. Serum ve akciğer dokusunda BLM'ye bağlı olarak artan; DNA hasarı (comet assay ve 8-OHdG), malondialdehit (MDA), myeloperoksidaz (MPO), protein karbonil (PC), hidroksiprolin (HPR), total oksidant durum (TOS) ve oksidatif stress indeksi (OSI) gibi oksidant parametreler ile süperoksit dismutaz (SOD), total antioksidant durum (TAS) ve glutatyon (GSH) gibi antioksidan paremetreleri incelenerek, resveratrolün BLM'ye bağlı olarak gelişen, pulmoner toksisite ve genotoksisitenin önlenmesindeki potansiyel rolü araştırıldı.

Bulgular: Çalışma sonucunda resveratrolün (RES); bleomisine (BLM) bağlı olarak artış gösteren MDA, PC, TOS, MPO OSI, HPR ve DNA hasar seviyesini azaltırken ($p < 0.05$), GSH, TAS ve SOD gibi antioksidanların aktivitelerini artırmıştır ($p < 0.05$). Ayrıca histolojik bulgularda'da RES'in BLM kaynaklı fibrozisi engellediği tespit edilmiştir.

Sonuç: Bu sonuçlar EAT taşıyan farelerde BLM'nin akciğerde neden olduğu DNA hasarı, lipit ve protein oksidasyonu sonucu oluşan fibrozisin RES uygulamasıyla azaltılabileceği ve RES'in akciğerdeki DNA hasarına ve pulmoner fibrozisin konvansiyonel tedavi yöntemlerine destekleyici tedavi sağlayabileceğini göstermektedir.

Anahtar kelimeler: Bleomisin, Ehrlich asit karsinoma, Akciğer fibrozis, Resveratrol, DNA hasarı

Corresponding Author: İsmail KOYUNCU

E-mail: ismailkoyuncu1@gmail.com

Received : 20.07.2017 Accepted: 11.09.2017 Available Online Date: 30.04.2018

BEZMİÂLEMscience

Abstract

Objective: Oxidative stress plays an important role in the pathogenesis and genotoxicity of bleomycin (BLM)-origin lung fibrosis. The aim of this study was to investigate the protective effect of resveratrol (RES) on lung fibrosis, DNA damage and genotoxicity developing associated with BLM in mice carrying Ehrlich ascites carcinoma.

Methods: A total of 60 balb/c mice were randomly separated into 6 groups. With the exception of the Sham group, mice in the other groups were injected intraperitoneally (ip) with 3 x 10^5 live EAT cells, then after 24 hours, BLM only (10 mg/kg, i.p), RES only (50 mg/kg, i.p), BLM (10 mg/kg, i.p)+RES (25 mg/kg, i.p) and BLM (10 mg/kg, i.p)+RES (50 mg/kg, i.p) were applied to the mice for 6 days. With examination in serum and lung tissue of oxidant parameters such as DNA damage (comet assay and 8-OHdG), malondialdehyde (MDA), myeloperoxidase (MPO), protein carbonyl (PC), hydroxyproline (HPR), total oxidant status (TOS) and oxidative stress index (OSI) and antioxidant parameters such as superoxide dismutase (SOD), total antioxidant status (TAS) and glutathione (GSH), evaluation was made of BLM-related pulmonary toxicity and genotoxicity and the potential role of resveratrol (RES) in preventing damage.

Results: The results determined that RES reduced MDA, PC, TOS, MPO OSI, HPR and DNA damage levels which showed an increase related to BLM ($p<0.05$) and increased GSH, TAS and SOD activities ($p<0.05$). It has also been found that RES inhibits BLM-induced fibrosis in histological findings.

Conclusion: These results have shown that fibrosis formed as a result of lipid and protein oxidization and DNA damage caused by BLM in the lungs of EAT-carrying mice could be reduced with the application of RES. Therefore, RES could be considered as an adjuvant therapy to conventional treatment methods for pulmonary fibrosis and DNA damage of the lungs.

Keywords: Bleomycin, Ehrlich ascites tumor, pulmonary fibrosis, Resveratrol, DNA damage

Introduction

Bleomycin is a cytotoxic antibiotic obtained from Streptomyces verticillus strain and is used as an anti-cancerogenic drug in many cancer types such as some lymphoma types, germ cell tumours and head and neck cancers (1, 2). However, when used in cancer treatment, just as in other chemotherapeutic drugs, there may be significant unwanted side-effects. As hydrolase enzyme, which inactivates BLM, is found at a very low level in lung tissue in particular compared with other tissues, it is known that widespread fibrosis in the lungs and interstitial pneumonia is formed, the antioxidative capacity is reduced and free radical production is increased, leading to DNA damage and pulmonary fibrosis (3-6). Therefore, in patients using BLM, the rate of damage developing in the lungs can vary from 3%-40% and 10% of patients have been reported to be under life-threatening risk (7, 8).

These findings indicate that oxidative stress plays an important role in bleomycin toxicity. However, in several studies made on animal models, it has been shown that antioxidant substances such as molsidomine (9), quercetin (10), berberine (11), atorvastatin (5), N-acetylcysteine (12), resveratrol

(13), ginkgo biloba (14), melatonin (15) and dexpanthenol (16) have a protective effect of varying degrees on bleomycin-origin pulmonary fibrosis and damage (17).

Resveratrol (trans-3, 4', 5-trihydroxystilbene) is a phytoalexin obtained from various plant sources such as grape skins in particular, cornelian cherries, mulberries, strawberries, cranberries and other fruits and is known to have strong antioxidative, anti-inflammatory and chemotherapeutic properties (18, 19). The antioxidant mechanism of resveratrol can be explained by the inhibiting effects on the complex chain of ROS formation, capturing superoxide radicals produced in the cell and the inhibition of lipid peroxidation (MDA) induced with oxidative stress (19).

Recently, resveratrol has been found to have protective and healing effects on some cardiovascular diseases, lipid metabolism modulation, an increase in cellular nitric oxide levels, vascular endothelium protection and the preventative effects of platelet aggregation (18, 20-23). Robust data produced by several studies have focused on the beneficial effects of resveratrol in the prevention of cancer and coronary heart diseases. However, there have been few studies which have tested its potential use as a therapeutic substance against genotoxicity in oxidative organs and peripheral blood mononuclear cell (PBMC) associated with the use of various drugs.

Previous studies have shown that resveratrol is extremely effective in the prevention of bleomycin-related pulmonary fibrosis and the treatment of fibrosis (24, 25). However, there has been no evaluation of whether or not it is effective in the prevention of pulmonary toxicity and the level of DNA damage caused by BLM in the lymphocyte cells and pulmonary tissue in mice carrying Ehrlich ascites carcinoma.

In the majority of previous studies, BLM has been applied to healthy rats or mice to form an idiopathic pulmonary fibrosis model similar to that in humans. In the current study, a different model was applied to be different from previous studies for the first time. In contrast to previous studies, in the current study model, BLM was not used as an agent forming pulmonary damage, but was used as an antineoplastic agent for treatment purposes in mice with cancer. Thus, the genotoxic effects shown in PBMC and pulmonary tissue and the protective efficacy of RES on this toxicity were examined when BLM was used for treatment purposes in cancer patients. As the physiological environment of normal mice and mice with cancer show a great difference from each other, it was considered that the actual toxic effect of BLM and the actual protective effect of the antioxidant substance would be able to be better examined in this model.

Materials and methods

Materials

BLM was obtained from Onko Medical Company (Bleocin®-S 15 mg, Istanbul, Turkey). BLM was administered intraperitoneally (i.p.) at the dose of 10 mg/kg b.w. BLM was dissolved in normal saline (0.9% NaCl). Before each administration solutions were thoroughly vortexed to obtain homogenous suspension.

Experimental Methods

Female and male Balb/C mice (n = 60), weigh 25-30 g, were taken from Bezmi-alem university (İstanbul, Turkey). The mice were kept in a place which was environmentally controlled at constant temperature (21 ± 1°C) and humidity (70 ± 5%) with a 12 h light/dark periods until experiments. The

mice were accustomed to the climate conditions for a week prior to study procedure and accessed freely to standard laboratory feed and water ad libitum. This research has the approval of Ethics Review Committee for Ethics in Animal Experiments of the Bezmi-alem university and strictly followed standards for the Guide for the Care and Use of Laboratory Animals (Ethics Committee Decision Number: 2016/66).

Mice were randomly assigned to six equal groups (n = 10). All mice, except those of the sham group, were inoculated intra peritoneal (i.p.) with 3×10^5 cells in 0.5 mL of Hank's balanced salt solution (HBBS) on day 0 in accord with a modified method of Özkol et al., (26). After 24 hours of inoculation, mice were exposed for 6 days as follows: 1) sham group: mice were treated with 0.5 ml of normal saline daily i.p., 2) EAT group: mice were injected with 0.5 mL of normal saline by i.p Daily, 3) BLM (alone) group: mice were injected with 10 mg/kg b.w. of BLM by i.p Daily, 4) RES (alone) group: mice were injected with 50 mg/kg b.w. of RES by i.p Daily, 5) BLM+RES_{25} group: mice were treated with 10 mg/kg b.w. BLM and 25 mg/kg b.w. of RES by i.p Daily, 6) BLM+RES_{50} group: mice were treated with 10 mg/kg b.w. of BLM. and 50 mg/kg b.w. of RES by i.p daily;

Blood Collection and Isolation of mice PBMC

After experimental process (6 days), blood samples were taken from each group while the mice were under anesthesia. After decapitation of the animals, blood samples were taken according to cardio-puncture method, and they were put into blood collection tubes with (for *PBMC isolation*) and without heparin (for serum). By the difference of density gradient, the peripheral blood mononuclear cells (PBMC) were isolated by a gradient medium (Hystopaque 1077). Cell viability was assessed by using trypan blue dye exclusion technique. The ratio of vital cells was at least 90% before comet assay.

Preparation of Lung Tissue Homogenates

Lung tissues were rinsed with ice-cold phosphate buffered saline (PBS) and kept at −80°C until analysis. To produce 10% (w/v) homogenate, tissues were homogenized with ice-cold Tris-HCl buffer (0.15 M, pH 7.4) for 5 minutes by a manual homogenizer. Then the homogenate was centrifuged at 7.000 × g for 15 min. The pellet was separated and the clear supernatant was used for the analysis. All procedures were performed at 4 °C.

Measurement of Biochemical Parameters

Blood urea nitrogen (BUN), creatinine (CRE), alanine trans aminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) levels were evaluated by relying on an enzymatic technique carried out by an autoanalyzer (Cobas integra 800, Roche). Myeloperoxidase (MPO) activity of lung homogenates was tested by the method defined by Bradley *et al*. (27).

Hydroxyproline level was assessed in lung homogenates with commercially available kits. (Hangzhou Eastbiopharm Co., Ltd., China) by using an via microplate reader (Spectra max M5, USA), absorbance was read at 450 nm. Hydroxyproline levels were expressed as µg/ml.

Glutathione (GSH) level was assessed through reaction with OPA (1mg/ml o-phthaldialdehyde in methanol) following to the modified technique of Kandar *et al*.(28). GSH was used as a standard. GSH samples were assessed via microplate reader (Spectra max M5, USA), with excitation at 345 nm

and emission at 425 nm. Results were expressed as nmol/ml and nmol/g in serum and in wet tissue, respectively.

Malondialdehyde (MDA) levels in lung homogenates and serum were assessed following the technique defined by Ohkawa *et al*.(29). ELISA plates were read by a microplate reader (Spectra max M5), at 532 nm. The results were obtained as nmol/ml in serum and nmol/g in wet tissue respectively.

Protein carbonyl (PC) level was assessed by Cayman's Protein Carbonyl Assay Kit as carbonyl content in the samples. This kit is based on the principle that utilizes the 2,4-dinitrophenylhydrazine reaction to measure protein carbonyl content in homogenate (9). The amount of protein hydrozone produced is quantified spectrophotometrically at an absorbance of 360 nm by a 96-well plate reader (M5; SpectraMax, Sunnyvale, California, USA). Carbonyl content was standardized to protein concentration. Protein content in the samples was measured by the method of Lowry *et al.* (30)**,** with bovine serum albumin as the standard.

Superoxide dismutase (SOD) is one the very significant antioxidant enzyme. Activity of SOD was measured at 505 nm and 37°C, and calculated utilizing the inhibition percentage of formazan formation method of McCord and Fridovich (31) **.** Quantification of SOD was done by using Randox commercial kits (Randox Laboratories, Crumlin, UK). Protocol was followed as described in the manufacturer instructions.

Total oxidant status (TOS) and total antioxidant status (TAS) were detected in serum and tissue homogenates by using commercially available kits (Rel Assay, Turkey) with an autoanalyzer (Cobas integra 800, Roche). TOS and TAS results were presented in µmol H_2O_2 equivalent/L (32) and mmol Trolox equivalent/L, respectively (33). The ratio of the TOS to the TAS revealed the oxidative stress index (OSI), which is used as an indicator for total oxidative stress (34)**.**

Comet Assay

The alkaline single cell gel electrophorese analysis (comet assay) was used to study the potential preventive effects of the RES on BLM-induced DNA damage in PBMC genotoxicity in mice. Comet assay was performed according to Kocyigit *et al*., (35) as follows: approximately 2×10^4 cells were suspended in low melting point agarose (LMA) (75 µl of 1.0%), and stratified onto semi-frozen slides previously covered with a slim stratum of normal melting point agarose (1.0%). Another stratum of 0.5% LMA was put over the second layer. The cells were dissolved for 2 h at 4°C in a solution (100 mM EDTA, 2.5 M NaCl, 10% DMSO, 1% Triton X-100, 10mMTris, pH 10.0). Following dissolution, the slides were exposed to electrophoresis in buffers (0.3 M NaOH, 1mM EDTA, pH 13.1) for 30 min. Then, the slides were neutralized within a Tris buffer (0.4 M Tris-HCl, pH 7.5). The slides were carefully dried at 25 ^{o}C in an incubator and marked with ethidium bromide (10 µg/ml in distilled water, 70 µl/ slide). The slides were screened by using fluorescence microscope (Leica DM 1000, Solms, Germany) imaging system. A hundred cells were randomly scored in each sample on a scale of 0–4 based on fluorescence beyond the nucleus. The used scale scores were as follows: 0, no tail; 1, comet tail, half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus; and 4, comet twice the width of the nucleus. Scoring cells in this way have been shown to be as accurate and precise as using computerized image analysis.

Measurement of 8-OHdG in Serum and lung tissue

8-Hydroxydeoxyguanosine is one the very significant sign of oxidant-induced DNA damage. Quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was done by using OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA). Protocol was followed as described in the manufacturer instructions.

Histopathological Method

The mice were decapitated and pulmonary tissue samples were fixed in 10% buffered formalin. Then, to remove the formaldehyde from the tissues, the samples were washed overnight in running water. Routine pathology tissue procedures were applied with the tissues passed through a graded series of alcohol (50%, 75%, 96%, 100%) and xylol, then embedded in paraffin blocks. Slices of 4 micron thickness were cut from the blocks for examination under light microscope. The first 3 slices and every 10^{th} slice were taken with a Leica RM 2125 RT onto slides.

The slideswere passed through a series of graded alcohol and xylol, then stained with hematoxylin and eosin (H-E). All the samples were examined at x40 – x100 magnification under a high-resolution light microscope (Olympus DP 73 camera, Olympus BX53-DIC microscope; Tokyo, Japan) and the findings were scored with a modified semi-quantitative grading system as used by Ashcroft et al (36). The evaluations according to this system were Grade 0: normal lung, Grade 1: minimal or moderate increase in thickness of the alveolar or bronchus wall, Grade 2: increased fibrosis and small fibrous formations together with mild damage to the lung structure, Grade 3:severe structural distortion and large fibrous areas (honeycomb appearance in the lung) and Grade 4: total fibrosis.

Statistics

Data obtained from study samples were presented as means and standard deviation of means (SD) and analyzed by one-way analysis of variance (ANOVA). TUKEY post hoc test on the SPSS (11.5) program were performed. Differences among the average values according to $p < 0.05$ were evaluated to be statistically significant.

Results

Results for oxidant status are shown in Table 1. MDA, PC, TOS and OSI level are commonly used as an indicator for measuring free-radical induced by oxidants. A statistically significant ($p <0.05$) increase were detected MDA, PC, TOS and OSI levels of lung and serum of mouse administered with BLM when compared to the normal and EAT control group. On the contrary, administration of RES in a dose-dependent way demonstrated a significant ($p <0.05$) decrease MDA, PC, TOS and OSI levels in lung and serum when compared to the BLM group (Table 1).

Table 1. Effect of Resveratrol on oxidant status of Bleomycin-induced, EAT-bearing mice.

	MDA		**PC**		**TOS** (µmol H_2O_2 Eq/L)		**OSI (**arbitrary unit)	
	Serum (nmol/ml)	**Lung** (nmol/g)	**Serum** (nmol/mg protein)	**Lung** (nmol/mg protein)	**Serum**	**Lung**	**Serum**	**Lung**
Normal	24,9±6,5	28,2±3,7	0,65±0,18	1,25±0,37	10,3±1,7	11,1±1,9	0,6±0,16	0,6±0,11
EAT Control	30,7±12,2	40,8±5,2 [a]	0,88±0,28	1,72±0,35	14,7±1,6 [a]	14,1±1,6	1,1±0,13 [a]	1,0±0,26 [a]
RES (alone)	23,8±6,1	32,9±4,8 [b]	0,79±0,29	1,49±0,35	12,2±1,9	11,9±1,7	0,7±0,22	0,7±0,11 [b]
BLM (alone)	43,7±5,1 [ab]	60,5±6,7 [ab]	1,09±0,32 [a]	3,43±0,46 [ab]	19,8±1,3 [ab]	17,3±1,8 [ab]	1,6±0,29 [ab]	1,6±0,50 [ab]
BLM+RES$_{25}$	34,4±9,4	46,0±4,5 [ac]	0,85±0,20	2,62±0,45 [abc]	12,5±1,5 [c]	13,1±2,1 [c]	0,7±0,14 [bc]	0,9±0,18 [c]
BLM+RES$_{50}$	30,6±8,4 [c]	37,5±4,1 [ac]	0,77±0,21 [c]	2,25±0,68 [ac]	11,4±2,9 [bc]	11,8±1,6 [c]	0,6±0,17 [bc]	0,7±0,10 [c]

Values are given as the means ± SD. There were no significant differences between EAT control and normal groups in terms of any parameter ($P > 0.05$). [a]$P < 0.05$, compared with the EAT control group; [b]$P < 0.05$, compared with the EAT normal group; [c]$P < 0.05$, groups in which BLM and RES were treated together, compared with the BLM group

EAT: Ehrlich ascites carcinoma, RES:Resveratrol, BLM:Bleomycin, MDA: Malondialdehyde, PC: Protein Carbonyl, TOS: Total Oxidant Status, OSI: Oxidative Stress Index

Influence of RES on antioxidant defense systems level in lung and serum of mouse shown in Table 2, TAS, GSH level and SOD activity of normal groups were significantly higher than those of EAT control. In the BLM (alone) group, all antioxidant defense systems decreased markedly, compared with normal and EAT control groups, and applying RES in a dose dependent way caused an inversion in BLM-mediated changes of TAS, GSH and SOD and TOS activities. The RES_{25} and RES_{50}-treated rats significantly ($p < 0.05$) increased SOD, TAS and GSH level in the lung and serum compared to the BLM group. Treatment with only RES is not affected the TAS, GSH level and SOD activity of serum and lung tissues compared with the normal group. But treatment with only RES significantly increased TAS, GSH level and SOD activity of serum and lung tissues compared with the EAT control group.

Table 2. Effect of Resveratrol on anti-oxidant status of Bleomycin-induced, EAT-bearing mice

	TAS (µmol Trolox Eq/L)		**GSH**		**SOD**	
	Serum	**Lung**	**Serum** (nmol/ml)	**Lung** (nmol/g)	**Serum (U/g)**	**Lung (U/g)**
Normal	1,7±0,28	1,8±0,19	7,83±1,51	3,29±0,73	93,8±19,5	160,5± 19,3
EAT Control	1,4±0,14	1,4±0,23 [a]	6,39±0,64 [a]	2,20±0,78 [a]	75,1±13,4 [a]	108,6± 22,4 [a]
RES (alone)	1,8±0,40 [b]	1,8±0,21 [b]	7,19±0,62	2,62±0,66	65,5±18,8	137,2± 29,4
BLM (alone)	1,3±0,23 [a]	1,2±0,30 [ab]	4,25±0,75 [ab]	1,53±0,37 [a]	38,7±11,1 [ab]	80,1± 20,6 [a]
BLM+RES_{25}	1,8±0,17 [bc]	1,5±0,17 [ac]	6,10±1,07 [ac]	2,06±0,34 [a]	51,4±9,3 [ab]	114,4± 27,5[c]
BLM+RES_{50}	1,9±0,24 [bc]	1,6±0,11[c]	6,57±1,03[c]	2,38±0,27 [ac]	59,8±13,6 [ac]	148,6± 30,3 [ac]

Values are given as the means ± SD. There were no significant differences between EAT control and normal groups in terms of any parameter ($P > 0.05$). [a]$P < 0.05$, compared with the EAT control group; [b]$P < 0.05$, compared with the EAT normal group; [c]$P < 0.05$, groups in which BLM and RES were treated together, compared with the BLM group

EAT: Ehrlich ascites carcinoma, RES:Resveratrol, BLM:Bleomycin, TAS:Total Antioxidant Status, GSH: Glutathione SOD:Superoxide dismutase

The comet assay and 8-OHdG results which indicate protective effect of RES on BLM-mediated DNA damage are presented in table 3. In the BLM group, 8-OHdG levels were observed to be significantly high in compared with the sham and EAT control group ($p < 0.05$). Also, 8-OHdG levels in RES_{25}, and RES_{50} treatments can significantly reduce 8-OHdG levels in comparing with the EAT control and BLM group. Moreover, RES prevented BLM-mediated change of 8-OHdG in a dose depending way compared to the BLM group.

The protective effect of the RES on mouse PBMCs was also investigated using the comet assay. The comet assay was applied to determine the ratio of DNA in the tail. The results are showed in table 3. Only RES treatment showed no genotoxic influence, and BLM treatment with RES_{50} drastically decreased DNA migration compared to the BLM group ($p< 0.05$). Taking the genotoxicity into consideration, RES treatment itself did not trigger DNA damage compared to the normal group. There was an important increase in DNA fragmentation in BLM group when compared to the sham and EAT control group. Treatment of the animals with RES along with BLM led to a statistically significant decline ($p < 0.05$) in DNA migration to the tail compared to the sole BLM treatment.

Hydroxyproline level was evaluated in lung homogenates. BLM elicited a statistically significant increase in hydroxyproline level lung ($p < 0.05$) compared with the EAT control group. However, combination of BLM with RES_{50} was able to create a significant decrease of hydroxyproline levels lung comparing with the BLM group ($p < 0.05$) (Table 4). Moreover, MPO activity was observed to be high in BLM group. The findings showed that RES treatments decreased MPO activity previously increased caused by the use of BLM.

Table 3. Effect of Resveratrol on DNA damage of Bleomycin-induced, EAT-bearing mice

	8-OHdG (ng/ml)		**COMET (**arbitrary unit)
	Serum	**Lung**	**PBMC**
Normal	195,3±34,1	347,1±106,2	6,60±1,7
EAT Control	292,9±52,5	538,0±66,1 [a]	13,50±3,0 [a]
RES (alone)	246,3±90,7	456,8±88,1	10,40±4,0
BLM (alone)	630,2±108,9 [ab]	1314,7±142,5 [ab]	35,30±6,4 [ab]
BLM+RES_{25}	435,6±90,2 [abc]	889,4±180,6 [abc]	21,13±4,1 [ab]
BLM+RES_{50}	348,8±69,3 [ac]	727,1±180,1 [ac]	17,67±5,4 [ac]

Values are given as the means ± SD. There were no significant differences between EAT control and normal groups in terms of any parameter ($P > 0.05$). [a]$P < 0.05$, compared with the EAT control group; [b]$P < 0.05$, compared with the EAT normal group; [c]$P < 0.05$, groups in which BLM and RES were treated together, compared with the BLM group.

EAT: Ehrlich ascites carcinoma, RES:Resveratrol, BLM:Bleomycin, 8-OHdG:8-hydroxy-2' -deoxyguanosine, COMET: Comet assay, PBMC: Peripheral blood mononuclear cell,

The lung tissues taken from the mice in the study were evaluated histopathologically in figure 1. In the comparison between the groups, the descriptive statistics and comparisons are shown in Table 4. Fibrosis in the pulmonary samples of the BLM group was seen to be statistically significantly high compared to the other groups ($p<0.05$). An evident reduction in fibrosis was seen in the BLM+RES group, and the level in the RES_{25} group in particular was statistically significantly high compared to the pulmonary tissues of the sham group ($p<0.05$). A statistically significant reduction was determined in fibrosis in the pulmonary tissues of the RES_{50} group but no statistically significant difference was determined in the sham group pulmonary tissue ($p<0.05$). From the results of the study it was determined that resveratrol (25 mg/kg and 50 mg/kg) partially reversed the cycle of bleomycin-induced pulmonary fibrosis, evaluated with the increased semi-quantitative fibrosis score, hydroxyproline content and MPO activity in the pulmonary tissue.

Serum levels of biochemical parameters measured are shown in Table 4. Though the levels of liver enzymes (AST, ALT, and LDH) and renal function markers (BUN and CRE) increased significantly between the BLM (alone) and EAT control groups, each dose of RES supplementation in BLM-treated mice resulted in marked decreases of these biochemical parameters.

Table 4. Grades of lung fibrosis, lung hydroxyproline and MPO contents

	Hydroxyproline (µg/ml)	MPO (U/g)	Grade of fibrosis
Normal	163,7± 34,6	6,29±1,79	0,36±0,09
EAT Control	220,6± 64,8	11,48±1,46	0,49±0,13
RES (alone)	171,8± 24,0	6,77±1,87	0,41±0,11
BLM (alone)	315,6± 67,9 [ab]	19,63±2,21 [ab]	1,65±0,40 [ab]
BLM+RES_{25}	251,8± 85,0 [a]	14,64±2,43 [abc]	0,85±0,27 [abc]
BLM+RES_{50}	197,1± 37,5[c]	10,87±2,34 [c]	0,68±0,22 [ac]

Values are given as the means ± SD. There were no significant differences between EAT control and normal groups in terms of any parameter ($P > 0.05$). [a]$P < 0.05$, compared with the EAT control group; [b]$P < 0.05$, compared with the EAT normal group; [c]$P < 0.05$, groups in which BLM and RES were treated together, compared with the BLM group

EAT: Ehrlich ascites carcinoma, RES:Resveratrol, BLM:Bleomycin, MPO:Myeloperoxidase

Table 5. Effect of Resveratrol on serum liver enzymes and renal function markers of BLM- induced EAT-bearing mice

	BUN (mg/dL)	CRE (mg/dL)	AST (U/L)	ALT (U/L)	LDH (U/L)
Normal	15,8±3,8	0,2±0	39,6±3,2	26,2±7,9	1,7±0,3
EAT Control	19,6±3,8	0,2±0,1	40,8±6,1	30,2±9,1	1,9±0,4
RES (alone)	15,9±3,8	0,2±0,1	37,2±5,2	23,9±2,4	1,6±0,6
BLM (alone)	35,5±2,2[ab]	0,4±0,1[ab]	70,9±13,1[ab]	52,2±7,1[ab]	3,5±1 [ab]
BLM+RES_{25}	20,8±3 [ac]	0,2±0,1[c]	44,2±12,5 [c]	39,7±7,3 [ac]	2,4±0,3 [c]
BLM+RES_{50}	18,7±3[c]	0,2±0,1 [c]	41,6±7,4 [c]	32,5±7,2 [c]	2,2±0,4 [c]

Values are given as the means ± SD. There were no significant differences between EAT control and normal groups in terms of any parameter ($P > 0.05$). [a]$P < 0.05$, compared with the EAT control group; [b]$P < 0.05$, compared with the EAT normal group; [c]$P < 0.05$, groups in which BLM and RES were treated together, compared with the BLM group

EAT: Ehrlich ascites carcinoma, RES:Resveratrol, BLM:Bleomycin,
BUN: Blood Urea nitrogen, CRE: creatinine, AST: aspartate transaminase, ALT: alanine trans aminase, LDH: lactate dehydrogenase

BEZMİÂLEMscience

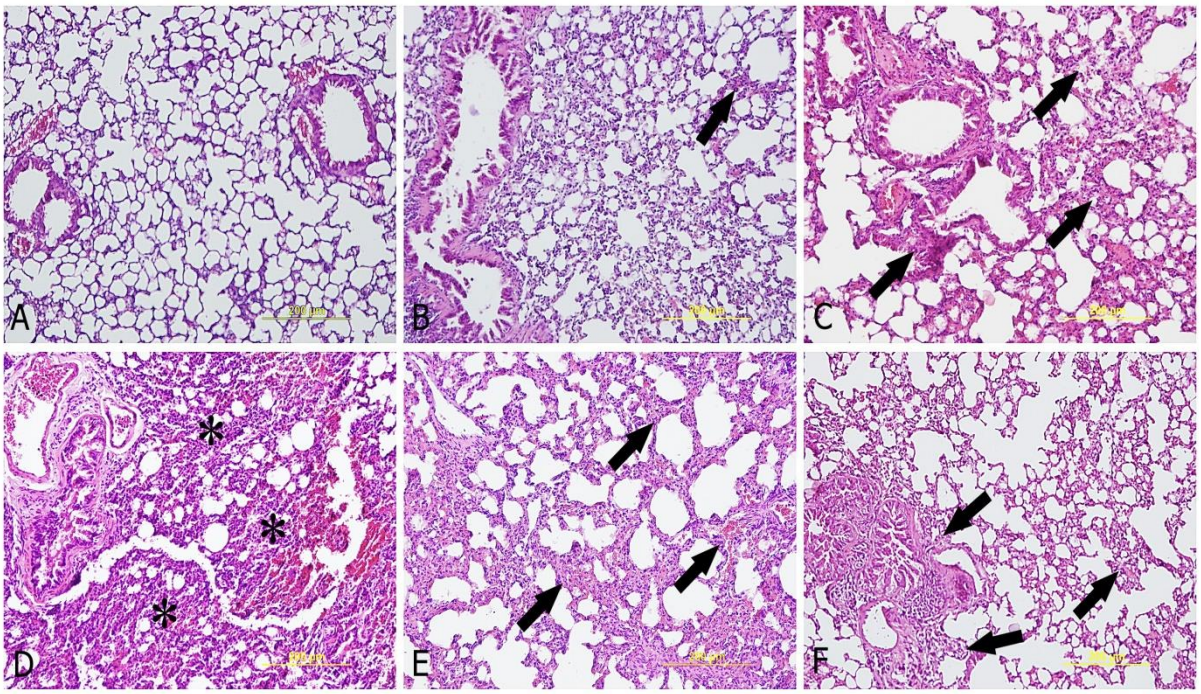

Figure 1. Histopathological changes in the lungs caused by BLM exposure and its amelioration by RES treatment: **A)** Sham group showing normal histology of the lung. (H & E), **(B)** RES treated animal's lung shows the to mild fibrosis (arrows) normal alveolar architecture which resembles to control, (H & E), **(C)** EAT group animal show mild thickening of alveolar and inflammatory cells (arrows). (H & E), **(D)** BLM group showing severe diversification fibrous and inflammation (stars). (H & E), **(E)**Lung sections of BLM + RES-25 group animal shows moderate inflammation (arrows). (H & E), **(F)** Lung sections of BLM + RES-50 group animal shows moderate fibrosis and inflammation (arrows). (H & E).

Discussion

Bleomycin (BLM) is an anticancer drug widely used in the treatment of different kinds of cancers. However, when BLM molecules interact with oxygen and ion, this leads to an increase in superoxide and other ROS (1). By showing a cytotoxic and genotoxic effect, an increase in BLM-related ROS production in the pulmonary tissue forms unwanted side-effects such as pulmonary fibrosis. Therefore, the protective effects of a series of antioxidant substances have been researched to provide protection against BLM toxicity (2-8, 10, 11).

In this study, for the first time an examination was made of the protective effect of RES on lung histopathology and oxidant parameters such as DNA damage (comet assay and 8-OHdG), MDA, MPO, PC, TOS and OSI, which increased related to BLM in the serum and pulmonary tissue of mice carrying EAT. In addition, the potential role of resveratrol was investigated in the prevention of BLM-related pulmonary toxicity and genotoxicity, such as the increase in SOD, TAS and GSH in the antioxidant defence system. The results of the study showed that RES reduced the level of MDA, PC, TOS, OSI, HPR and DNA damage which show an increase related to BLM and corrected the decline in the antioxidant system related to BLM in the serum and pulmonary tissue of mice.

In studies by Akgedik et al (13) and Sener et al (25), similar to the current study, it was determined that RES administered after BLM prevented pulmonary fibrosis to a significant degree in experimental rats and this effect was due to antioxidant and anti-inflammatory properties. It was also shown that RES lowered the MDA level in serum and pulmonary tissue and increased the reduction in TAS level in pulmonary tissue related to BLM exposure in rats. The depletion of the TAS level in the serum and pulmonary tissue and the reverse effect of the increase in MDA level in pulmonary tissue could be due to the possible antioxidant activity of RES (20).

By leading to an increase in free radical production, BLM causes single or double chain breakages in DNA (37-39). Furthermore, damage seen in pulmonary tissue after BLM treatment, which results in increased ROS and RNS production in increased active inflammatory cells further increases DNA damage (3, 40). In a study by Yasmina et al, (41) the chemotherapeutic agent BLM showed a cytotoxic and genotoxic effect on isolated rat lymphocytes and bee toxin that was administered as protection reduced the level of BLM-related DNA damage.

In the current study, the level of DNA damage caused by BLM in the serum and pulmonary tissue was determined with 8-OHdG and the level of damage in PBMC cells with the comet assay. The biomarker, 8-OHdG, released secondary to DNA damage, is regarded as the most significant indicator of DNA damage (42)**.** Hydroxyl radicals eliminate hydrogen from nucleic acids or react with double bonds, leading to 8-OHdG (43). Yasmina r*et al*. and Tayebeh et al (40, 41) suggested that DNA damage is increased by following BLM in rat PMBC and sperm. Our results showed that 8-OHdG score is also a good indicator of tissue damage in BLM induced genotoxicity in experimental mouse. The results of the study showed that RES at doses of 25 and 50 mg/kg effectively protected against DNA damage stimulated by BLM treatment in mice carrying EAT.

Previous studies have suggested that DNA damage was decreased by anti-oxidant supplementation (44, 45) and the results of the present study supported these data. RES exerted its anti-oxidant effects by reducing ROS-mediated 8-OHdG levels which indicates the prevention of oxidative DNA damages. This finding was the most important restorative effect of RES treatment. The protective effect of RES on PBMCs of mouse of which DNA damage was overt due to BLM induction was studied using comet assay. Findings of the comet assay demonstrated enhancements in the amount of DNA damage in BLM-administrated PBMCs. Treatment with BLM substantially diminished BLM-induced DNA damage in the comet assay. These results strongly demonstrate that RES possess genoprotective efficiency.

Oxidative stress disrupts the equilibrium among the production and elimination of oxidants. This can be owing to an extreme level of oxidants produced in the body or weak anti-oxidant defense mechanisms that is mostly seen at chemotherapy regimens, especially with BLM treatments. In this respect, the present study assessed the overall effects of the use of BLM on oxidative and anti-oxidative status via different perspectives. OSI reveals the redox equilibrium among oxidation and anti-oxidation, and it has been used as the major indicator for body redox balance**.** Separate evaluation of various oxidant molecules like superoxide radical, hydroxyl radical and hydrogen peroxide (H_2O_2) is not practical, however their oxidant impacts are incremental. So, we measured TOS in lung tissue and serum in accordance with the previous descriptions by Erel (32)**.** Likewise, rather than individually determining antioxidant molecules, we measured TAS, according to the techniques of Erel, as well (33)**.** In previous studies, it was suggested that OSI could show the oxidative state more precisely than the sole levels of TOS or TAS (46)**.** Hence, OSI could be regarded as an approximate indicator for identifying both BLM mediated genotoxicity, lung toxicity and preservation provided by RES in subjects under BLM treatment. In the BLM administrated group, the

levels of TOS and OSI were dramatically enhanced compared with sham, ETA-control and sole-RES administrated groups, whereas TAS levels were clearly reduced when compared to the control group. However, applying RES with BLM decreased the levels of TOS and OSI, while it enhanced TAS levels when compared with the BLM group. These results demonstrate that exposure to BLM caused an increase in oxidative stress in the tissues, and elevated oxidative stress was hampered by the RES treatment.

MPO is a pro-oxidant enzyme widely used as a neutrophil infiltration index. The increase in MPO secretion, which is an enzyme that reacts with H_2O_2, from the increased active neutrophils in the lungs following the application of BLM, leads to ROS formation causing damage in the lungs (2, 4). In the current study, the evident increase in MPO activity in the lungs of the BLM group suggests that neutrophil activation plays a critical role in the harmful effect of BLM. In a study conducted on rats by Kilic et al (4), a similar high level of MPO activity in the lungs was observed following BLM treatment. An increase in MPO activity was determined to have been prevented in both the BLM+RES groups compared to the BLM group. The role of RES in weakening lung MPO activity has been shown to be related to the capability of the preventative effect of reducing neutrophil infiltration (25).

Lipid peroxidation through ROS causes loss of membrane stability and integrity leading to increased trans epithelial permeability and fibrosis in the lungs (47). Malondialdehyde is a reactive carbon component which is used as a marker of lipid peroxidization (48). Consistent with the findings of previous studies, an increase was observed in the MDA levels in the serum and pulmonary tissue of mice given BLM (49). The application of RES significantly reduced BLM-induced oxidative stress, as can be understood from the low MDA levels. This protective effect of RES is thought to be related to the effect of sweeping free radicals and antioxidant activity.

From the interaction of ROS and proteins, protein carbonyl (PC) products occur as a result of damage occurring in many amino acid remnants and/or the peptide backbone. Protein carbonyls are the most widely measured product of protein oxidization. The determination of PC levels is a sensitive method in the identification of oxidative protein damage (50). High PC contents of the BLM group may originate from an increase in free radicals as a result of BLM toxicity, because while BLM increases the amount of ROS, it also decreases antioxidant production. The application of RES, with its sweeping of free radicals, may block the inducing effect of BLM on protein oxidation and the reduced lipid and TOS level caused by BLM.

SOD is one of the antioxidant endogenous enzymes which plays an important role in the cellular antioxidant system against oxidative damage by transforming superoxide radicals to H_2O_2 (51). By entering into molecular interaction with oxygen and iron, BLM produces superoxide anion and other oxygen metabolites (38, 39). A decrease in SOD activity which will neutralize these radicals leads to a genotoxic and cytotoxic effect seen in PMBC and the lungs by other ROS molecules, primarily hydroxyl radical associated with an increase in superoxide radicals. Increasing damage in the lungs in particular causes fibrosis.

GSH is an important antioxidant molecule, which is a non-protein thiol and by directly reacting with O_2, peroxyl roots and singlet oxygen, the thiol in proteins and other neutrophylic groups protects against the toxic effects of ROS. The intracellular GSH concentration is an important marker of the degree of BLM-related pulmonary damage (51). It has been shown that the cellular GSH concentration could be affected by the exogenous application of antioxidants (47-52). In a study by Sener et al (25), the depletion of GSH reserves following the administration of BLM was corrected with RES treatment.

In the current study, a significant increase in the GSH level and SOD activity was determined only in the RES group. In comparison with the EAT control group, the SOD and GSH levels of the BLM group were significantly reduced. Both dosage levels of RES and all the doses of combined treatment of BLM significantly increased GSH content and SOD activity. Excessive ROS production and SOD and GSH depletion in toxicity caused by BLM has been shown in previous studies (53). Therefore, the GSH depletion and decrease in SOD activity may have caused the increase in lipid and protein oxidization in the BLM group. Thus, the modulation of GSH metabolism with resveratrol may be considered a useful adjuvant therapy in bleomycin treatment.

Liver and kidney damage do not always show increases in BUN and CRE levels in addition to serum AST, ALT and LDH activities. These increases may originate from other causes such as muscle trauma and stress (53). However, in the current study, the increase in the above-mentioned parameters was most likely from the BLM application. As seen in Table 1, although the AST, ALT, LDH, BUN and CRE levels were increased in the BLM group compared to the EAT control group, the levels were significantly reduced with the support of each RES dose. In some previous studies, increased serum liver enzymes have been associated with increasing reactive oxygen species (ROS) (26).

The results obtained in the study were supported by histopathological evaluation. Consistent with the findings of previous studies, the HPR level was determined to be high in the BLM group of the current study and there was a high level of damage in the Ashcroft scoring of the histological findings. There are several studies showing that several antioxidant agents play a protective role in pulmonary fibrosis caused by BLM (2-7). In these studies, it has been determined that according to the semi-quantitative histopathological evaluation and the lung HPR content.

Antioxidant substances generally prevented or reduced pulmonary fibrosis induced by BLM in rats. Similarly, in the current study RES was determined to have reduced pulmonary fibrosis.

In this study, the treatment of mice with EAT which included 25 mg/kg and 50 mg/kg doses of RES was seen to be effectively protective against pulmonary fibrosis stimulated with BLM. These results are in accordance with previous research of the protective role of RES against pulmonary damage (25, 26). All the doses of RES played an antioxidant role against the application of BLM in the mice with EAT (Table 2). In the groups where BLM and RES were applied together, there was a significant reduction in serum and pulmonary tissue TOS, MDA, PC, MPO and HPR concentrations. In both the RES+BLM groups, the TOS, MDA, PC, MPO and HPR levels reduced almost to the level of the EAT control group.

The high TOS, MDA and PC content of the BLM only group could have originated from an increase in free radicals as a result of BLM toxicity, because while BLM increases the amount of ROS, it also reduces antioxidant production. The application of RES, with its sweeping of free radicals, may block the inducing effect of BLM on protein oxidation and the reduced lipid and TOS level caused by BLM. Similar reducing effects of RES on LPO have been previously reported (19, 25, 26).

Conclusion

In conclusion, the results of this study showed that the DNA damage and the fibrosis formed as a result of lipid and protein oxidization caused by BLM in mice with EAT could be reduced with the application of resveratrol. This protective effect of RES can be explained by the strong antioxidant

effect eliminating or preventing the formation of free oxygen radicals. RES can therefore be considered as a potential therapeutic agent that could be used in the treatment of pulmonary fibrosis, DNA damage in the lungs and genotoxicity.

Ethics Committee Approval: Ethics committee approval was received for this study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

References

1. Kilic T, Ciftci O, Cetin A, Kahraman H. Preventive effect of chrysin on bleomycin-induced lung fibrosis in rats. Inflammation. 2014 Dec;37(6):2116-24. PubMed PMID: 24973983.
2. Schroll S, Lange TJ, Arzt M, Sebah D, Nowrotek A, Lehmann H, et al. Effects of simvastatin on pulmonary fibrosis, pulmonary hypertension and exercise capacity in bleomycin-treated rats. Acta physiologica. 2013 Jun;208(2):191-201. PubMed PMID: 23527830.
3. Della Latta V, Cecchettini A, Del Ry S, Morales MA. Bleomycin in the setting of lung fibrosis induction: From biological mechanisms to counteractions. Pharmacological research. 2015 Jul;97:122-30. PubMed PMID: 25959210.
4. Kilic T, Parlakpinar H, Polat A, Taslidere E, Vardi N, Sarihan E, et al. Protective and therapeutic effect of molsidomine on bleomycin-induced lung fibrosis in rats. Inflammation. 2014 Aug;37(4):1167-78. PubMed PMID: 24526289.
5. Zhu B, Ma AQ, Yang L, Dang XM. Atorvastatin attenuates bleomycin-induced pulmonary fibrosis via suppressing iNOS expression and the CTGF (CCN2)/ERK signaling pathway. International journal of molecular sciences. 2013 Dec 16;14(12):24476-91. PubMed PMID: 24351828. Pubmed Central PMCID: 3876122.
6. Sausville E, Peisach, J, and Horwitz, SB. Effect of chelating agents and metalionson the degradation of DNA bybleomycin. Biochemistry. 1978;17(2740–2746).
7. Zitnik R, Respir J. Drug-induced lung disease: cancer chemotherapy agents 1995;16(855-865.).
8. Reinert T. Bleomycin-Induced Lung Injury. Hindawi Publishing Corporation Journal of Cancer Research. 2013.
9. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. Methods in enzymology. 1990;186:464-78. PubMed PMID: 1978225.
10. Verma R, Kushwah L, Gohel D, Patel M, Marvania T, Balakrishnan S. Evaluating the Ameliorative Potential of Quercetin against the Bleomycin-Induced Pulmonary Fibrosis in Wistar Rats. Pulmonary medicine. 2013;2013:921724. PubMed PMID: 24396596. Pubmed Central PMCID: 3875129.
11. Chitra P, Saiprasad G, Manikandan R, Sudhandiran G. Berberine attenuates bleomycin induced pulmonary toxicity and fibrosis via suppressing NF-kappaB dependant TGF-beta activation: a

biphasic experimental study. Toxicology letters. 2013 May 23;219(2):178-93. PubMed PMID: 23523906.
12. Covvey JR, Mancl EE. Recent evidence for pharmacological treatment of idiopathic pulmonary fibrosis. The Annals of pharmacotherapy. 2014 Dec;48(12):1611-9. PubMed PMID: 25202034.
13. Akgedik R, Akgedik Ş, Karamanlı H, Uysal S, Bozkurt B, Ozol D, et al. Effect of resveratrol on treatment of bleomycin-induced pulmonary fibrosis in rats. Inflammation. 2012;35(5):1732-41.
14. Iraz M, Erdogan H, Kotuk M, Yagmurca M, Kilic T, Ermis H, et al. Ginkgo biloba inhibits bleomycin-induced lung fibrosis in rats. Pharmacological research. 2006 Mar;53(3):310-6. PubMed PMID: 16459098.
15. Yildirim Z, Kotuk M, Erdogan H, Iraz M, Yagmurca M, Kuku I, et al. Preventive effect of melatonin on bleomycin-induced lung fibrosis in rats. Journal of pineal research. 2006 Jan;40(1):27-33. PubMed PMID: 16313495.
16. Ermis H, Parlakpinar H, Gulbas G, Vardi N, Polat A, Cetin A, et al. Protective effect of dexpanthenol on bleomycin-induced pulmonary fibrosis in rats. Naunyn-Schmiedeberg's archives of pharmacology. 2013 Dec;386(12):1103-10. PubMed PMID: 23995256.
17. Moeller A, Ask K, Warburton D, Gauldie J, Kolb M. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? The international journal of biochemistry & cell biology. 2008;40(3):362-82. PubMed PMID: 17936056. Pubmed Central PMCID: 2323681.
18. Signorelli P, Ghidoni R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. The Journal of nutritional biochemistry. 2005 Aug;16(8):449-66. PubMed PMID: 16043028.
19. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, et al. Resveratrol: a review of preclinical studies for human cancer prevention. Toxicology and applied pharmacology. 2007 Nov 01;224(3):274-83. PubMed PMID: 17306316. Pubmed Central PMCID: 2083123.
20. Wood LG, Wark PA, Garg ML. Antioxidant and anti-inflammatory effects of resveratrol in airway disease. Antioxidants & redox signaling. 2010 Nov 15;13(10):1535-48. PubMed PMID: 20214495.
21. Bradamante S, Barenghi L, Villa A. Cardiovascular protective effects of resveratrol. Cardiovascular drug reviews. 2004 Fall;22(3):169-88. PubMed PMID: 15492766.
22. Wolter F, Ulrich S, Stein J. Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in colorectal cancer: key role of polyamines? The Journal of nutrition. 2004 Dec;134(12):3219-22. PubMed PMID: 15570015.
23. de la Lastra CA, Villegas I. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochemical Society transactions. 2007 Nov;35(Pt 5):1156-60. PubMed PMID: 17956300.
24. Akgedik R, Akgedik S, Karamanli H, Uysal S, Bozkurt B, Ozol D, et al. Effect of resveratrol on treatment of bleomycin-induced pulmonary fibrosis in rats. Inflammation. 2012 Oct;35(5):1732-41. PubMed PMID: 22707284.
25. Sener G, Topaloglu N, Sehirli AO, Ercan F, Gedik N. Resveratrol alleviates bleomycin-induced lung injury in rats. Pulmonary pharmacology & therapeutics. 2007;20(6):642-9. PubMed PMID: 17035056.
26. Ozkol H, Musa D, Tuluce Y, Koyuncu I. Ameliorative influence of Urtica dioica L against cisplatin-induced toxicity in mice bearing Ehrlich ascites carcinoma. Drug and chemical toxicology. 2012 Jul;35(3):251-7. PubMed PMID: 21939360.

27. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. The Journal of investigative dermatology. 1982 Mar;78(3):206-9. PubMed PMID: 6276474.
28. Kandar R, Hajkova N. Assay of total glutathione and glutathione disulphide in seminal plasma of male partners of couples presenting for a fertility evaluation. Andrologia. 2014 Dec;46(10):1079-88. PubMed PMID: 24124871.
29. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry. 1979 Jun;95(2):351-8. PubMed PMID: 36810.
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75. PubMed PMID: 14907713.
31. McCord J, Fridovich I. Superoxide dismutase, an enzymatic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244(6049–6053).
32. Erel O. A new automated colorimetric method for measuring total oxidant status. Clinical biochemistry. 2005 Dec;38(12):1103-11. PubMed PMID: 16214125.
33. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clinical biochemistry. 2004 Apr;37(4):277-85. PubMed PMID: 15003729.
34. Harma M, Harma M, Erel O. Increased oxidative stress in patients with hydatidiform mole. Swiss medical weekly. 2003 Nov 01;133(41-42):563-6. PubMed PMID: 14691728.
35. Kocyigit A, Koyuncu I, Taskin A, Dikilitas M, Bahadori F, Turkkan B. Antigenotoxic and antioxidant potentials of newly derivatized compound naringenin-oxime relative to naringenin on human mononuclear cells. Drug and chemical toxicology. 2016;39(1):66-73. PubMed PMID: 25826180.
36. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. Journal of clinical pathology. 1988 Apr;41(4):467-70. PubMed PMID: 3366935. Pubmed Central PMCID: 1141479.
37. Vig B, Lewis R. Genetic toxicology of bleomycin. Mutat Res 1978;55(121–145).
38. Povirk L, Austin M. Genotoxicity of bleomycin. Mutat Res 1991;257(127–143).
39. Lim L, Neims A. Mitochondrial DNA damage by bleomycin. Biochem Pharmacol. 1987;36(2769–2974).
40. Amirshahi T, Najafi G, Nejati V. Protective effect of royal jelly on fertility and biochemical parameters in bleomycin-induced male rats. Iranian journal of reproductive medicine. 2014 Mar;12(3):209-16. PubMed PMID: 24799882. Pubmed Central PMCID: 4009575.
41. Abd-Elhakim YM, Khalil SR, Awad A, Al-Ayadhi LY. Combined cytogenotoxic effects of bee venom and bleomycin on rat lymphocytes: an in vitro study. BioMed research international. 2014;2014.
42. Stepniak J, Karbownik-Lewinska M. 17beta-estradiol prevents experimentally-induced oxidative damage to membrane lipids and nuclear DNA in porcine ovary. Systems biology in reproductive medicine. 2016;62(1):17-21. PubMed PMID: 26677908.
43. Cadet J. Oxidative degradation pathways of cellular DNA: product formation and mechanistic insights. Free radical biology & medicine. 2014 Oct;75 Suppl 1:S2. PubMed PMID: 26461303.
44. Abraham SK, Khandelwal N, Hintzsche H, Stopper H. Antigenotoxic effects of resveratrol: assessment of in vitro and in vivo response. Mutagenesis. 2016 Jan;31(1):27-33. PubMed PMID: 26152226.
45. Bingol G, Gulkac MD, Dillioglugil MO, Polat F, Kanli AO. Effect of resveratrol on chromosomal aberrations induced by doxorubicin in rat bone marrow cells. Mutation research Genetic toxicology and environmental mutagenesis. 2014 May 15;766:1-4. PubMed PMID: 24713549.

46. Karafakioglu YS, Bozkurt MF, Hazman O, Fidan AF. Efficacy of safranal to cisplatin-induced nephrotoxicity. The Biochemical journal. 2017 Mar 20;474(7):1195-203. PubMed PMID: 28188255.
47. Venkatesan N, Punithavathi V, Chandrakasan G. Curcumin protects bleomycin-induced lung injury in rats. Life sciences. 1997;61(6):PL51-8. PubMed PMID: 9250724.
48. Lei Y, Wang K, Deng L, Chen Y, Nice EC, Huang C. Redox regulation of inflammation: old elements, a new story. Medicinal research reviews. 2015 Mar;35(2):306-40. PubMed PMID: 25171147.
49. Zhao L, Wang X, Chang Q, Xu J, Huang Y, Guo Q, et al. Neferine, a bisbenzylisoquinline alkaloid attenuates bleomycin-induced pulmonary fibrosis. European journal of pharmacology. 2010 Feb 10;627(1-3):304-12. PubMed PMID: 19909737.
50. Evans P, Lyras L, Halliwell B. Measurement of protein carbonyls in human brain tissue. Methods in enzymology. 1999;300:145-56. PubMed PMID: 9919518.
51. Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. Journal of amino acids. 2012;2012:736837. PubMed PMID: 22500213. Pubmed Central PMCID: 3303626.
52. Cortijo J, Cerda-Nicolas M, Serrano A, Bioque G, Estrela JM, Santangelo F, et al. Attenuation by oral N-acetylcysteine of bleomycin-induced lung injury in rats. The European respiratory journal. 2001 Jun;17(6):1228-35. PubMed PMID: 11491169.
53. Hemmati AA, Rezaie A, Darabpour P. Preventive effects of pomegranate seed extract on bleomycin-induced pulmonary fibrosis in rat. Jundishapur journal of natural pharmaceutical products. 2013 Spring;8(2):76-80. PubMed PMID: 24624192. Pubmed Central PMCID: 3941905.