Cysteinyl-leukotriene Receptor Antagonist Montelukast Ameliorates Acute Lung Injury following Hemorrhagic Shock in Rats

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الخلاصة

ضرر الرئة الحاد الناتج عن الصدمة النزفية وإنعاشها بالسوائل الوريدية يعد مساهم مهم في الامراضية ونسبة الوفيات المتأخرة لدى مرضى الصدمة. الصدمة النزفية المتبوعة بالإنعاش يعد كذريعة تؤدي وبصورة متكررة إلى متلازمة الاستجابة الالتهابية الشاملة والإجهاد ألتأكسدي الذي ينتج عنه متلازمة الاختلال الوظيفي للأعضاء المتعددة المتضمن ضرر الرئة الحاد. المونتيلوكاست هو مثبط لمستقبل السيستينيل ليكوتراين يبدي فعالية تضاد الالتهاب والإجهاد ألتأكسدي. تم أخذ ثمانية عشر جرذا أبرصا بالغا في الدراسة وتم توزيعهم على ثلاثة مجاميع:

المجموعة الأولى: هي المجموعة المزيفة التي تمر بها جميع ظروف التجربة دون تعريضها الى الصدمة النزفية وإنعاشها وعددها ستة جرذان. المجموعة الثانية: هي مجموعة السيطرة التي تم تعريضها إلى الصدمة النزفية لمدة ساعة واحدة وإنعاشها بالرنگر لاكتيت لمدة ساعة أيضا ولم يتم علاجها وعددها ستة جرذان. المجموعة الثالثة: وهي المجموعة التي تم إعطائها المونتيلوكاست (montelukast) بجرعة ٧ ملغ\ كغم داخل البريتون نصف ساعة قبل تعريضها للصدمة ومباشرتا قبل إنعاشها وعددها ستة جرذان. الصدمة النزفية تحدث بواسطة تعريض الجرذان الى ٥٠% من فقدان الدم (٣٠مل\كغم) عن طريق سحب الدم مباشرتا من القلب من الجهة اليسرى من الصدر في غضون دقيقتين وتترك بحالة الصدمة لمدة ساعة واحدة ثم يتم إنعاشها بالرنگر لاكتيت وريديا عن طريق الذيل بحجم يساوي مرتين من حجم الدم المفقود أي (٦٠مل\كغم) خلال ساعة واحدة. عند نهاية التجربة (ساعتين بعد إكمال الإنعاش بالسوائل) تم أخذ نماذج من الدم وتم قياس عاملي الالتهاب TNF-α و IL-6. بعد ذلك تم إزالة الرئة و أخذ الرئة اليسرى ومجانستها وتم قياس عامل التأكسد المالون ثنائي الالديهايد (MDA) وكذلك مستوى الكلوتوثايون المختزل (GSH) فيها وتم أخذ الرئة اليمنى و فحص التغيرات النسيجية الحاصلة فيها. الصدمة النزفية سببت زيادة معنوية ($P < ٠.٠٥$) في مستوى عاملي الالتهاب TNF-α و IL-6 في مصل الدم وكذلك ارتفاع بمستوى عامل التأكسد المالون ثنائي الالديهايد (MDA) في الرئة وسببت الصدمة أيضا انخفاض معنوي ($P < ٠.٠٥$) بمستوى الكلوتوثايون المختزل (GSH) في الرئة بالمقارنة مع المجموعة المزيفة. أما بالنسبة للنتائج النسيجية فان جميع الجرذان التي تعرضت للصدمة أظهرت أضرار معنوية في الرئة ($P < ٠.٠٥$). إن العلاج بالمونتيلوكاست أظهر تأثيرا معنويا ($P < ٠.٠٥$) على الالتهاب من خلال منع ارتفاع عاملي الالتهاب TNF-α و IL-6 وعلى الإجهاد التأكسدي بالرئة من خلال منع ارتفاع عامل التأكسد المالون ثنائي الالديهايد ($P < ٠.٠٥$). كذلك ان العلاج بالمونتيلوكاست والأم كي ٨٨٦ منع النقصان بمستوى الكلوتوثايون المختزل بصورة معنوية ($p< ٠.٠٥$). تحليل النتائج نسيجيا أظهر ان العلاج بالمونتيلوكاست سبب انخفاضا معنويا ($P < ٠.٠٥$) من حدة أو شدة ضرر الرئة الحاصل بالجرذان المتعرضة للصدمة.

نتائج دراستنا أظهرت ان المونتيلوكاست قد حسن من ضرر الرئة الحاصل لدى الجرذان المتعرضة للصدمة النزفية من خلال تثبيط الالتهاب والإجهاد التأكسدي مبينا دورها في نشوء التهاب الرئة الناتج عن الصدمة النزفية.

Abstract

Acute lung injury following hemorrhagic shock/resuscitation is an important contributor to late morbidity and mortality in trauma patients. Hemorrhagic shock followed by resuscitation is considered as an insult frequently induces a systemic inflammatory response syndrome and oxidative stress that results in multiple-organ dysfunction syndrome including acute lung injury. Montelukast is a cysteinyl leukotriene receptor antagonist exerts an anti inflammatory and antioxidant activity.

Objectives

The objective of present study was to assess the possible protective effect of montelukast against hemorrhagic shock-induced acute lung injury via interfering with inflammatory and oxidative pathways.

Materials and Methods

Eighteen adult Albino rats were assigned to three groups each containing six rats: group I, sham group, rats underwent all surgical instrumentation but neither hemorrhagic shock nor resuscitation was done; group II, Rats underwent hemorrhagic shock (HS) for 1hr then resuscitated with Ringer's lactate (1hr) (induced untreated group, HS); group III, HS + montelukast (7 mg/kg i.p. injection 30 min before the induction of HS, and the same dose was repeated just before reperfusion period). At the end of experiment (2 hr after completion of resuscitation), blood samples were collected for measurement of serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). The lungs were harvested, excised and the left lung was homogenized for measurement of malondialdehyde (MDA) and reduced glutathione (GSH) and the right lung was fixed in 10% formalin for histological examination.

Results

Montelukast treatment significantly reduced the total lung injury score compared with the HS group ($P < 0.05$). Montelukast also significantly decreased serum TNF-α & IL-6; and lung MDA as compared with the HS group ($P < 0.05$). Montelukast treatment significantly prevented the decrease in the lung GSH levels compared with the HS group ($P < 0.05$).

Conclusions

The results of the present study reveal that montelukast may ameliorate lung injury in shocked rats via interfering with inflammatory and oxidative pathways implicating their role in the pathogenesis of hemorrhagic shock-induced lung inflammation.

1. Introduction

Hemorrhagic shock (HS) is a commonly encountered complication within a blunt traumatic or surgical injury. Hemorrhagic shock followed by resuscitation (HSR) is conceived as an insult frequently induces a systemic inflammatory response syndrome (SIRS) that results in multiple-organ dysfunction syndrome (MODS) [1, 2] including acute lung injury (ALI), which is a major clinical problem, leading to significant mortality and morbidity [1, 3]. The mechanism of pathogenesis of SIRS in the field of HS is complex and a variety of mechanisms are implicated. The most widely recognized mechanisms are ischemia and reperfusion (I/R) and stimulation of cells of the innate immune system [4]. Ischemia and reperfusion is mainly participating in oxidative stress and SIRS arising during post-ischemic resuscitation. I/R injury is, by itself, a potent inflammatory trigger, increasing cytokine release, reactive oxygen species generation, and endothelial activation, with consequent nitric oxide production and expression of adhesion molecules [5]. Neutrophils are the major cellular elements involved in acute lung inflammation after resuscitated hemorrhagic shock [6]. Studies have shown that neutrophils are activated following HS [7] and that lung injury is associated with an increased neutrophils accumulation in the lungs after HS [8]. The activated neutrophils appear to infiltrate the injured lung in parallel with increased expression of adhesion molecules on endothelial cells and elevated local chemokines/cytokines levels following HS [7].

A selective reversible CysLT1 receptor antagonist, montelukast is used in the treatment of allergic rhinitis and asthma [9]. Montelukast has gastroprotective effect on indomethacin-induced ulcerations and alendronate -induced lesions of the rat gastric mucosa attributed to its ameliorating effect on oxidative damage and myeloperoxidase (MPO) activity [10,11]. Montelukast ameliorates burn- and sepsis-induced multiorgan damage by a neutrophil-dependent mechanism [12,13]. Furthermore, montelukast has been shown to reduce I/R-induced oxidative damage in the liver, intestine, kidney, testes and bladder, through its anti-inflammatory and antioxidant properties [14,15,16,17]. However, to our knowledge, the effect of montelukast on ALI caused by HSR has not been reported.

2. Materials and Methods

A total of eighteen adult male Albino rats weighing 150-220 g were purchased from Animal Resource Center, the Institute of embryo research and treatment of infertility, Al-Nahrain University. They were housed in the animal house of Kufa College of Medicine in a temperature-controlled (25°C) room with alternating 12-h light/12-h dark cycles and were allowed free access to water and chow diet until the start of experiments. After the 1st week of acclimatization the rats were randomized into three groups as follow:

I.Sham group: this group consisted of 6 rats; rats underwent the same anesthetic and surgical procedures for an identical period of time as shock animals, but neither hemorrhage nor fluid resuscitation was performed.

II. Control group: (induced untreated group): this group consisted of six rats; rats underwent hemorrhagic shock (for 1hr) then resuscitated with Ringer's lactate (RL) (for 1hr), and left until the end of the experiment.

III.Montelukast treated group: this group consisted of 6 rats; Rats received montelukast 7 mg/kg i.p. injection 30 min before the induction of HS, and the same dose was repeated just before reperfusion period.

The drug was purchased from (Cayman chemical, USA) and prepared immediately before use as a homogenized solution in 2% ethanol [14]. Each dose was homogenized in 1ml ethanol and injected via i.p [14]. Both sham and induced untreated rats received the same volume of the vehicle.

Animals were intraperitoneally anesthetized with 80 mg/kg ketamine and 8 mg/kg xylazine [18] and subjected to a 50% blood loss (30 ml/kg) via intracardiac puncture from the left side of the chest over 2 min and left in shock state for 1hr. The animals were then resuscitated with two times blood loss (60 ml/kg) using i.v lactated Ringers via tail over 1 hr [19]. The sham group underwent all instrumentation procedures, but neither hemorrhage nor resuscitation was carried out. Animals were allowed to breathe spontaneously throughout the experiment. Two hour after the completion of resuscitation, rats were again anesthetized and sacrificed by exsanguinations, where the chest cavity was opened and blood samples were taken directly from the heart, sera were removed, and analyzed for determination of serum TNF-a and IL-6 using enzyme-linked immunosorbent assay (ELISA) kits (IMMUNOTECH. France). The lungs were harvested, excised and the left lung was homogenized and supernatant was used for determination of GSH and MDA [18]. The MDA levels were assayed for products of

lipid peroxidation by monitoring thiobarbituric acid reactive substance formation according to the method of Buege and Aust in 1978 [20]. GSH measurements were performed using a colorimetric method at 412nm (BioAssay Systems' QuantiChrom™ Glutathione Assay Kit). The right lung was fixed in 10% formalin for histological examination. The sections were examined by microscope then the histological changes were determined. The degree of lung injury was assessed using the scoring system described by **Matute-Bello *et al.* (2001)** that graded congestion of alveolar septae, intra-alveolar cell infiltrates, and alveolar hemorrhage [21]. Each parameter was graded on a scale of 0-3. The total lung injury score was calculated be adding the individual scores for each category and lung injury was categorized according to the sum of the score to normal (0), mild (1-3), moderate (4-6) and severe injury (7-9). The histological sections were evaluated by a pathologist without prior knowledge of the treatment given to the animals.

Statistical analyses were performed using SPSS 12.0 for windows.lnc. Data were expressed as mean ± SEM. Analysis of Variance (ANOVA) was used for the multiple comparisons among all groups followed by post-hoc tests using LSD method. The histopathological grading of lung changes is a non-normally distributed variable measured on an ordinal level of measurement; therefore non-parametric tests were used to assess the statistical significance involving this variable. The statistical significance of difference in total score between more than 2 groups was assessed by Kruskal-Wallis test, while Mann-Whitney U test was used for the difference between 2 groups. In all tests, $P< 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect on Proinflammatory Cytokines (TNF-α and IL-6)

At the end of the experiment, the serum TNF-α and IL-6 levels were significantly higher in the HS group when compared with the sham group ($P < 0.05$). Treatment with montelukast significantly decreased the serum TNF-α and IL-6 levels when compared with the HS group ($P < 0.05$). The TNF-α and IL-6 values for the different groups are shown in table (1) and figure (1A&B).

Table (1): Serum TNF-α and IL-6 levels (pg/ml) of the three experimental groups at the end of the experiment (N = 6 in each group).

Group	TNF-α (pg/ml)	IL-6 (pg/ml)
1.Sham	19.4±2.12	21.16±2.61
2.HS	93.3±6.48*	44.84±2.33*
3.Montelukast treated group	42.66±2.42†	31.88±1.65†

* $P < 0.05$ vs. sham group, † $P < 0.05$ vs. HS (induced untreated) group

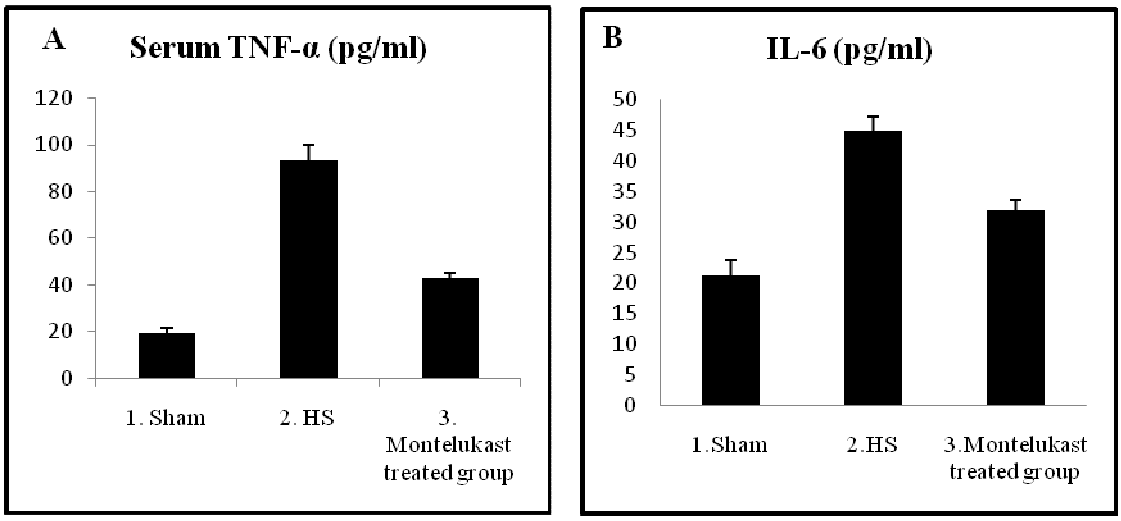

Figure (1): The mean of serum TNF-α (A) and IL-6 (B) level in the three experimental groups at the end of the experiment.

3.2. Effect on Lung MDA and GSH Levels

The MDA levels, measured as a major degradation product of lipid peroxidation in the pulmonary tissue, were found to be significantly higher in HS group as compared to those of the sham group ($P < 0.05$), while treatment with montelukast abolished these elevations ($P < 0.05$). The HS caused a significant decrease in lung GSH level ($P < 0.05$) when compared with the sham group, while in the montelukast treated group, the lung GSH level was found to be preserved ($P < 0.05$) and not significantly different from that of the sham group. The MDA and GSH values for the different groups are shown in table (2) and figure (2A&B).

Table (2): Lung MDA and GSH levels of the three experimental groups at the end of the experiment (N = 6 in each group).

Group	Lung MDA (nmol/g)	Lung GSH (µmol/g)
1.Sham	95±2.78	4.36±0.27
2.HS	157±6.15*	2.12±0.25*
3.Montelukast treated group	115.1±5.18†	3.54±0.4†

* $P < 0.05$ vs. sham group, † $P < 0.05$ vs. HS (induced untreated) group

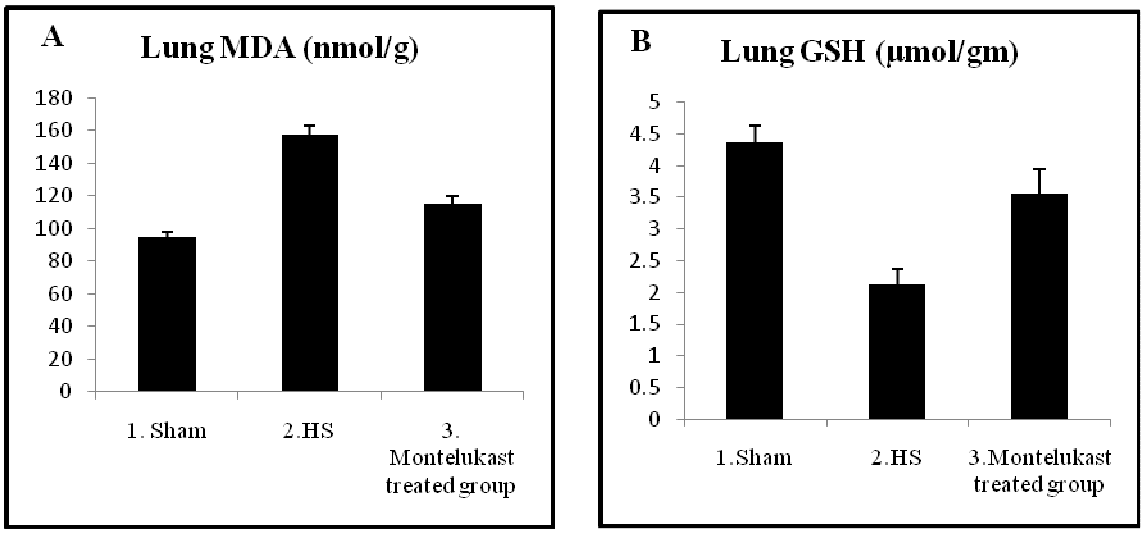

Figure (2A): The mean of lung MDA (A) and GSH (B) level in the three experimental groups at the end of the experiment.

3.3. Histological finding

A cross section of sham rat's lung showed the normal appearance of all three parameters. All rats in this group showed normal lung appearance (100%). There was statistically significant difference between induced untreated (HS) group and sham group ($P < 0.05$) and the total score mean of the HS group showed moderate lung injury. 66.7% of the group had moderate lung injury and 33.3% had severe lung injury. Treatment of rats with montelukast ameliorated the lung injury significantly ($P < 0.05$) as compared with induced untreated group and the total score mean of this group showed mild lung injury. 16.7% of the group had normal histopathological appearance, 66.6% of the group had mild lung injury and 16.7% of the group had moderate lung injury as shown in table (5) & (6).

Table (5): The differences in histopathological grading of abnormal lung changes among the three experimental groups.

Histopathological grading	Study group					
	Sham		Control (HS)		Montelukast	
	N	%	N	%	N	%
Normal	6	100	0	0	1	16.7
Mild	0	0	0	0	4	66.6
Moderate	0	0	4	66.7	1	16.7
Severe	0	0	2	33.3	0	0
Total	6	100	6	100	6	100

Table (6): Acute lung injury score.

Study group	Congestion of alveolar septae	Intra-alveolar cell infiltrates	Alveolar hemorrhage	Total score	Total score grade
Sham	0	0	0	0	Normal
HS	1.5±0.34	2.5±0.22	1.83±0.16	5.83±0.60*	Moderate
Montelukast treated group	1±0.25	1.33±0.42	0.5±0.34	2.33±0.56†	Mild

* $P < 0.05$ vs. sham group, † $P < 0.05$ vs. HS (induced untreated) group

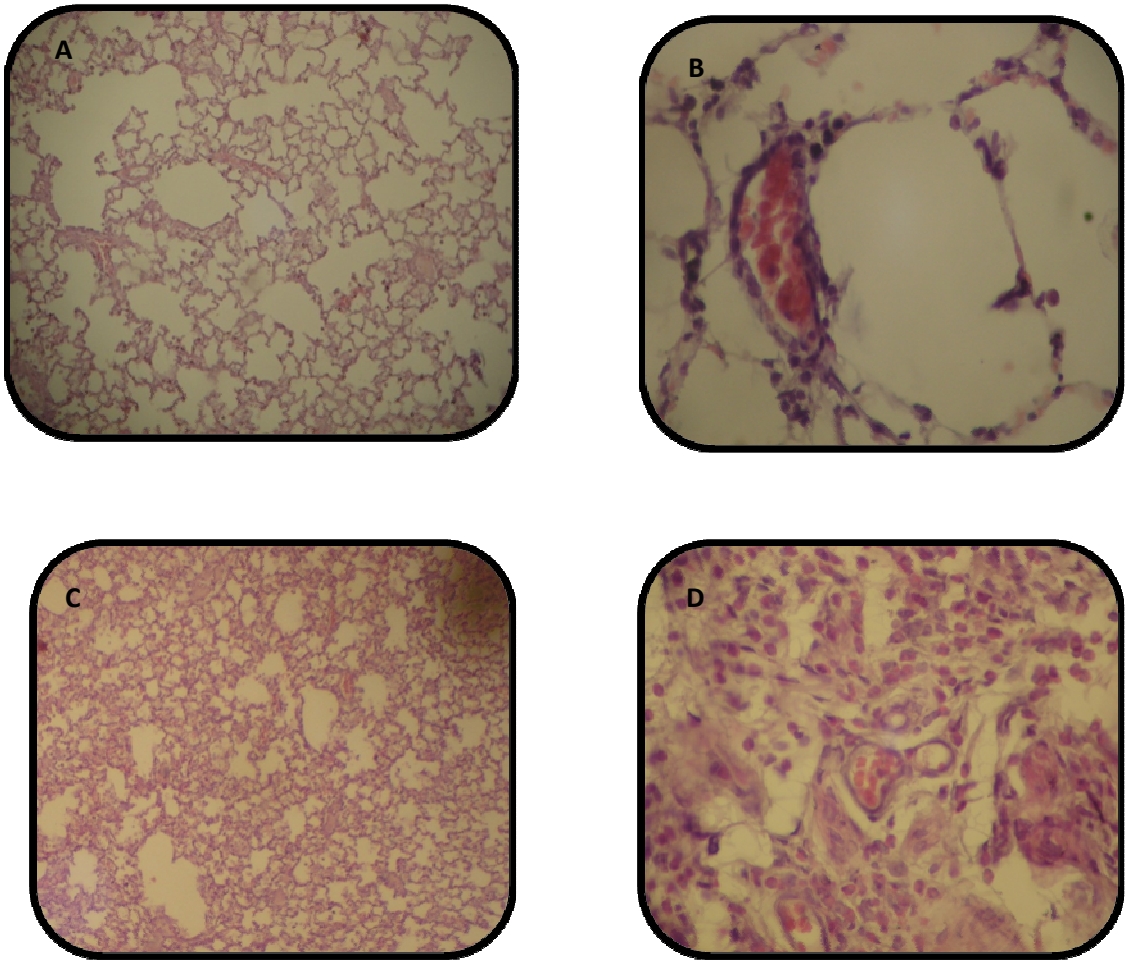

Figure (6): Photomicrographs of lung section stained with Haematoxylin and Eosin shows the normal architecture (X10) (A), mild injury (X40) (B), moderate injury (X10) (C), and severe injury (X40) (D).

Discussion

The present study demonstrates that HS causes ALI, as evidenced by biochemical and histologic changes. Montelukast prevented the biochemical changes and protected the lung morphology after HS. Hemorrhagic shock is considered as an insult frequently leading to systemic inflammatory response syndrome including the systemic release of proinflammatory cytokines which is central in the inflammatory response. Previous studies have shown that levels of IL-6 and TNF-α significantly increased following trauma-hemorrhage and remain elevated for several hours [22]. The results in present study are consistent with that reported by **Vincenzi *et al.* (2009)** [23] who found that a significant increase in the TNF-α and IL-6 levels in shocked rats in comparison with sham group. Activated inflammatory cells, especially macrophages and neutrophils have been shown to play a pivotal role in the propagation of SIRS following resuscitated shock and could be considered the main source of inflammatory cytokines including TNF-α and IL-6. In this study montelukast significantly reduced the elevation of IL-6 and TNF-α level in the shocked rats as compared with induced untreated group suggesting that montelukast has protective effect in hemorrhagic shock-induced acute lung injury. **Maeba *et al.* (2005)** showed that high doses of montelukast modulate the production of IL-6 and TNF-α through the inhibition of NF-κB activation [24]. **Şener *et al.* (2006)** demonstrated that montelukast significantly decreases the plasma TNF-α and IL-6 level in the rats as compared with induced untreated group in a renal I/R injury model [15]. The inhibitory action of CysLTs receptor blockers on the generation of pro-inflammatory cytokines was shown in intestinal I/R injury model, where the treatment with CysLTs receptor antagonist significantly markedly suppressed IL-6 levels [25]. Montelukast was found to decrease serum TNF-α level in burn and sepsis-induced multiple-organ injury in the rats [12,13]. Furthermore, **Kabasakal *et al.* (2005)** demonstrated that montelukast significantly decreases the serum TNF-α level in the rats that underwent burn-induced oxidative injury of the gut [26]. Through examination of metabolic processes, GSH has been shown to be important in host defenses against oxidative stress [27]. Another important agent showing oxidative stress is MDA, a marker of free radical activity [27]. It was reported that oxidative stress significantly elevated MDA levels and reduced GSH levels [28]. Oxidative stress has been implicated as an important cause of HSR pathogenesis [2,27]. The result in present study are consistent with that reported by **Kilicoglu *et al.* (2006)** who found that a significant increase in lung MDA level and significant decrease in lung GSH level were found in hemorrhagic shock group as compared to sham group in a rat model of hemorrhagic shock-induced acute lung injury [18]. In this study montelukast significantly reduced the elevation of lung MDA level and significantly elevates the lung GSH level in the shocked rats as compared with induced untreated group suggesting that montelukast has protective effect in hemorrhagic shock-induced oxidative injury of the lung. **Şener *et al.* (2005 & 2007)** found that montelukast significantly reduces lung MDA level and elevates lung GSH level in the rats that underwent burn and chronic renal failure-induced oxidative injury of remote organs [13,29]. Furthermore, montelukast has been shown to reduce I/R-induced oxidative damage in the rat liver, bladder, testes and kidneys, through its anti-inflammatory and antioxidant properties (by balancing oxidant-antioxidant status) [14,15,16,17]. The antioxidant effect of montelukast was further supported by its action and largely based on its anti-inflammatory effect, where proinflammatory cytokines, chemokines, and activated complement factors are responsible for neutrophil recruitment and the subsequent neutrophil-induced oxidant stress during the reperfusion phase [30]. **Wang *et al.* (2008)** demonstrated that LTC_4

affects the GSH/GSSG ratio by activating signals to increase IL-8 production while pretreatment with a leukotriene receptor antagonist, montelukast, significantly suppressed LTC_4-induced time-dependent changes in the intracellular redox state, and also suppressed upregulation of IL-8 production by suppressing NF-κB activation [31]. On the other hand, CysLTs have been implicated as inflammatory mediators in various studies [15] based on their potent chemotactic and chemokinetic properties (including recruitment of neutrophils), and because of their ability to increase vascular permeability which are common features of I/R injury. Morphologically, there was a statistically significant difference between induced untreated group and sham group and the total score mean of the HS group shows moderate lung injury. Treatment of rats with montelukast ameliorates the lung injury significantly as compared with induced untreated group and the total score mean of the control group shows mild lung injury. Although there is no data available about the protective effect of montelukast on the lung parenchyma in HS rats, but **Şener *et al.* (2007)** showed that treatment with montelukast ameliorated the degenerated lung epithelium and significantly decreased the number of inflammatory cells in the lung of rats that underwent chronic renal failure-induced multiple-organ injury [29]. Furthermore, montelukast protects against burn induced lung injury reflected by amelioration of massive alveolar structural disturbance and disappearance of interstitial hemorrhage [13]. In the context of I/R models, various studies showed that montelukast can retard histopathological changes in different organs including liver, kidney and testes [14,15,16]. In present study, the amelioration effect of montelukast can be attributed to its ability to balance oxidant-antioxidant status and to reduce the generation of pro-inflammatory mediators as well as its inhibitory effect on neutrophil activation and infiltration that have been reported in various studies [12,13,15].

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