EFFECT OF MONTELUKAST ON ACUTE LUNG INJURY INDUCED BY INTESTINAL ISCHEMIA AND REPERFUSION IN RATS

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ABSTRACT
Aims: Lungs are the most affected remote organs after intestinal ischemia/reperfusion. The aim of our study is to investigate the effects of montelukast on intestinal ischemia/reperfusion-induced acute pulmonary injury, mast cells, and apoptosis.

Materials and methods: Wistar Albino rats were used in the study. Control, intestinal ischemia/reperfusion (IIR), and IRR+montelukast groups, each comprising six rats, were constructed. Ischemia was induced by ligating the superior mesenteric artery for 60 min, followed by 60 min of reperfusion. Montelukast at a dose of 7 mg/kg was administered intraperitoneally 10 min before reperfusion. Lung tissues were fixed in neutral formalin. Paraffin sections were stained with hematoxylin-eosin and toluidine blue to assess mast cells. Pulmonary damage was graded, and mast cells were counted. Apoptotic cells were examined by terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining.

Results: Thickening of the inter-alveolar wall, capillary congestion, haemorrhage, edema, and accumulation of numerous inflammatory cells around the vessels were observed in the lung specimens of the IIR group. Wall thickness, edema, haemorrhage, and inflammatory cell infiltration were less severe in the montelukast-treated group. The number of mast cells and apoptotic cells with positive TUNEL staining was found to be increased in the IRR group and decreased in the montelukast-treated group.

Conclusion: Montelukast alleviated tissue damage and reduced the number of mast cells and apoptotic epithelial cells caused by IIR-induced acute pulmonary injury. These results suggest a new approach for the prevention or treatment of secondary lung injury.

Key words: Intestinal ischemia/reperfusion, lung, montelukast, apoptosis, mast cells.

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Introduction
Intestinal ischemia and reperfusion (IIR) is a serious clinical problem that can cause necrotizing enterocolitis, midgut volvulus, mesenteric ischemia, haemorrhage, and septic shock and results in mortality in 60-80% of the cases. Viscera other than the intestines are also affected. The lungs are amongst the most affected remote organ, with ensuing acute lung injury, which is a major cause of mortality1-3. The molecular pathway that results in acute lung injury induced by IIR remains unclear. It has been shown that lung damage is due to endotoxins of bacterial origin and proinflammatory cytokine release from the ischemic tissues; moreover, both the number of inflammatory cells and level of free oxygen radicals increase4-5. Leukotrienes, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, Toll-like receptor 4, and NF-kappa B p65 expression increase in response to IIR6-8. There is no widely accepted effective treatment for the alleviation or prevention of lung injury due to IIR, although there is a growing body of research using different medications and substances1,8,9-14.

Granules of the mast cells contain more than 50 mediators, including neuropeptides. It is acknowledged that neurogenic inflammation is important in the pathophysiology of chronic lung diseases and that neuropeptides, mast cells, and mast cell mediators also play roles15. Mast cells are present in the bronchi, bronchioles, alveolar parenchyma, pleura, and around the pulmonary ves-
mast cells are activated during as well as play roles in allergic and inflammatory processes of the respiratory system. Cytokines such as IL-1 and TNF-α, chemokines, histamine/serotonin, proteases, eicosanoids, and lipid mediators are secreted from the mast cells. Leukotrienes are important mediators of inflammation, vascular injury, ischemia, and obstructive pulmonary disease. Produced by the oxidation of arachidonic acid, cysteinyl leukotrienes (CysLTs), which comprise the proinflammatory mediator leukotrienes C4, D4, and E4, affect leukocyte migration and activation. CysLTs receptors, which are coupled to G proteins, have two subtypes, namely CysLT1 and CysLT2. Leukotriene receptors are found in neutrophils, monocytes, macrophages, mast cells, basophils, dendritic cells, and lymphocytes.

Montelukast is a leukotriene receptor antagonist (CysLT1) that prevents the activation of mast cells that are increased in allergic diseases such as asthma, atopic dermatitis, and psoriasis and exerts an anti-inflammatory effect.

To our knowledge, no study has investigated the protective effects of montelukast on acute lung damage caused by IIR. Here, we aimed to examine the impact of montelukast on mast cell count and apoptosis, which are believed to play roles in the pathological physiology of acute lung damage caused by experimental IIR in rats.

Materials and methods

Animals

All experimental protocols were performed according to the guidelines for the ethical treatment of experimental animals and were approved by the Animal Care and Use Local Ethics Committee of Abant Izzet Baysal University, School of Medicine (Bolu, Turkey). Eight-week-old male Sprague-Dawley rats (200-250 g) were housed at a constant room temperature (22 ± 2°C) under a 12-h light/dark cycle. They were fed standard rat chow (210 kcal/100 g/day) and drank tap water. The animals were fasted for 12 h before starting the experiments but had free access to water. Anaesthesia was induced by intramuscular injection of ketamine hydrochloride (50 mg/kg, Ketalar®; Parke Davis, Eczacibasi, Istanbul, Turkey) and xylazine (10 mg/kg, Rompun®; Bayer AG, Leverkusen, Germany), and all procedures were performed without mechanical ventilation. The animals were placed on the operating table in the supine position, immobilized at four points, and subjected to abdominal trichotomy and antisepsis with a povidone detergent. A midline abdominal laparotomy with exposure of the abdominal cavity was performed to expose the superior mesenteric artery (SMA).

Treatment groups

Animals were randomly divided into three treatment groups (n = 6 per group). In the sham control group, a laparotomy was performed to expose the SMA with no further treatment. In the ischemia-reperfusion (I-R) group, the SMA was isolated, and a 60-min ischemic period was maintained by complete occlusion of the SMA using microvascular clips to interrupt the mesenteric blood flow. To exclude the collateral blood supply from the right colic and jejunal arteries, we used the technique developed by Megison et al. The clips were carefully removed after the ischemic period to allow 60 min of reperfusion. In the I-R/montelukast (I-R/M) group, ischemia and reperfusion proceeded as for the I-R animals, except the rats received an intraperitoneal injection of montelukast (7 mg/kg), 10 min before the reperfusion period. Sixty minutes after the procedure, all animals were euthanized by intracardiac puncture, and tissue samples were obtained.

Histology

Lung tissue specimens from the right middle lobe were fixed in neutral formalin for 48 h, embedded in paraffin, and cut into 5-μm sections. Hematoxylin-eosin staining was carried out to assess the general structure of the lung and grade the injury. The lung tissue was examined with respect to damage, haemorrhage, perivascular and peribronchial cellular infiltration, alveolar wall thickness, alveolar exudate, interstitial fibrosis, and interstitial neutrophil infiltration and graded on a four-point scale as follows: 0: normal; 1: minimal; 2: moderate; 3: severe changes. To demonstrate the mast cell distribution, samples were stained using toluidine blue, and mast cells were counted in six fields under 400× magnification. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was carried out to demonstrate apoptosis, and TUNEL-positive cells were counted. Two blinded observers performed histologic analyses.
Statistical analysis

Data pertaining to the groups were compared using the non-parametric Kruskal–Wallis test, whereas the groups were compared with each other using the Mann-Whitney U-test. A p value of 0.05 was deemed to be statistically significant.

Results

The lung injury scores of the groups are presented in Figure 1. The differences in perivascular and peribronchial cellular infiltration, alveolar wall thickness, haemorrhage and increased neutrophil count between the IIR and control groups were statistically significant, whereas those between the control and montelukast-treated groups were not. When the IIR- and montelukast-treated groups were compared, cellular infiltration, alveolar wall thickness, and haemorrhage were reduced significantly.

Comparison with the lung tissue of the control group (Figure 2a) showed an increase in inflammatory cells around the vessels and bronchi, edema, an increase in alveolar wall thickness, capillary congestion and haemorrhage (Figure 2b, Figure 2c), fibrotic connective tissue in one subject (Figure 2d), and haemorrhage within the septum and alveoli septum (Figure 2e) in the IIR group. Conversely, cellular infiltration, wall thickness, and haemorrhage were significantly less severe in the montelukast-treated group (Figure 2d). Histological examination showed that the areas of perivascular and peribronchial infiltration, edema, and alveolar wall thickness were considerably reduced and that there was no intra-alveolar haemorrhage in the montelukast-treated group, although capillary congestion persisted in some regions (Figure 2f).

Fig. 1: Lung Injury Score. a p<0.05 versus control group (p=0.003), b p<0.05 versus IIR group (p=0.003), c p<0.05 versus control group (p=0.002), d p<0.05 versus IIR group (p=0.007), e p<0.05 versus control group (p=0.002), f p<0.05 versus control group (p=0.019) and p<0.05 versus IIR group (p=0.007), g p<0.05 versus control group (p=0.006).

Fig. 2: Lung tissue sections of the control, Intestine ischemia/reperfusion (IIR) and IIR+Montelukast groups. (2a) Control group, (2b) increased inflammatory cells in IIR group, thickened alveolar walls, capillary congestion, (2c) edema and haemorrhage, (2d) increased fibrotic connective tissue, (2e) alveolar septum and intra-alveolar haemorrhage was observed. Cellular infiltration, wall thickness, edema and haemorrhage were significantly decreased in the montelukast-treated group. Capillary congestion persisted in some regions, intra-alveolar haemorrhage not seen in the montelukast-treated group (2f). H&E staining.

Fig. 3: Mast cells and TUNEL-positive cells in the rat lung. * p<0.05 versus control group (p=0.004), ** p<0.05 versus control group (p=0.005) and p<0.05 versus IIR group (p=0.015), # p<0.05 versus control group (p=0.004), ## p<0.05 versus control group (p=0.003) and p<0.05 versus IIR group (p=0.003).

The number of mast cells and apoptotic cells with positive TUNEL staining is presented in Figure 3. Apoptotic epithelial cells in both the IIR and IIR+montelukast groups were significantly increased compared with the control group. However, the number of apoptotic epithelial cells in the montelukast-treated group was less than that in the IIR group. We noted that the number of mast cells in the IIR group was higher than that in the control group, whereas montelukast treatment reduced the number of mast cells, although not to the level of the control group. Copious amounts of mast cells around the vessels (Figure 4a), usually degranulated (Figure 4b), were observed in the IIR group; however, the density of the mast cells in the montelukast-treated group was decreased (Figure 4c). Few TUNEL-positive epithelial cells were detected in the control group (Figure 4d), whereas more were present in the IIR group (Figure 4e), and less in the montelukast-treated group than in the IIR group (Figure 4f).
Reducing TNF-α production is an important strategy for preventing IIR damage\(^\text{50}\). Montelukast lowers TNF-α secretion from the mast cells\(^\text{40}\) and myeloperoxidase (MPO) and lipid peroxidase (LPO) levels in the lungs, liver, heart, and kidneys during septic shock\(^\text{41}\). Induction of leukocyte infiltration and apoptosis in the lungs, liver, and aortic tissues by TNF-α, a pro-inflammatory cytokine, has been demonstrated following IIR (50). MPO activity, haemorrhage, edema, and vascular permeability increase significantly in the lungs\(^\text{52-55}\). Furthermore, increased expression of intercellular adhesion molecule (ICAM)-1, integrins, and fibronectin in lung tissue has been observed\(^\text{14, 34, 35, 50}\). ICAM expression was shown to be increased to prevent neutrophil migration, accumulation, and activation\(^\text{47}\). Additionally, increased mast cells have been shown in lung tissues of dogs following IIR\(^\text{58}\).

It has been reported that the increase in mast cells in lungs following IIR could be reduced by chloro- melyn sodium, ketotifen, and protamine\(^\text{2}\). In the present study, we also observed that montelukast exerted a lung injury attenuating effect and lowered the number of mast cells. Cell infiltration, haemorrhage, and wall thickness were attenuated in subjects who received the treatment. The increase in the number of mast cells in the IIR group as opposed to the decrease in the number of mast cells in the montelukast group suggested that mast cells play a role in the pathological physiology of lung injury following IIR.

It has been reported that apoptosis, mediated by Fas, Fas ligand, and caspase-3, was increased in bronchial and type 2 alveolar epithelia and that cytokines played roles in IIR-induced lung injury\(^\text{1,3, 33, 56, 59, 60}\). Another study reported significant increases in Bax and Bcl-2 expression, with Bax being expressed twice as much as Bcl-2\(^\text{41}\). Pro-apoptotic and anti-apoptotic gene expression has been evaluated, and an anti-apoptotic gene was found to be significantly underexpressed\(^\text{2}\). It has been argued that TUNEL-positive epithelial cells increase in the lungs after IIR, but oncotic cell death was more common than apoptotic or necrotic cell death\(^\text{63}\). In the present study, we demonstrated apoptotic cell death by the TUNEL method. Apoptosis was increased in the epithelial cells of the IIR group, but was lower in the montelukast-treated group. Montelukast alleviates injury by inhibiting the release of CysLTRs and TNF-α from mast cells, thereby reducing the number of apoptotic cells.

**Discussion**

IIR frequently causes dysfunction of distant organs, including the lung, liver, heart, and kidney, and is also referred to as “multiple organ dysfunction syndrome” (MODS)\(^\text{34, 35}\). Lungs come at the forefront of organs affected by IIR, and the injury is particularly sustained during reperfusion\(^\text{33}\). A significant amount of cytokines released during the inflammatory response is secreted by the mast cells\(^\text{36}\). Secondary to the local inflammatory response, increased vascular permeability of small vessels, congestion of the alveoli, effusion, haemorrhage, edema, and an increase in the number of inflammatory cells, particularly neutrophils, occur\(^\text{37, 38}\). A significant amount of reactive oxygen species (ROS) is released by neutrophils\(^\text{39, 40}\). Antioxidant substances/medications have been reported to alleviate lung injury and decrease ROS in IIR\(^\text{41}\). Cysteinyl leukotrienes are mediators secreted from mast cells that play roles in increased leukocyte migration and activation in the injured lung following IIR, causing capillary dilation and edema\(^\text{40, 41}\). CysLT1 receptor expression increases following IIR\(^\text{41}\). Montelukast is a CysLT1 receptor antagonist, lowering its expression and alleviating inflammation\(^\text{26, 30, 42, 43}\). Montelukast primarily inhibits CysLTRs in allergic rhinitis and atopic asthma. Additionally, montelukast decreases degranulation of eosinophils in asthmatic patients\(^\text{44, 45}\). Furthermore, it is inhibited secondarily in cystic fibrosis, viral bronchiolitis, paranasal sinus disease, nasal polyposis, allergic conjunctivitis, atopic dermatitis, chronic urticaria, pulmonary fibrosis, atherosclerosis, sepsis, haemorrhagic shock, and hepatic ischemia/reperfusion damage\(^\text{46-49}\).
One of the limitations of our study is limited number of animals which may lead to conflicting results in the groups. Therefore, further experimental studies are necessary to confirm our findings.

In conclusion, Montelukast alleviates lung injury following IIR. The severity of injury is related to the increase in the number of mast cells. The number of apoptotic epithelial cells decreases as the number of mast cells decreases. These results, which are presented for the first time, introduce a new approach to the prevention or treatment of secondary lung injury.

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