

The evaluation of different treatment protocols for trauma-induced lung injury in rats

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Background: Lung contusion is an important factor that affects mortality and morbidity of lung injury after blunt chest trauma (BCT). The present study aims to evaluate the effectiveness of different treatment regimens on BCT-induced lung injury.

Methods: A total of 35 Sprague Dawley rats were divided into five experimental groups (n=7): sham, control; BCT; BCT + MP, BCT group treated with methylprednisolone (MP; 30 mg/kg on first day and 3 mg/kg/d on the following days); BCT + Q, BCT group treated with quercetin (Q; 50 mg/kg/d for seven days); and BCT + MP + Q, BCT group treated with the same doses of MP and Q. Serum Clara Cell Protein-16 (CC-16), thiobarbituric acid reactive substances (TBARS), and superoxide dismutase (SOD) levels were analyzed to determine histopathological changes in the lung tissues.

Results: Elevated serum CC-16 and TBARS levels and reduced serum SOD levels were found in the BCT group compared to the Sham group. There was a significant change in the serum CC-16 levels in the BCT + MP group compared to the Sham group. Serum TBARS levels were significantly lower in the BCT + MP and BCT + Q group compared to the BCT group. The combined therapy regimen yielded significantly decreased CC-16 levels and increased serum SOD levels compared to the individual treatment groups. Serum TBARS levels did not significantly differ between the BCT + MP + Q group and the other treatment groups. Compared to the BCT + MP + Q group, the BCT + MP group showed significantly lower alveolar edema (AED) and alveolar exudate (AEX) scores, while the BCT + Q group showed significantly lower peribronchial inflammatory cell infiltration (PICI) and AED scores.

Conclusions: The combined usage of quercetin and low dose MP treatment after initial high dose MP at the early stage of lung injury after BCT is more effective.

Keywords: Blunt; chest; trauma; quercetin; steroid



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Introduction

Traumas injury are the leading cause of death for young people (<45 years) and the fourth leading cause of death in western countries (1). Blunt chest trauma (BCT), responsible for 25% of trauma-related deaths, is seen in patients with approximately 10% (1-3). The most common

type of injury in BCT, which is often associated with other systemic injuries, is pulmonary contusion; its incidence varies between 17% and 25% (3,4).

BCT-induced lung injury depends on trauma severity and alveolocapillary membrane damage (4,5). A better understanding of the pathophysiological mechanisms and cellular events in lung contusion can help in determining

the prognosis and treatment methods for this important clinical condition (4). Therefore, many studies have been performed to clarify the pathophysiological mechanisms, cellular changes, and inflammatory processes occurring in lung consiont (4-8).

Imbalance between defense mechanisms involving antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase with free radical formation and increased levels of oxygen metabolites during inflammatory processes play an important role in lung damage. In other words, oxidative stress is an important pathophysiological event leading to tissue damage (9,10). The end product of lipid peroxidation, thiobarbituric acid reactive substances (TBARS), is used as a common marker of oxidative stress (11). Several studies have indicated that a significant relationship exists between TBARS levels and the extent of tissue damage (12,13). Moreover, Clara Cell Protein-16 (CC-16) is also a marker of acute lung injury (ALI). This protein which is secreted from the tracheobronchial tree and particularly from the terminal bronchioles plays role as an anti-inflammatory effect on lung tissue after inflammatuar process (10).

In these previous studies, effective anti-inflammatory agents preventing and/or reducing inflammation in lung tissues and medicines with antioxidant and histological healing effects on damaged tissues were used for the treatment of ALI. Steroids are known as the strongest anti-inflammatory agents; they function via intracellular receptors by affecting prostaglandin synthesis (14). In particular, administration of high doses of steroids in the early stage of lung injury was found to be effective (15). Another therapeutic agent with anti-inflammatory and antioxidant properties is quercetin (Q). Several studies have shown that Q prevents tissue damage because of its specific effect on the pathophysiological processes induced by free radical formation (16-18).

The purpose of this study is to determine the effects of the antioxidant and anti-inflammatory properties of Q and methylprednisolone (MP) on damaged lung tissue in a lung contusion model of rats and evaluate whether their combined use improves treatment efficacy.

Materials and methods

Experimental protocol

This study was approved by the Ethics Committee of Ondokuz Mayis University for Experimental Animal

Studies. A total of 35 healthy female Sprague Dawley rats weighing 250-300 g were included. Food and tap water were provided ad libitum. All rats were kept in windowless animal quarters with temperature automatically maintained at 24 °C and controlled lighting (12 h light/12 h dark cycle) and humidity (55-60%).

The rats were divided into five groups as follows: Sham (control); BCT; BCT + MP, BCT group treated with i.p. MP (20 mg Prednol-L; Mustafa Nevzat, Turkey); BCT + Q, BCT group that received oral gavage administration of Q (Sigma Chemical Co., St. Louis, MO, USA); and BCT + MP + Q, BCT group treated with MP and Q. Each group had seven animals.

The rats in the BCT groups were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and subjected to chest trauma with 1.96 J of impact energy as described by Raghavendran K. *et al.* (4). The impact energy (E) of the falling weight was calculated from the following equation: $E = m (0.4 \text{ kg}) \times g (9.8 \text{ m/s}^2) \times b (50 \text{ cm})$ (19). The rats in the BCT + MP group were injected with MP i.p. once a day (30 mg/kg injected 5 min after the trauma on day 1 and 3 mg/kg from days 2 to 7) (15). The rats in the BCT + Q group were administered Q once a day orally (50 mg/kg administered 5 min after the trauma on day 1 and 50 mg/kg from days 2 to 7) (20). The rats in the BCT + MP + Q group were administered the same doses of MP and Q for the same duration. All rats were kept under observation until they recovered from the experimental procedure.

After seven days of treatment, all rats were killed with i.p. ketamine hydrochloride and xylazine injections. Their lungs were removed from the thorax for histopathological and immunohistochemical analyses.

Histopathological evaluation

Lung tissue samples were immersed in neutral-buffered 10% formalin solution for 24 h and then embedded in paraffin. For histopathological analysis, 5- μm -thick tissue sections were stained with the hematoxylin-eosin (HE) stain. Pathologists analyzing the samples were blinded, and the samples from all study groups were examined microscopically to determine the extent of peribronchial inflammatory cell infiltration (PICI), alveolar septal infiltration (ASI), alveolar edema (AED), alveolar exudate (AEX), alveolar histiocytes (AHI), and interstitial fibrosis (IF) formation using a 4-point scale (Table 1) (21).

Table 1 All parametres for histopathologic evaluation (4 point scale)

	0	1	2	3
PICI	No	Prominent germinal centers of lymphoid follicles	Infiltration between lymphoid follicles	Confluent bandlike form
ASI	No	Minimal	Moderate	Severe, impending of lumen
AED	No	Focal	In multiple alveoli	Widespread, involving lobules
AEX	No	Focal	In multiple alveoli	Prominent, widespread
AHI	No	Scattered in a few alveoli	Forming clusters in alveolar spaces	Filling the alveolar spaces
IF	No	Focal, minimal	Focal, prominent fibrous thickening	Widespread, prominent fibrous thickening
Abbreviations: PICI, peribronchial inflammatory cell infiltration; ASI, alveolar septal infiltration; AED, alveolar edema; AEX, alveolar exudate; AHI, alveolar histiocytes; IF, interstitial fibrosis.				

Biochemical analysis

Sample preparation

After the rats were sacrificed, blood samples were collected in sterile test tubes. Whole blood was allowed to clot at room temperature for 30 min. Then, the samples were centrifuged at 3,000 $\times g$ for ten minutes at 4 °C. Following centrifugation, the serum was removed and transferred into a clean tube. All samples were stored at –80 °C until analysis. A day before the study, all samples were dissolved at 2–8 °C.

Measurement of serum CC-16 levels

CC-16 levels were determined using a sandwich ELISA plate as per the manufacturer's instructions (Usen Life Science Inc., Wuhan, China). The plate was pre-coated with an antibody specific to CC-16. Then, samples with a biotin-conjugated antibody specific to CC-16 were added to the wells. Next, avidin-conjugated to horseradish peroxidase was added to the wells and the plate was incubated. After the TMB substrate solution was added, the enzyme-substrate reaction was terminated by adding sulphuric acid solution, and the CC-16 levels were determined colorimetrically at 450 nm. The concentration of CC-16 concentration was then determined from the standard curve, and the results have been presented in picograms per milliliter.

Measurement of serum TBARS levels

TBARS were measured using the TBARS Assay Kit (Catalog No. 10009055, Cayman Chemical Company, Ann Arbor, MI, USA). The principle of the test is the formation of the Malondialdehyde-Thiobarbituric acid (MDA-TBA) adduct by the reaction of MDA and TBA under high temperature (90–100 °C) and acidic conditions. The concentration of the MDA-TBA adduct was measured colorimetrically at 530 and 540 nm. The results have been presented in micromoles per liter.

Measurement of serum SOD levels

SOD levels were measured using the Superoxide Dismutase Assay Kit (Catalog No. 706002, Cayman Chemical Company, Ann Arbor, MI, USA). The principle of the test is based on the utilization of a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to cause 50% dismutation of superoxide radicals. The results have been presented in units per milliliter.

Statistical analysis

Biochemical results and histopathological scores were analyzed using IBM SPSS 21.0 for Windows. The results were presented as median (minimum/maximum) or mean \pm standard deviation. All values were evaluated with the non-parametric Mann-Whitney *U* test. Differences were considered significant at $P < 0.05$.

Results

Biochemical examination

Increased serum CC-16 and TBARS levels were found in the BCT group compared to the Sham group ($P = 0.001$ and $P = 0.001$, respectively) (Table 2). However, serum SOD levels were decreased in the BCT group compared to the Sham group ($P = 0.001$). With MP and Q treatment, serum CC-16 levels were decreased in the BCT group compared to the Sham group, but only the BCT + MP group showed a significant difference ($P < 0.05$). Serum TBARS levels were significantly lower in the BCT + MP and BCT + Q group than the BCT group ($P = 0.001$ and $P = 0.001$, respectively). However, serum SOD levels were not significantly difference

Table 2 The comparison of serum troponin I and TNF- α levels in all experimental groups

	Sham	BCT	BCT + MP	BCT + Q	BCT + MP + Q
CC-16 (pg/mL)	24.86 \pm 2.75	72.79 \pm 16.29 [†]	50.98 \pm 14.00 [‡]	58.08 \pm 11.70	29.97 \pm 6.30 ^{‡,§,¶}
SOD (U/mL)	1.28 \pm 0.06	1.02 \pm 0.13 [†]	1.11 \pm 0.05	1.12 \pm 0.05	1.24 \pm 0.07 ^{§,¶}
TBARS (μ mol/mL)	6.72 \pm 1.76	19.11 \pm 1.71 [†]	12.61 \pm 1.61 [‡]	11.49 \pm 2.46 [§]	9.77 \pm 5.33 [§]

The values were represented as mean \pm SD. Sham, control; BCT, blunt chest trauma; BCT + MP, BCT group treated with methylprednisolone; BCT + Q, BCT group treated with quercetin; BCT + MP + Q, BCT group treated with methylprednisolone and quercetin; CC-16, Clara Cell Protein-16; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; MP, methylprednisolone; Q, quercetin. [†], P=0.001 compared to Sham group; [‡], P<0.05 compared to BCT group; [§], P=0.001 compared to BCT group; [¶], P=0.001 compared to BCT group; [§], P=0.001 compared to BCT group; [‡], P=0.001 compared to BCT group; [§], P<0.01 compared to BCT group; [¶], P<0.01 compared to BCT + MP group; [§], P=0.001 compared to BCT + Q group.

between these treatment groups (BCT + MP and BCT + Q) (Table 2). In the BCT + MP + Q group, serum CC-16 and TBARS levels were decreased compared to the BCT group (P=0.001 and P<0.01, respectively). On the other hand, serum SOD levels were the highest in the BCT + MP + Q group among the BCT groups (P<0.01) (Table 2). The combined therapy regimen significantly decreased CC-16 levels compared to the individual treatments with MP and Q (P<0.01 and P=0.001, respectively). Similarly, combined MP and Q treatment significantly increased the serum SOD levels compared to the other two treatment groups (P<0.01 and P<0.05, respectively) (Table 2). Serum TBARS levels were not significantly different in the BCT + MP + Q group compared to the other treatment groups (Table 2).

Histopathological findings

Images of histopathological sections from all study groups are shown in Figure 1. All histopathological scores including PICI, ASI, AED, AEX, AHI, and IF were higher in the BCT group than in the Sham group (P<0.01, P=0.001, P=0.001, P<0.01, P<0.01, and P=0.001, respectively) (Figures 1,2).

The MP caused a significant decrease in the PICI, ASI, AED, and IF scores compared to the BCT group (P<0.05, P<0.05, P=0.001, and P=0.001, respectively) (Figures 1,2). However, Q treatment only caused a significant decrease in the AED and IF scores in BCT + Q group compared to the BCT group (P=0.001 and P<0.01, respectively) (Figure 2). On the other hand, combined MP and Q combined treatment caused a significant decrease in the scores of all the above histopathological parameters (Figure 2).

Compared to all treatment groups; the combined treatment group (BCT + MP + Q) showed significantly lower AED and AEX scores compared to the BCT+MP

group (P<0.05 and P<0.05, respectively) (Figure 2). In addition, PICI and AED scores in combined therapy group showed significantly lower than the BCT + Q group (P<0.05 and P<0.05, respectively). There was no significant difference in the histopathological scores between the BCT + MP and BCT + Q groups (Figure 2).

Discussion

In this study, the effects of the antioxidant and anti-inflammatory properties of Q and MP on damaged lung tissue in a lung contusion model of rats were evaluated. Combined MP and Q treatment significantly reduced AED and AEX formation, which is particularly important in the progression of ALI to acute respiratory distress syndrome (ARDS) according to alone usage of MP. In addition, this combined therapy significantly reduced PICI and AED, which is regarded as an important marker of inflammatory reaction according to alone treatment of Q. Apart from its histopathological effectiveness, the ability of combined therapy to reduce serum CC-16 and to increase SOD levels are considered particularly effective in alleviating lung injury according to the other treatment groups.

ALI developing after BCT is among the most important factors that determine prognosis. This injury occurs in about 50% to 60% of patients with BCT (22). Today, despite the widespread availability of intensive care facilities and advanced respiratory devices, the BCT-related mortality rate is 15.5%, and BCT is the third most common cause of death among young people (23,24).

Lung contusion developing after BCT is a clinical condition accompanied by histopathological findings such as alveolar congestion, alveolar hemorrhage, AED, leukocyte infiltration and ventilation-perfusion defects

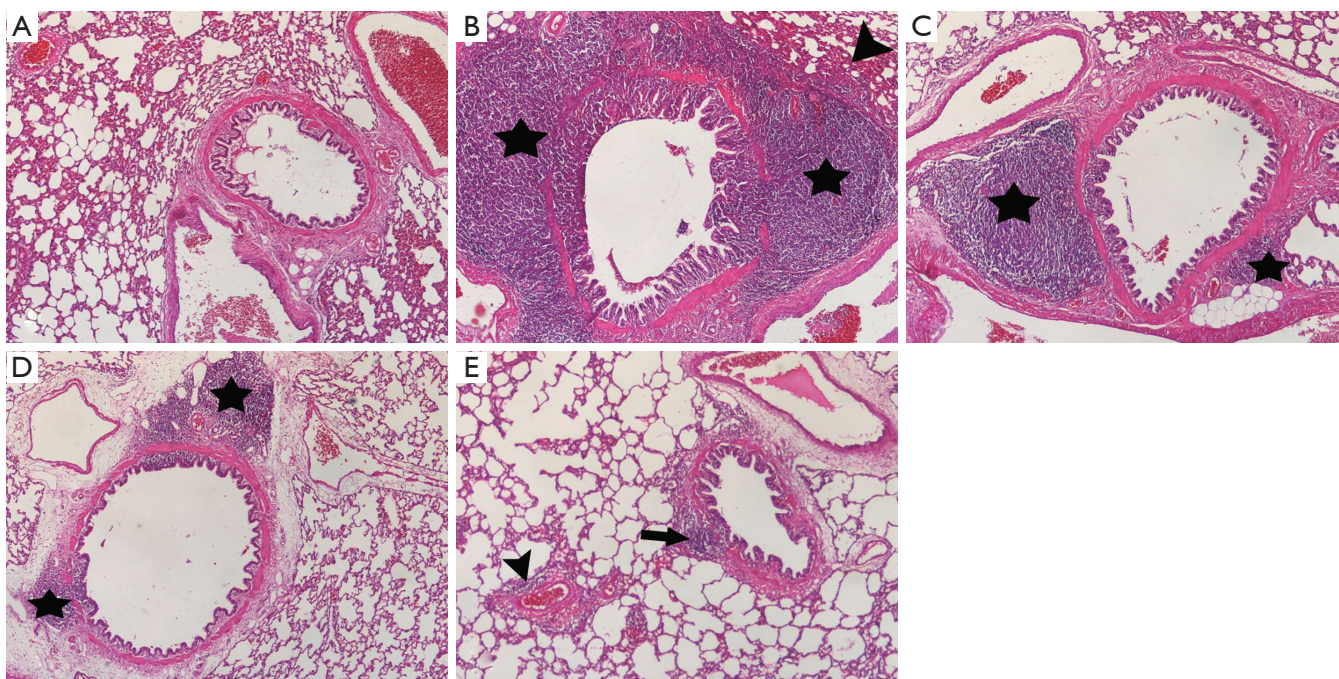


Figure 1 Histopathological evaluation of lung tissues. (A) Sham group, there is no peribronchial inflammatory cell infiltration and alveolar septal infiltration HE $\times 4$; (B) BCT group, severe peribronchial inflammatory cell infiltration showing infiltration between lymphoid follicles, (stars) and severe alveolar septal infiltration collapsing the lumen of the alveols (arrow head) HE $\times 4$; (C) BCT + Q group, there is weak germinal centres of lymphoid follicles (stars) HE $\times 4$; (D) BCT + MP group, moderate peribronchial inflammatory cell infiltration (stars) HE $\times 4$; (E) BCT + MP + Q group, weak peribronchial inflammatory cell infiltration (star) and weak mononuclear cell infiltration in the perivascular area (arrow head). Sham, control; BCT, blunt chest trauma; BCT + MP, BCT group treated with methylprednisolone; BCT + Q, BCT group treated with quercetin; BCT + MP + Q, BCT group treated with MP and Q.

caused by pathological changes, hypoxemia, hypercapnia caused by intrapulmonary shunt development and reduction in compliance, and increased respiratory workload with variable weight and duration (25,26). ARDS occurs because of the disruption of endothelial and epithelial cell functions as a result of lung injury developing after BCT. The most significant histopathological event in the lung tissues of ARDS patients is noncardiogenic high-permeable pulmonary edema (27). Alveolocapillary damage, occurring with fibrosis following structural disorder of type 2 alveolar pneumocytes and increase in myofibroblasts after edema in the lungs, leads to ARDS (28). In our study, similar to the histopathological findings mentioned in the literature, there was a significant increase in the PICI, ASI, AED, AEX, AHI, and IF scores after BCT. We also found that MP and Q combination therapy was more effective than MP or Q alone in reducing AED, which is accepted as a trigger in the process leading to ARDS after lung contusion.

The underlying molecular and physiological changes in lung injury developing after BCT have still not been

clearly elucidated. However, bacterial toxins often increase free oxygen radical release by activating inflammatory cells, proteases, and microembolies, thus leading to expansion of lung pores and increased permeability. Further, apoptosis causes pulmonary edema, which is accepted as the basic mechanism underlying ALI (27,29). Under normal physiological conditions, there is a balance between free oxygen radicals, which are effective in pathophysiology, and the antioxidant enzyme system (SOD, catalase, GSH-Px, glutathione, etc.). An imbalance between oxidants and antioxidants causes DNA and RNA damage due to oxidation of structural lipids, proteins, and polysaccharides at the cellular level (9). Increased TBARS (end product of lipid peroxidation) levels in the damaged tissue is recognized as a marker of oxidative damage (12,13). An experimental study performed by Mokra *et al.* showed that serum TBARS levels correlated with the severity of lung injury and increased significantly in lung injury occurring after meconium aspiration (30). Further, Goraca *et al.* demonstrated that TBARS levels in the BAL fluid increased after oxidative

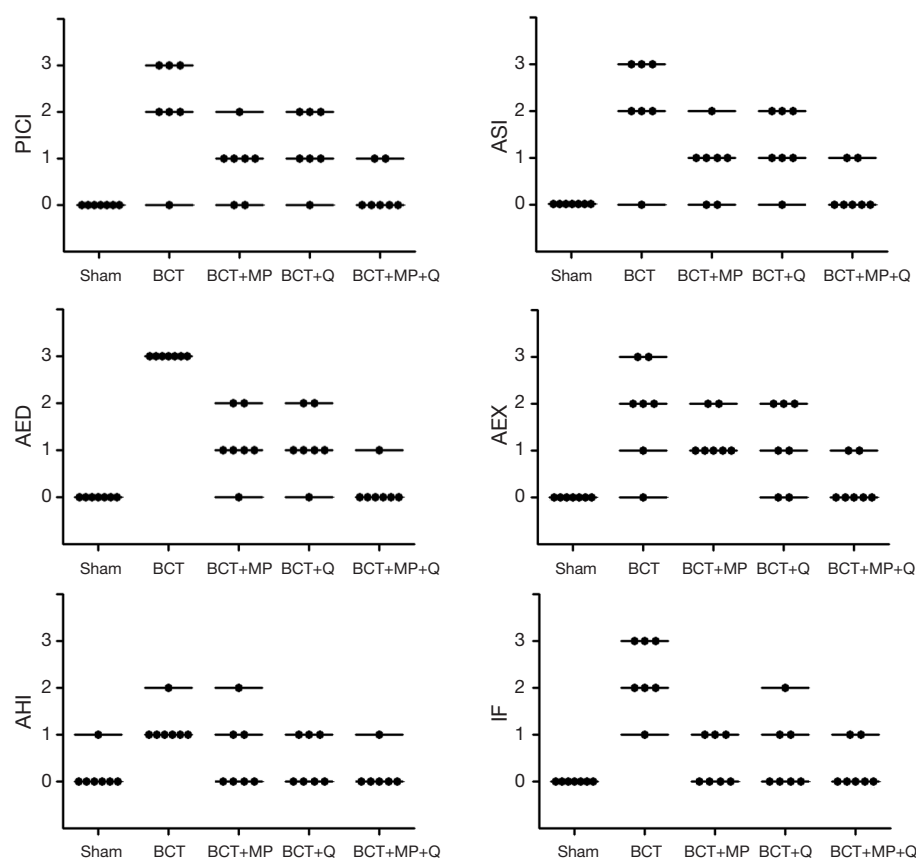


Figure 2 All histopathological scores in study groups were represented. PICI, peribronchial inflammatory cell infiltration; ASI, alveolar septal infiltration; AED, alveolar edema; AEX, alveolar exudate; AHI, alveolar histiocytes; IF, interstitial fibrosis; Sham, control; BCT, blunt chest trauma; BCT + MP, BCT group treated with ethylprednisolone; BCT + Q, BCT group treated with quercetin; BCT + MP + Q, BCT group treated with methylprednisolone and quercetin.

stress and decreased after treatment with alpha-lipoic acid in one of their studies (31). In our study, we measured serum TBARS and SOD levels for determining the oxidant-antioxidant balance. Significant decrease was found in the level of serum SOD after BCT, while there was an increase in serum TBARS levels. We found that while MP and Q therapies when used alone did not cause significant changes in serum SOD level, they only caused significant increase in TBARS levels. We also found that the combined use of MP and Q was the most effective treatment for maintaining the balance of oxidants and antioxidants. This result demonstrated that the combined therapy was significantly effective in reducing oxidative damage and particularly effective in maintaining the oxidant-antioxidant balance.

CC-16 is a protein secreted from tracheobronchial tree and particularly from the terminal bronchioles where the Clara cells reside and plays a protective role against oxidative stress and inflammatory response in the respiratory

tract (10,32). It is considered as a biomarker to determine the severity of lung injury (10,33). In a study performed by Wutzler *et al.*, it has been reported that there was a significant correlation between the level of serum CC-16 and increased respiratory complications after chest trauma (33). In our study, in accordance with histopathological findings, significant increase in serum CC-16 levels were detected after contusion. Moreover, it was determined that the combined therapy of MP and Q decreased serum CC-16 level significantly compared to the other treatment groups.

The pathophysiological mechanism of lung contusion has not been clearly elucidated. Symptomatic and supportive approach is usually considered in the treatment of BCT. These treatment strategies are respiratory failure treatment, post-traumatic pain management, chest physiotherapy, and fluid support (34). In addition to these treatment strategies, medications such as antioxidants, steroids diuretics, and surfactants are used in reducing lung injury (3,5,34-36). The

beneficial effect of steroid administration in lung injury has been found to reduce proinflammatory cytokine production, expression of leukocyte adhesion proteins, and prevention of excessive collagen accumulation in alveolar tissues because of antifibrotic activity (27). Some studies have reported that early administration of high-dose steroids after BCT significantly reduced lung injury and in-hospital mortality; the incidence of post-traumatic complications was also reduced in patients treated with steroids (37,38). Glucocorticoids and antioxidants were used together for treating lung and heart lesions in a variety of experimental studies (39,40).

In conclusion, high-dose MP therapy should be administered in the early stage of lung injury developed after BCT. The initial high-dose MP therapy should be maintained on lower doses. In addition, we suggest that the use of combined quercetin and this MP treatment regimen is more effective for treating lung injury after BCT.

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