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The role of resveratrol on mast cell and chymase and tryptase expression in blunt-chest-trauma-induced acute lung injury in rats

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Abstract: Acute lung injury is a clinical symptom that can cause morbidity, acute respiratory failure, and risk of developing pneumonia. Mast cells are found more commonly in places where antigens can enter the body, such as skin and respiratory and digestive systems, which enables them to be among the first groups of cell to act in the defense mechanism against foreign matter entry. Resveratrol is an active substance which is found in the structure of many plants and which can be used against pathogens due to its antimicrobial effect. The aim of our study is to show the degranulation and heterogeneity of mast cells and the presence of tryptase and chymase secreted from their granules immunohistochemically in acute lung injury which occurs after blunt trauma and to research what kind of an effect resveratrol has on mast cells in this process. Twenty adult female Sprague–Dawley rats were used in the study. The rats were randomly assigned to four groups. The first group was the control group, the second was the contusion group, the third was resveratrol group, and the fourth was the treatment group in which contusion was induced following 30 mg/kg intraperitoneal resveratrol administration. In the present study, acute lung injury was created with trauma and the effects of resveratrol in this process on the distribution, heterogeneity, degranulation, and immunohistochemical characteristics of mast cells were examined. It was concluded that resveratrol, which caused a significant decrease in mast cell degranulation and increase in number following trauma, had an influential role in this process.

Key words: Blunt chest trauma, rat modeling of lung injury, mast cell, resveratrol, tryptase, chymase

1. Introduction

Acute lung injury is a clinical symptom that can cause morbidity, acute respiratory failure [1,2], and risk of developing pneumonia [3]. It generally develops as a result of blunt thoracic trauma that occurs in traffic accidents [4,5]. Besides causing local and systemic changes that may result in septic complications, lung contusion can also influence inflammatory response that occurs as a result of injury [6]. Following trauma, neutrophil increase in intraalveolar areas and deterioration in alveolar capillary circulation and lung parenchymal tissue integrity can develop [7]. It has been reported that susceptibility to infections increases as a result of changes in natural and adaptive immune system due to trauma [8] and a significant increase is observed in the expression of inflammatory mediators such as IL-6 and TNF [7].

Mast cells are found more commonly in places where antigens can enter the body such as skin and respiratory and digestive systems [9], which enables them to be among the first groups of cell to act in the defense mechanism against foreign matter entry [10]. In addition to functioning as cells presenting antigens by processing antigens [11], they are also reported to play a significant role in wound healing [12] and developing immune response against sepsis [13]. Secretory granules of various sizes in the cytoplasm of mast cells [14] include primary mediators, such as stored histamine, tryptase, and chymase, and secondary mediators which are synthesized after stimulation, such as IL-6 and TNF-α [15]. When these cells are stimulated by immunological factors such as cytokines and physical factors such as trauma and sunshine, they can be activated by discharging granule content [14]. Mast cells, which have effects in inflammatory and allergic reactions, are common in lung tissue [16] and they play a role in maintaining homeostasis of respiratory function [17]. Studies conducted have shown an association between the severity of acute lung injury and mast cell density [18,19].

Resveratrol is an active substance which is found in the structure of many plants and which can be used against pathogens due to its antimicrobial effect [20]. Resveratrol, which is a polyphenolic antioxidant, has an antiinflammatory effect [21]. It is reported to have

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antioxidant [22], cancer preventing, and blood sugar lowering effects [23]. Studies conducted have shown that resveratrol has an inflammation suppressing feature in many phases of the inflammatory process [24], antihistaminic effect in the lungs in allergic asthma [25], and a decreasing effect on sepsis which develops during acute lung injury [26].

The most common type of damage in rib cage blunt trauma is lung contusion and acute pulmonary injury occurs as a result of it. Drug therapy can be applied for systemic septic complications which develop as a result of trauma and inflammatory response, in order not to affect the whole body and to minimize tissue damage. At this stage, resveratrol is a commonly preferred active substance used in suppressing inflammatory reactions. Studies conducted have reported the presence of mast cells following trauma and during healing process. Although there are studies showing the association between resveratrol and mast cells in acute lung injury, the number of studies investigating their immunohistochemical characteristics and heterogeneity is limited. The aim of our study is to show the degranulation and heterogeneity of mast cells and the presence of tryptase and chymase secreted from their granules immunohistochemically in acute lung injury which occurs after blunt trauma and to research what kind of an effect resveratrol has on mast cells in this process.

2. Materials and methods

The experimental protocol and all animal procedures were approved by the Experimental Ethics Committee (Experimental protocol number No: 23.07.2014/17).

Twenty female adult Sprague–Dawley rats with an average weight of 250–300 g were used in the study. The rats were randomly assigned to four groups. The first group was the control group in which no intervention was made. The second group was the contusion (trauma) group in which chest contusion was induced. The third group was resveratrol group, which was given 30 mg/kg resveratrol intraperitoneally (i.p.). The fourth group was the treatment group in which contusion was induced following 30 mg/kg i.p. resveratrol administration. All the groups except the first group were given intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) anesthesia, and chest trauma was induced with the help of a standard mechanism. Rats were fixed on a platform with their chests looking up and thoracic trauma was induced with a free fall of a 0.3-kg aluminium weight which would strike an average of 2.2 J energy in an aluminium pipe [27]. The rats in all groups were sacrificed after 24-h follow-up and their lung tissues were taken. The lungs of the rats were fixed in 10% formaldehyde solution for histological examination. Following this, they were blocked in paraffin after undergoing routine tissue tracking procedures.

2.1. Mast cell histochemical staining

In order to examine the normal histological structure of the tissues, 5-µm sections taken from paraffin blocks were stained with Crosmon trichrome staining technique [28]. Ten series of 5-µm thick sections taken from the blocks at 30-µm intervals with 5% Toluidin Blue dye solution prepared in citric acid disodium buffer were stained to determine the mast cells [29]. In order to determine subtypes of mast cells, 5-µm thick sections with 30-µm intervals were taken from each block on the same lame and stained with Alcian Blue (AB)/Safranin O (SO) (AB/SO) combined staining method [29].

In the serial sections prepared to find out the numerical distribution of mast cells, cell counts were performed with a 100 square ocular micrometer (eyepiece graticule). The mast cells at 100 square units of the ocular micrometer were counted with a magnification of ×40. Cell count was performed at 10 randomly chosen different areas of the sections taken from lungs and the arithmetic mean of the results was taken. All the data obtained by calculating the square of 100 square ocular micrometer for ×40 objective magnification with the help of micrometric lame were turned into mast cell number within a unit area of 1 mm^2 [30]. SPSS program was used in the comparison of mast cell counts between groups and one-way ANOVA was conducted [31]. The results were assessed with a minimum 5% error margin.

2.2. Immunohistochemical staining

Five-micrometer lung sections taken from paraffin blocks were stained immunohistochemically by using mouse monoclonal tryptase (Abcam, ab2378, England) and rabbit polyclonal chymase (Biorbyt, orb11030, England) primary antibodies with Streptavidin biotin complex method [32]. Histostain Plus (Zymed kit: 85-6743, United States) kit was used as secondary antibody. After deparaffinization, sections were heated in a microwave oven of 700 W within citrate buffer (pH:6) solution for proteolysis. In order to block endogenous peroxidase activity, the tissues were incubated in 3% hydrogen peroxide solution. Following washing with phosphate buffer solution (PBS), serum in the kit was instilled to prevent nonspecific protein binding in sections. 1/100 dilution of primary antibody was applied to sections and they were stored at +4 °C for one night. Only PBS solution was instilled on negative control group tissues. Following the washing procedure, biotinylated secondary antibody was instilled into sections and incubated at streptavidin-HRP complex after washing. As the last stage, 3,3'-diaminobenzidine (DAP) (The Thermo Scientific, United States) was used as chromogen and the slides were covered with entellan after counterstaining was performed with hematoxylin.

In preparations which were stained histochemically and immunohistochemically, chymase-positive and

tryptase-positive mast cell distribution was evaluated semiquantitatively. Semiquantitative assessment was made as no positive cells in the scanned area (-), 1–2 cells (±), 3–4 cells (+),5–6 cells (++), 7 and more cells (+++) [33].

2.3. Statistical evaluation of data

In this study, variance analysis was performed for analysis of differences between the averages of mast cell count in trial groups, and Duncan multiple comparison tests were conducted for analysis of differences between groups [30].

3. Results

3.1. Histological results

Mast cells stained metamacromasically with toluidin blue in lung tissues of the control and experimental groups were distinguished significantly. In alcian blue/safranin O combined staining conducted to find out the subtypes of mast cells, blue AB (+), red SO (+), and red-blue AB/SO (+) (mixed) mast cells were observed in all groups in the lung tissue (Figures 1 and 2).

Mast cells were observed to be located around sacculus alveolaris, in interalveolar septal connective tissue and around capillaries. Mast cells were found to be scattered in visceral pleura of the lung, bronchial wall, and connective tissue in the wall of terminal and respiratory bronchioles. In addition, mast cells were found among smooth muscles. No difference was found between groups in terms of mast cell localization. Differences were found in numbers and degranulation depending on the application. Mast cells and degranulation were found to be in small numbers in the control group (Figure 2a). A significant increase was found in mast cell number and degranulation in the contusion (trauma) group (Figure 2b). When the resveratrol group was assessed, while increase was found in the cell number and degranulation when compared with the control group, a decrease was found when compared with the treatment group. In the treatment group, a decrease was found in mast cell number and especially in degranulation when compared with the contusion group (Figures 1and 2).

When the groups were evaluated within themselves, the highest increase in mast cell number and degranulation was found in the contusion group. In the treatment group, a significant decrease was found when compared with the contusion group. The suppressive role of resveratrol on the mast cell count and degranulation in contusion of lung tissue was found to be a significant result of our study (Figure 3, Table 1).

3.2. Immunohistochemical results

The immunohistochemically found localizations of chymase- and tryptase-positive mast cells in lung tissue are in parallel with the results we obtained with histochemical stain. The lowest number of mast cells was found in the control group, while the highest number of mast cells was

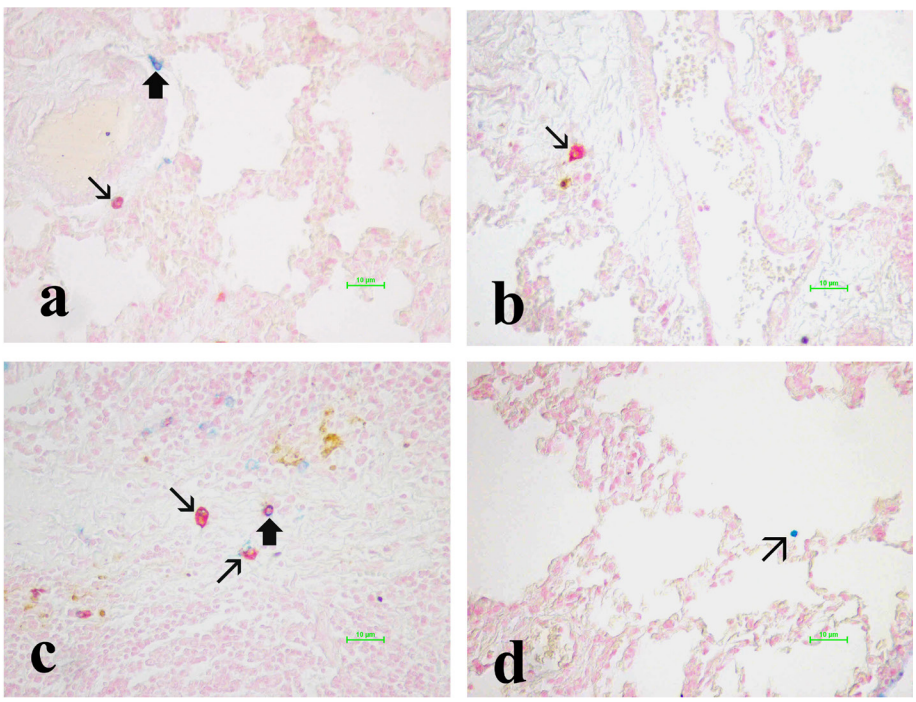

Figure 1. Lung tissues were stained by Alcian blue Safranin O. Control group (a), trauma group (b), resveratrol group (c), and resveratrol+trauma (treatment) group (d). Arrow: mast cells; thick arrow: mast cells of mixed type ×40.

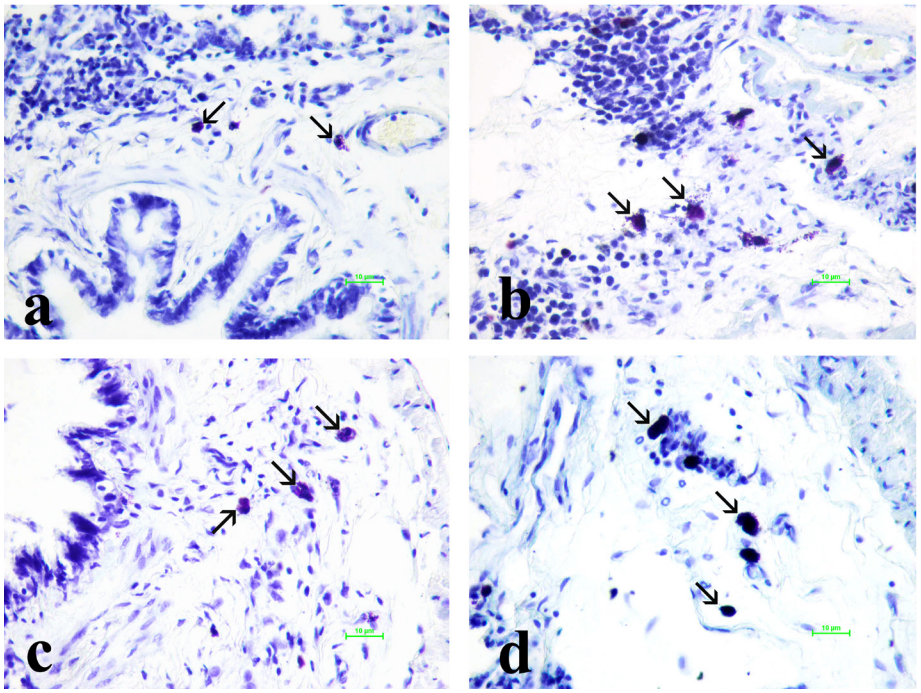

Figure 2. Lung tissues were stained by Toluidine blue. Control group (a), trauma group (b), resveratrol group (c), and resveratrol+trauma (treatment) group (d). Arrow: mast cells, ×40.

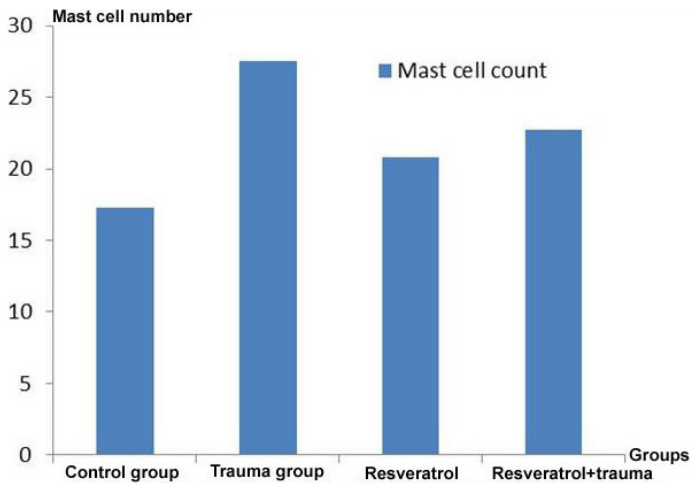

Figure 3. Statistical evaluation of mast cells.

found in the trauma group. Chymase-positive mast cells were found to show a higher increase in number in the trauma group when compared with tryptase-positive cells. Chymase- and tryptase-positive mast cells were found to be less in number in the treatment group when compared with the trauma group. It was found that resveratrol caused decrease in posttrauma chymase- and tryptase-positive mast cell degranulation and number of cells. (Figures 4 and 5, Table 2).

4. Discussion

It has been found that complications can develop after lung contusion and that it can affect inflammatory response with local and systemic changes [6]. It is known that the immune system is stimulated due to trauma [8]. It is reported that mast cells, which are commonly found in lung tissue [16], discharge their granule content when they are stimulated by trauma and immunological content [14] and play a significant role in inflammatory

Table 1. Mast cell counts after staining with toluidin blue in lung tissue.

Group	Mast cell count (× ±Sx /mm^2)	Minimum	Maximum
Contol	17.28±0.93^a	14.40	19.20
Trauma (contusion)	27.52±0.93^c	25.60	30.40
Resveratrol	20.80±0.71^b	19.20	22.40
Resveratrol + Trauma (Treatment)	22.72±1.17^b	19.20	25.60
P	***		

a, b, c: Means with different letters in the column differ significantly ($P < 0.5$)
***: $P < 0.001$

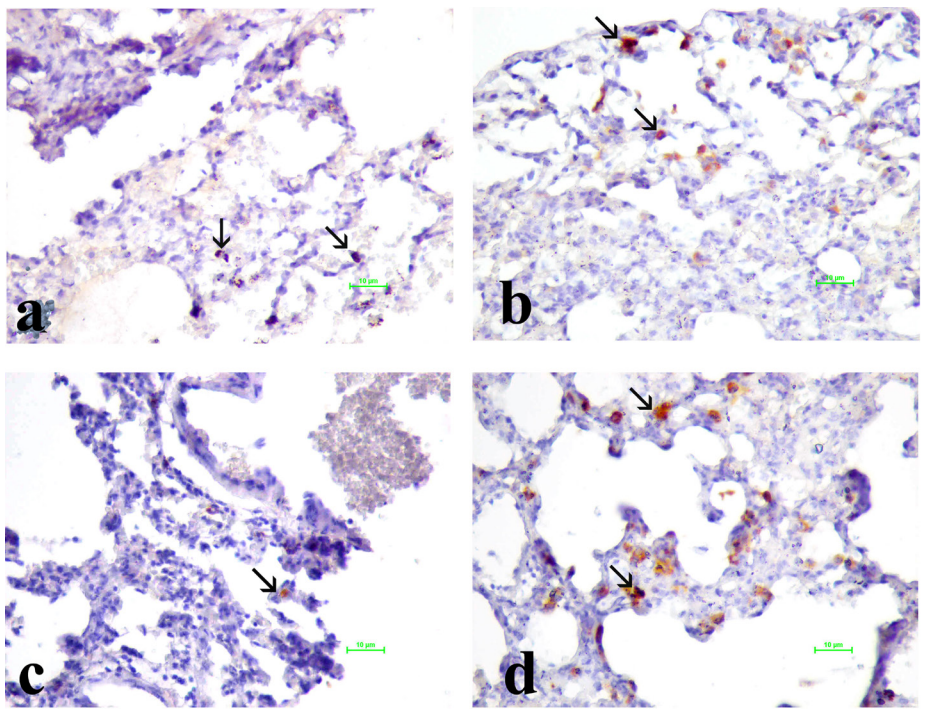

Figure 4. Lung tissues obtained from different treatment groups were immunostained with antibodies against chymase. Control group (a), trauma group (b), resveratrol group (c) and resveratrol+trauma group (d). Arrow: mast cells, ×40.

and allergic reactions [16], wound healing [12], and developing immune response against sepsis [13]. Studies conducted have reported resveratrol to have suppressive characteristics in many stages of the inflammation process [24] and a decreasing influence on sepsis which develops in acute lung injury [26].

Vander et al. reported in their study that the number of chymase-positive mast cells increased in lungs with chronic asthma [34]. Similarly, in another study, it was reported that the number of chymase-positive mast cells in lungs with interstitial pneumonia increased compared to the control group [35]. The significant increase in chymase-positive mast cell numbers in the trauma group in our study is consistent with other studies. In addition, an increase in the number of chymase-positive mast cells was reported in many studies in which viral infections in the lungs, asthma, chronic obstructive pulmonary syndrome, pulmonary hypertension, and lung fibrosis were established, similar to our study [36–39].

Tryptase released from mast cells is known to be a potent growth factor for epithelial cells, airway smooth muscle cells, and fibroblasts. [40]. In many studies conducted

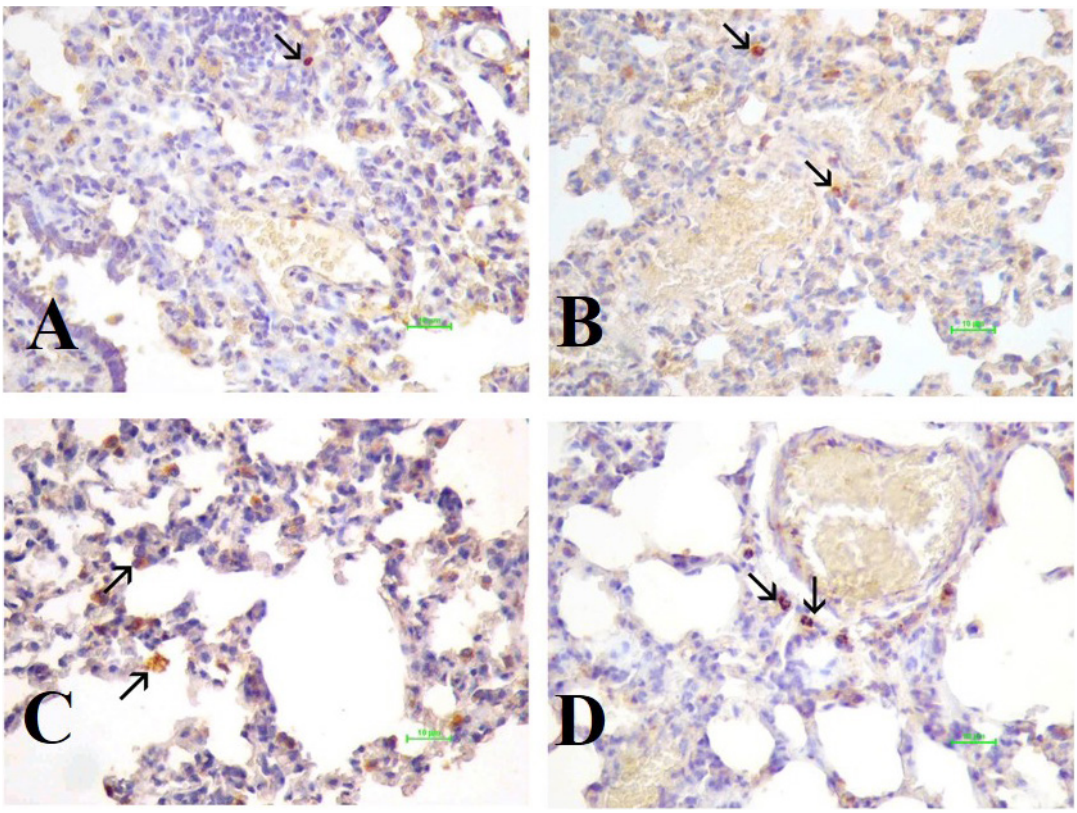

Figure 5. Lung tissues obtained from different treatment groups were immunostained with antibodies against tryptase. Control group (a), trauma group (b), resveratrol group (c), and resveratrol+trauma group (d). Arrow: mast cells, ×40.

Table 2. Tryptase- and chymase-positive cell reaction in lung tissue of different groups.

	Control	Trauma (contusion)	Resveratrol	Resveratrol + trauma (treatment)
Tryptase	±	++	+	++
Chymase	±	+++	+	+

No positive cell (-), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++)

with the lungs, it has been shown that tryptase-positive mast cells are found in the connective tissue around the capillaries [35], between the smooth muscles surrounding the bronchi [41], in the lung parenchyma tissue [42], and in the wall of the alveoli [43]. The findings of our study are similar to the studies in the literature. Gossman et al. reported that there is degranulation in tryptase-positive mast cells in lungs with chronic obstructions [44]. Increases in the number of tryptase-positive mast cells have also been reported in diseases such as acute lung injury, sepsis, pneumonia, and allergic respiratory tract [35,41–43,45,46]. In a different study, it was reported that tryptase-positive mast cell increase was formed in the cortical region of the brain in rats with head trauma [47]. In our study, tryptase-positive mast cell increase in trauma and resveratrol groups is in parallel with other studies in the literature [35, 41–43,45–47]

There are studies which have similar results to the ones we found in our study with Alcian blue/safranin O combined staining. In their study, Wilkes et al. [48] showed the presence of red-stained SO(+) and blue-stained AB(+) mast cells. In their study, Tomita et al. [49] reported a numerical increase in safranin-positive mast cells in rat lungs in helminth infection.

In studies conducted on acute lung injury in rat lungs [50], anaphylactic mouse skin [51], and humans [52],

resveratrol has been reported to show a decreasing effect on mast cell degranulation. Some studies have reported that while an increase was found in mast cell number in rats with allergic rhinitis [53], fibrosis prostate [54], and urinary bladder with fibrosis, there was a decrease in numerical increase in groups administered resveratrol. The most striking results of our study, i.e. the decrease in the increase in mast cell degranulation and mast cell numbers, are in parallel with those of other studies.

Mast cells in lung tissue have been found to increase in number in rats exposed to hyperoxia [55] and in mice with sistosoma infestation [56]. Mast cells which increase in acute lung injury have been found to decrease when given montelukast used in asthma treatment [57]. Degranulation has been reported in lung fibrosis [58] and in rats in which experimental migraine head ache was induced [59]. It has been found that less degranulation developed in groups administered herbal medical therapy when compared with rats in which chronic obstructive lung disease developed with cigarette smoke [60]. Our results that the number of mast cells increased due to trauma and complications and that this increase was suppressed with resveratrol are in parallel with those of the aforementioned studies [55–60].

Acute lung injury is a symptom which can result in death if not treated immediately and which can cause pneumonia and a systemic disorder that can influence the whole body, mostly in respiratory failure that develops as a result of chest traumas. Mast cells, which are known to have a role in the homeostasis of respiratory functions, contribute to defense and wound healing process. Resveratrol, an antioxidant, is known to be used in inflammatory reactions. In the present study, acute lung injury was created with trauma and the effects of resveratrol in this process on the distribution, heterogeneity, degranulation, and immunohistochemical characteristics of mast cells were examined. It was concluded that resveratrol, which caused a significant decrease in mast cell degranulation and increase in number following trauma, had an influential role in this process. As a conclusion, we believe that our results will contribute to the association between resveratrol, trauma, and mast cells; and that physiological, biochemical, and different immunohistochemical studies to be conducted will clarify the mast cell function in trauma and inflammatory response in more detail.

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