

Therapeutic effect of montelukast in hepatic ischemia-reperfusion injury in rats

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SUMMARY

Hepatic ischemia-reperfusion injury (IRI) occurs in a number of clinical conditions such as trauma, hypovolemic shock, hepatic resection, and liver transplantation. Although several drugs are effective in attenuating hepatic ischemia-reperfusion (I/R) injury, there are still no satisfying treatment strategies available to prevent hepatic I/R injury. Sixty rats were randomly assigned to 3 groups: control group, I/R model group, and I/R + M group (7 mg/kg montelukast pretreatment). The total hepatic ischemia was induced for 45 minutes by clamping the hepatic artery, portal vein, and bile duct using a vascular clamp. The rats were then allowed reperfusion at 1, 3, 6 and 12 hours. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) activity, and proinflammatory cytokines (i.e., tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-1 β)) were measured in blood samples. Malondialdehyde (MDA), and glutathione (GSH) levels and myeloperoxidase (MPO) and Na⁺, K⁺-ATPase activities were determined in the liver tissue samples while formation of reactive oxygen species was monitored by using the chemiluminescence (CL) technique with luminol and lucigenin probes.

Tissues were also analyzed histologically. Hepatic IRI induced a marked increase in CysLTR1, Caspase-8 and Caspase-9 protein expression in the liver, which were markedly reduced by preconditioning with a 7 mg/kg montelukast. Preconditioning with 7 mg/kg montelukast significantly attenuated liver tissue injury and liver damage and decreased plasma AST, ALT, LDH, TNF- α , IL-1 β , MDA and MPO levels after a hepatic IRI. In conclusion, preconditioning with montelukast could attenuate hepatic IRI and the subsequent systemic inflammatory response in rats.

Key words: Apoptosis – Gene expression – Ischemia-reperfusion – Liver – Montelukast

INTRODUCTION

Hepatic ischemia-reperfusion injury (IRI) occurs in a number of clinical conditions such as trauma, hypovolemic shock, hepatic resection, and liver transplantation (Zhai et al., 2013). Ischemia reperfusion (I/R)-induced oxidative stress and proinflammatory cytokines have been suspected to be contributors to the morbidity of clinical hepatic surgeries, especially for hepatic resections and liver transplantation (Penna et al., 2009; Zhou et al., 2009). Reactive oxygen species (ROS) generated in the liver as an inevitable consequence of hepatic I/R can directly disrupt the function and structure of mitochondria and thus lead to cellular death in the liver during ischemia (Clemens et al., 1985; McCord, 1985). An excessive inflammatory response is also recognized as a key mechanism of injury during reperfusion (Jaeschke et al., 2003).

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Various chemokines and a lipid mediator, cysteinyl leukotrienes (CysLTs), that is, one of the 5-lipoxygenase metabolites of arachidonic acid, are potent inflammatory mediators that are associated with I/R-induced tissue injury (Hughes et al., 1992). The infiltration of polymorphonuclear leukocytes in a tissue is characteristic of acute inflammation and indicates the collective action of chemotactic mediators (Ishikawa et al., 2005). Once neutrophils migrate into the ischemic area, they release ROS, proteases, elastase, myeloperoxidase (MPO), cytokines, and various other mediators, all of which are involved in tissue injury (Willerson, 1997). Hence, minimizing ischemia-reperfusion injury (IRI) is of great clinical significance to hepatopathy. Although our knowledge concerning the molecular and cellular pathophysiology of hepatic injury after I/R has improved greatly, there are still no satisfying treatment strategies available that prevent hepatic IRI (Wu et al., 2011). A selective reversible CysLT1 receptor antagonist, montelukast (MK-0476), was used in the treatment of asthma and was reported to reduce eosinophilic inflammation in the airways (Damtew et al., 1993; Wallace et al., 1998); while CysLT1 receptor antagonists or biosynthesis inhibitors have been reported to ameliorate ethanol-induced gastric mucosal damage (Carsin et al., 2002; Wallace et al., 2000).

Recently, it was reported that the CysLT1 receptor antagonist, montelukast, ameliorated I/R-induced liver, renal and intestinal injury in rat models (Daglar et al., 2009; Ozkan et al., 2010; Sener et al., 2006; Wu et al., 2015). In spite of these findings, this study aimed to elucidate the putative protective effect of montelukast against I/R-induced tissue damage in the liver. Therefore, this randomized study was performed to evaluate the efficacy and safety of montelukast in rats with IRI. I hypothesized that the inhibitory effects of montelukast on Caspase-8, Caspase-9, and CysLTR1 protect against hepatic I/R by decreasing apoptosis protein levels and injury that was induced by I/R in rats with hepatic IRI.

MATERIALS AND METHODS

Animals

Six-week-old male Sprague-Dawley rats, weighing approximately 180-220 g each, were used for this study. All rats were housed in climate-controlled conditions with a twelve-hour-light / twelve-hour-dark cycle, and had free access to food and water. All animal experiments were approved by the local Animal Ethics Committee of Seoul National University, and were carried out in accordance with the Institutional Animal Care and Use Committee Guidelines of Seoul National University Bundang Hospital.

Hepatic Ischemia Reperfusion (I/R) rat model

Rats were anesthetized (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine; intraperitoneally) during all surgical procedures. Under anesthesia, a midline laparotomy was made using minimal dissection. Total hepatic ischemia was induced for 45 min by clamping the hepatic artery, the portal vein, and the bile duct using a vascular clamp, and the rats were then allowed reperfusion at 1, 3, 6 and 12 hours. The rats were treated with montelukast, at 30 min prior to ischemia and immediately before the reperfusion period. The rats were grouped as follows: control (C) group (n=20), saline injection and normal diet; I/R group (n=20), ischemia-reperfusion and normal diet; I/R + M group (n=20), ischemia-reperfusion + montelukast injection and normal diet. The animals were sacrificed at 1, 3, 6 and 12 hours (each group, n=5) after reperfusion. Tissues were removed and immediately frozen at -70°C for enzyme analysis.

Drug

The montelukast was purchased from Merck (Whitehouse Station, NJ) and used as 7 mg/kg homogenized in 2% ethanol via intraperitoneal (ip) route. This dose was chosen on the basis of previous experiment showing that animals receiving it tolerated dose of montelukast (Daglar et al., 2009).

Biochemical analysis

The animals were decapitated at 1, 3, 6 and 12 hours of reperfusion, and trunk blood samples were collected to determine serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels as indicators of liver functions. Inflammatory cytokines, and lactate dehydrogenase (LDH) activity were also assayed in serum samples. In the hepatic tissue samples were stored at -70°C, malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) and Na⁺, K⁺-ATPase activities were measured at 3 hours of reperfusion. Formation of reactive oxygen species were monitored by chemiluminescence (CL) technique. The hepatic tissue samples were also placed in formaldehyde (4%) for histological evaluation. Serum AST, ALT (Moss et al, 1987), and LDH levels (Martinek, 1972) were determined spectrophotometrically using an automated analyzer. Plasma levels of tumor necrosis factor- α (TNF- α), and interleukin (IL)-1 β , were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay kits specific for the previously mentioned rat cytokines (Biosource Int., Nivelles, Belgium). To assess the role of ROS in I/R-induced tissue damage, lucigenin and luminol CL were measured as indicators of radical formation. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridiniumnitrate) were obtained from Sigma (St. Louis, MO). Measurements were made at room temperature using Junior LB 9509 luminometer (EG and G, Berthold, Germany).

Specimens were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers such as lucigenin or luminol for a final concentration of 0.2 mM. Luminol detects a group of reactive species (OH, H₂O₂, HOCl radicals), and lucigenin is selective for O²⁻ (Ohara et al., 1993). Counts were obtained at 1-min intervals, and the results were given as the area under curve for a counting period of 5 min. Counts were corrected for wet tissue weight (rlu/mg tissue) (Davies et al., 1994).

Assay of MDA and total GSH levels in liver

Liver tissue samples were homogenized with ice-cold 150 mM KCl for the determination of GSH and MDA levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (Beuge et al., 1978). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, and results were expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (Beutler et al., 1975). Briefly, after centrifugation at 2,000 g for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/l Na₂HPO₄·2H₂O solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of $1.36 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. Results were expressed in $\mu\text{mol GSH/g tissue}$.

Assay of MPO levels in liver

MPO activity was measured in tissues in a procedure similar to that documented by Hillegass et al. (1990). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze-and-thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H₂O₂ solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Na⁺, K⁺-ATPase Activity

Since the activity of Na⁺, K⁺-ATPase is sensitive to free radical reactions and lipid peroxidation. Measurement of Na⁺, K⁺-ATPase activity is based on the measurement of inorganic phosphate released by ATP hydrolysis during incubation of homogenates with an appropriate medium containing 3 mM ATP as a substrate. The total ATPase activity

was determined in the presence of 100 mM NaCl, 5 mM KCl, 6 mM MgCl₂, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4), while the Mg²⁺-ATPase activity was determined in the presence of 1 mM ouabain. The difference between the total and the Mg²⁺-ATPase activities was taken as a measure of the Na⁺, K⁺-ATPase activity (Atkinson et al., 1973; Reading et al., 1980). The reaction was initiated with the addition of the homogenate (0.1 mL), and a 5 min pre-incubation period at 37°C was allowed. Following the addition of Na₂ ATP and a 10 min re-incubation period, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3500 g, and Pi in the supernatant fraction was determined by the method of Fiske and Subbarow (Fiske et al., 1925). The specific activity of the enzyme was expressed as nmol Pi mg⁻¹ protein h⁻¹. The protein concentration of the supernatant was measured by the Lowry method (Lowry et al., 1951).

Histopathological analysis

For light microscopic investigations, liver tissue specimens were fixed in 10% formaldehyde, dehydrated in increasing alcohol series, clearing in toluene end embedding in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined under a photomicroscope (Olympus BX51, Tokyo, Japan).

Western blot analysis

Liver whole cell homogenates and nuclear extracts were obtained by lysis of hepatic tissue with the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to manufacturer instructions. Protein aliquots (50 μg) were subsequently separated by SDS-PAGE on 10% acrylamide gels and proteins were electrotransferred to PVDF membrane. The membrane was blocked in TBS with 5% non-fat dry milk at room temperature for 1 h in 0.1% Tween 20 and incubated with the appropriated primary antibodies, including CysLTR1 (Catalog number: 222033, United States Biological, Salem, MA), Caspase-8, Caspase-9 and Actin (sc-5263; sc-133109; sc-1616, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at 4°C overnight. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc., Danvers, MA, USA) for 1 h at room temperature. After washing, the membrane was visualized by a chemiluminescent reaction using an ECL-detection kit system from GE Healthcare (formerly Amersham Bioscience, Piscataway, NJ, USA).

Statistical analysis

Results were expressed as the mean \pm standard deviation. Differences between all other parameters for the three groups were evaluated by ANOVA followed by multiple-group comparisons. An unpaired two-tailed t-test and Mann-Whitney test

were used, and a P value of < 0.05 was considered statistically significant. SPSS version 14.0 for windows (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Serum results

Rats preconditioned with montelukast exhibited significantly less damage to the hepatic I/R. The Ischemia/reperfusion-induced liver injury significantly increased the ALT and AST levels, which indicated impairment of liver functions at 1, 3, 6, and 12 hours of reperfusion. Similarly, LDH activity, as an index of generalized tissue damage, and proinflammatory cytokines (TNF- α , and IL-1 β) increased in comparison with the control group. When montelukast was administered before ischemia and during the subsequent reperfusion period, these elevations significantly decreased at 1, 3, 6, and 12 hours of reperfusion ($P < 0.05$; Table 1).

Biochemical results

CL levels in the liver samples detected by both luminol and lucigenin probes exhibited significant increases in the saline-treated I/R group compared with the CL levels of the control group at 3 hours of reperfusion ($P < 0.05$; Fig. 1). On the other hand, the montelukast treatment in the I/R group decreased the elevations of the CL values of lucigenin and luminol significantly at 3 hours of reperfusion ($P < 0.05$). The liver tissue MDA content in the control group was elevated by IRI ($P < 0.05$); however, montelukast treatment significantly decreased the I/R induced in the liver tissue of the I/R group ($P < 0.05$), more so than it did in the control group at 3 hours of reperfusion (Fig. 2A). In accordance with this, I/R caused a significant de-

crease in liver GSH levels ($P < 0.05$) compared to that of the control group; while in the montelukast-treated I/R group, the liver GSH content was preserved significantly ($P < 0.05$), which was not significantly different from that of the control group at 12 hours of reperfusion (Fig. 2B). Myeloperoxidase activity, which was accepted as an indicator of neutrophil infiltration, was significantly higher in the liver tissue of the I/R group ($P < 0.05$) than that of the control group (Fig. 3A). On the other hand, montelukast treatment in the I/R group significantly decreased the liver tissue MPO level, which was found to be similar to that of the control group at 3 hours of reperfusion. The Na⁺, K⁺-ATPase activities in the liver tissue samples were significantly reduced in the I/R-induced rats ($P < 0.05$) which indicated impaired transport function and also membrane damage in the tissue at 3 hours of reperfusion (Fig. 3B). However, in the montelukast-treated I/R rats, the measured Na⁺, K⁺-ATPase activities was not different from those of the control rats.

Immunohistochemistry analysis

The light microscopic evaluation of the control group revealed a regular morphology of liver parenchyma with intact hepatocytes and sinusoids (Fig. 4A). In the I/R group, severe sinusoidal congestion and hemorrhage, dilation of the central vein, subendothelial edema, and degenerated hepatocytes with perinuclear vacuolization were observed (Fig. 4B). In the montelukast treated I/R group, the histological analysis demonstrated a well-preserved liver parenchyma. Despite the moderate sinusoidal dilatation and hemorrhage, which were in localized areas, the usual appearance of the central vein and hepatocytes was observed in most areas (Fig. 4C).

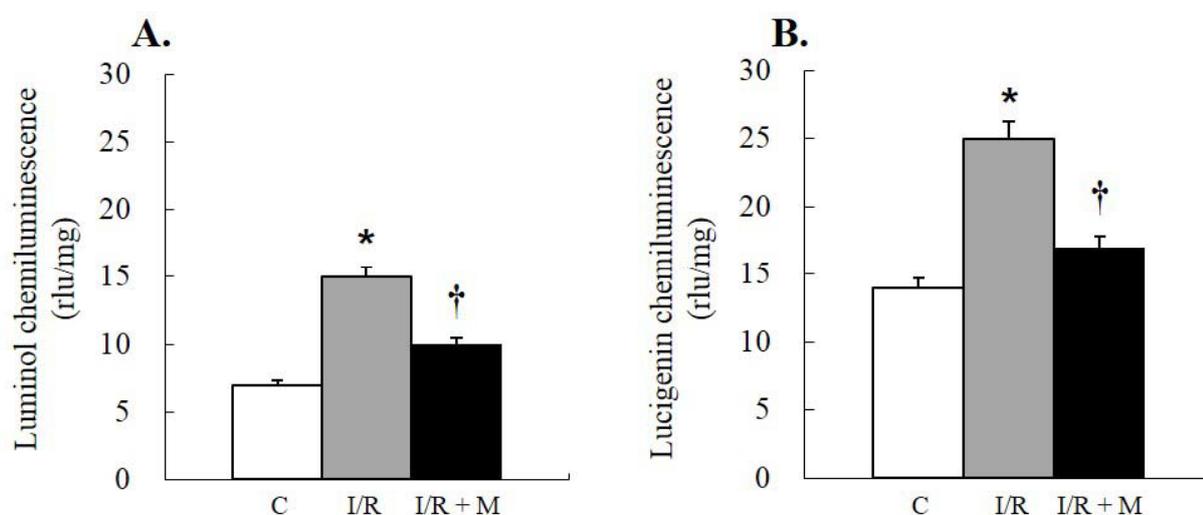


Fig. 1.- Luminol (A) and lucigenin (B) chemiluminescence (CL) values in the hepatic tissues of the I/R group or montelukast-treated I/R group and control group at 3 hours of reperfusion. Luminol and lucigenin were lower in the C group and the I/R + M group compared to the I/R group. C, control group; I/R, Ischemia-reperfusion injury group; I/R + M, Ischemia-reperfusion injury + montelukast group.

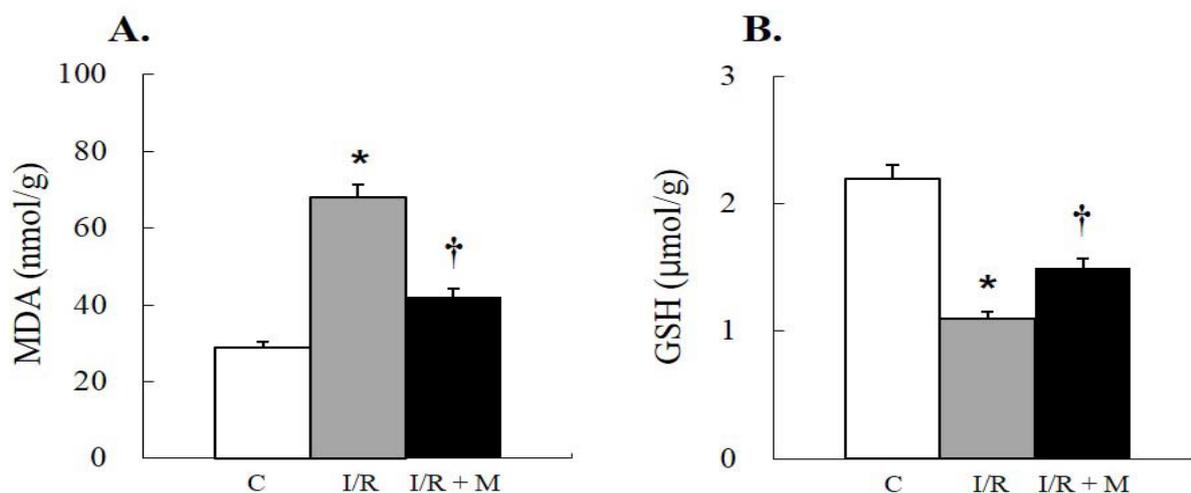


Fig 2. (A) Malondialdehyde (MDA) and (B) glutathione (GSH) levels in the liver tissue of the I/R group or montelukast-treated I/R group and control group.

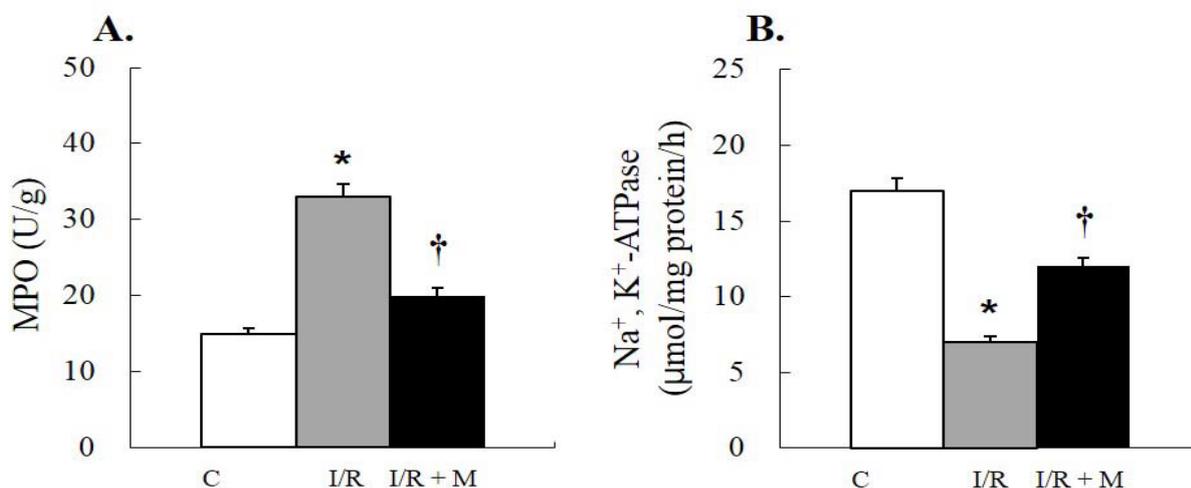


Fig. 3. (A) Myeloperoxidase (MPO) and (B) Na⁺, K⁺-ATPase activities in the liver tissue of the I/R group or montelukast-treated I/R group and control group. *P < 0.05 vs. C group, †P < 0.05 vs. I/R group. C, control group; I/R, Ischemia-reperfusion injury group; I/R + M, Ischemia-reperfusion injury + montelukast group.

Western blotting for apoptosis signaling and cysteinyl leukotrienes receptor-1 (CysLTR1) protein

This experiment was designed to investigate if montelukast changed the Caspase-8 and Caspase-9 protein expression levels in liver tissues. Caspase-8 and Caspase-9 protein immunoreactivity decreased significantly in the montelukast group compared with that in the IRI group (Figs. 5A, 5B). The actin bands indicated protein loading in the same sample (Figs. 5A, 5B). This experiment was designed to investigate if montelukast changed the CysLTR1 protein levels in liver tissues. CysLTR1 protein immunoreactivity decreased significantly in the montelukast group compared with that in the IRI group (Figs. 5C, 5D). The actin bands indicated protein loading in the same sample (Figs. 5C, 5D).

DISCUSSION

I/R injury is considered to play a key role in the pathogenesis of hepatic failure after hepatic resection or transplantation. It is responsible for triggering a systemic inflammatory response that may lead to liver organ dysfunction (Giakoustidis et al., 2002; Takamatsu et al., 2004). The protective effect of montelukast on hepatic I/R injury has been demonstrated in previous studies (Daglar et al., 2009; Ozkan et al., 2010). In addition, the protective effects of montelukast on renal damage and intestinal I/R have been reported (Sener et al., 2006; Wu et al., 2015). Consequently, extensive experimental research efforts are currently focusing on this area in order to elucidate the molecular pathophysiology of hepatic I/R, which presumably

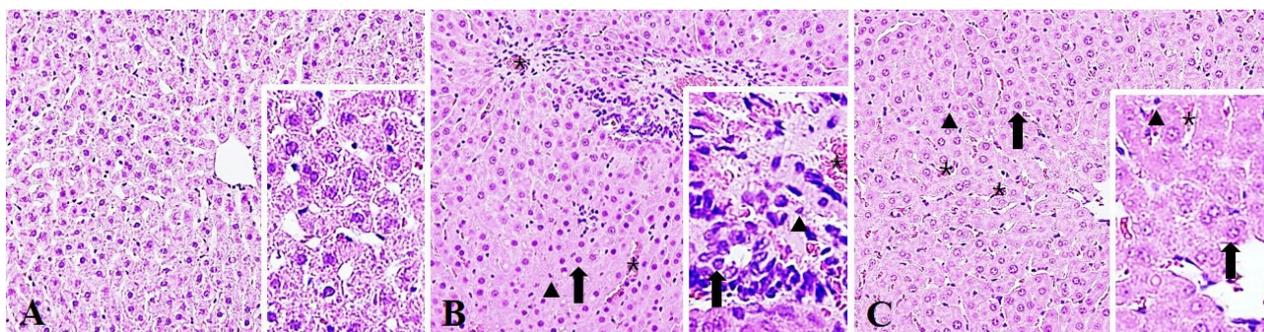


Fig. 4. (A) Control group of rats exhibiting regular liver parenchyma with a normal appearance of hepatocytes and sinusoids. (B) I/R group of rats exhibiting severe sinusoidal congestion (*) and hemorrhage, dilated central vein and degenerated hepatocytes (arrow head) showing perinuclear vacuolization and severe inflammatory cell infiltration (arrow). (C) Montelukast-treated group of rats displayed moderate sinusoidal dilatation (*) and hemorrhage in localized areas, mild inflammatory cell infiltration (arrow) with the usual appearance of central vein and hepatocytes (arrow head) in most areas. Original magnifications: x200, insets: x400.

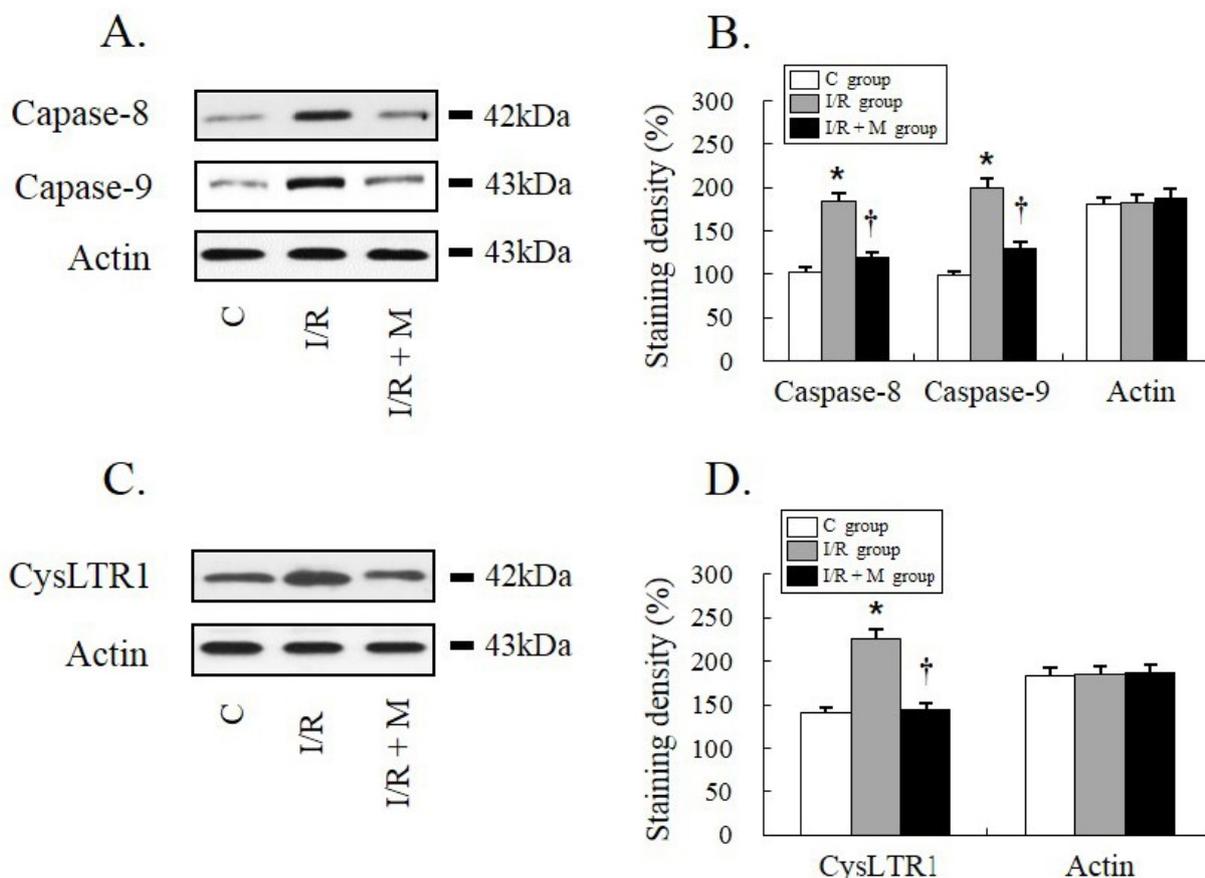


Fig. 5. Western immunoblot analysis of the levels of expression of Caspase-8, Caspase-9, CysLTR1 and Actin immunoreactivity after montelukast treatment in I/R mice. The semi-quantitative analysis confirmed that I/R mice exhibited increased levels of immunoreactivity of Caspase-8, Caspase-9 and CysLTR1 in liver tissues (n = 5 per group). However, the protein expressions of Caspase-8, Caspase-9 and CysLTR1 decreased in the I/R + M group compared to the I/R group.

will aid in designing advanced therapeutic strategies for better protection of the tissues. The present study investigated the therapeutic effects of montelukast, a selective antagonist of cysteinyl leukotriene receptor 1 (CysLT1), against I/R induced hepatic damage. I/R injury represents a condition whereby the restoration of blood flow in a previously ischemic liver leads to an accentuation

of damage (Ozkan et al., 2010).

In this study, plasma ALT, AST, and LDH levels increased following a hepatic IRI, which indicated impairment of liver functions and generalized tissue damage. At the same time, the increasing levels of plasma ALT, AST, and LDH levels were significantly decreased by montelukast treatment. Hepatic IRI is largely a result of an acute inflam-

Table 1. Serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Lactate Dehydrogenase, TNF- α , and IL-1 β Levels in the Control (C), Ischemia/Reperfusion (I/R), and Ischemia/Reperfusion + Montelukast (I/R + M) Groups.

Hour	Group	AST (U/L)	ALT (U/L)	LDH (U/L)	TNF- α (pg/m L)	IL-1 β (pg/m L)
1	C	90.2 \pm 4.3	49.3 \pm 3.2	506.6 \pm 2.8	1.3 \pm 0.5	9.2 \pm 1.3
	I/R	451.5 \pm 8.5*	153.2 \pm 8.9*	1051.5 \pm 6.8*	7.2 \pm 2.6*	15.5 \pm 2.5*
	I/R + M	107.1 \pm 5.4†	99.4 \pm 2.7†	639.4 \pm 6.2†	5.1 \pm 1.6†	10.1 \pm 2.2†
3	C	91.3 \pm 2.9	50.1 \pm 2.5	513.1 \pm 3.2	1.2 \pm 0.4	8.9 \pm 1.2
	I/R	687.6 \pm 10.1*	573.2 \pm 10.9*	1521.7 \pm 13.8*	8.4 \pm 3.9*	16.5 \pm 3.8*
	I/R + M	113.1 \pm 4.7†	109.4 \pm 4.3†	741.7 \pm 5.8†	5.6 \pm 2.4†	11.3 \pm 2.4†
6	C	90.8 \pm 3.1	49.7 \pm 2.8	511.2 \pm 3.7	1.2 \pm 0.5	9.1 \pm 1.4
	I/R	658.5 \pm 12.7*	623.2 \pm 12.9*	854.2 \pm 14.4*	9.5 \pm 4.1*	18.9 \pm 4.1*
	I/R + M	131.1 \pm 5.2†	122.4 \pm 4.8†	737.3 \pm 7.3†	6.2 \pm 3.2†	12.4 \pm 2.3†
12	C	89.8 \pm 2.6	49.9 \pm 3.1	509.2 \pm 3.3	1.3 \pm 0.3	9.3 \pm 1.2
	I/R	328.5 \pm 15.2*	253.2 \pm 13.9*	812.3 \pm 16.2*	8.2 \pm 3.2*	15.5 \pm 3.5*
	I/R + M	149.1 \pm 6.3†	139.4 \pm 5.7†	742.3 \pm 8.9†	6.3 \pm 2.9	13.9 \pm 3.1

Mean density is the sum of the gray values of all the pixels in the selection that was divided by the number of pixels within the selection. Values are presented as the mean (standard deviation). Student's t-test was performed (*P < 0.05 compared with the Control group, †P < 0.05 compared with the I/R group).

matory response characterized by the induction of a cascade of pro-inflammatory mediators that culminates in the recruitment of leukocytes to the post-ischemic tissue leading to cell injury. Several studies have demonstrated that treatment with montelukast resulted in an impressive reduction in the basal portal pressure and an attenuation of the activation of Kupffer cells in patients and rat models (Steib et al., 2010; Steib et al., 2015). Ischemia activates Kupffer cells, which are the main sources of vascular reactive oxygen formation during the initial reperfusion period (data not shown). Kupffer cells lead to the production of pro-inflammatory cytokines, such as TNF- α and IL-1 (Giakoustidis et al., 2002; Takamatsu et al., 2004). A recent study demonstrated that the tissue expression of TNF- α , IL-1 β , and IL-6 increased following renal I/R (Kher et al., 2005).

In accordance with these findings, in the present study, the plasma levels of the pro-inflammatory cytokines TNF- α , and IL-1 β significantly increased in an I/R-induced hepatic injury. Increased plasma TNF- α , and IL-1 β were decreased by montelukast treatment. This finding is in alignment with a previous study demonstrating that montelukast treatment reduced the elevations of the plasma TNF- α and IL-1 β levels in inflammatory conditions in hepatic IRI rat models (Daglar et al., 2009). ROS are known to be generated during I/R and may represent pivotal mediators of the ensuing pathological complications (Clemens et al., 1985; McCord, 1985). Accordingly, I evaluated the generation of

these molecules in the liver tissues by using enhanced CL assay using luminol and lucigenin probes. The two CL probes, luminol and lucigenin, differ in selectivity. Luminol detects H₂O₂, OH⁻, hypochlorite, peroxyxynitrite, and lipid peroxy radicals, whereas lucigenin is particularly sensitive to superoxide radicals (Ohara et al., 1993; Davies et al., 1994). In the current study, increases in luminol and lucigenin-CL levels supported the notion that liver injury induced by I/R involves toxic oxygen metabolites and montelukast treatment decreased these elevations by its antioxidant action. In accordance with the increases in toxic oxygen metabolites, the liver MDA level also significantly increased, which indicated the presence of enhanced lipid peroxidation due to I/R injury. At the same time, the levels of the tissue glutathione declined, which signified depletion of the antioxidant pool. Leukotrienes (LTs) are one of the most important mediators in the pathophysiology of asthma based on its anti-inflammatory effect. The results demonstrated that LTC₄ affected the glutathione (GSH)/oxidized glutathione (GSSG) ratio by activating signals to increase the IL-8 production, while pretreatment with a leukotriene receptor antagonist, montelukast, significantly suppressed the LTC₄-induced time-dependent changes in the intracellular redox state. It also suppressed the up-regulation of IL-8 production by suppressing NF- κ B activation (Wang et al., 2008). Pro-inflammatory cytokines, chemokines, and activated complement factors are responsible for neutrophil recruitment

and the subsequent neutrophil-induced oxidant stress during the reperfusion phase (Jaeschke et al., 1992). On the other hand, CysLTs have been implicated as inflammatory mediators based on their potent chemotactic and chemokinetic properties. Previous studies regarding the role of leukotrienes in hepatic I/R suggested that these lipid mediators enhanced the recruitment of neutrophils (Takamatsu et al., 2004). The neutrophils seemed to act as an amplifier of the reperfusion reaction, and were considered an important factor in the damaging cascade that takes place upon reperfusion. I/R elicits an acute inflammatory response characterized by activation of neutrophils (Heinzelmann et al., 1999).

In the present study, elevated levels of MPO activity, as an index of tissue neutrophil infiltration, were inhibited by montelukast treatment, which indicated that the therapeutic effect of montelukast is neutrophil-dependent. It is known that ischemia elevates the cytosolic calcium concentration, which, in turn, elevates phospholipase A2 and lipoxygenase activity that generates leukotrienes (Sener et al., 2006). Reduced blood flow or hypoxia that alters the mitochondrial respiratory chain function reduces the redox state of the mitochondrial enzymes. This reduction results in the inhibition of the oxidative phosphorylation process with a subsequent reduction in adenosine triphosphate (ATP) synthesis. Reduction of cellular ATP causes disturbances in membrane ion translocation by inhibiting the ATP dependent Na^+ , K^+ -ATPase, which results in sodium influx and intracellular sodium accumulation with corresponding cell swelling and death (Blum et al., 1991). Inhibition of the Na^+ , K^+ -ATPase pump also causes intracellular calcium accumulation, which is also implicated in the development of ischemic injury and thought to be a crucial step in the transition to irreversible damage. Furthermore, calcium also activates xanthine oxidoreductase (XOR), which has a role in oxygen free radical production following reperfusion (Ishii et al., 1990). In the current study, Na^+ , K^+ -ATPase activity in the liver tissue was inhibited due to I/R, while, at the same time, montelukast treatment maintained the redox state of the tissue through its anti-inflammatory and antioxidant effects. Several studies have shown that the protective role of montelukast in I/R injury is related to the inhibition of apoptosis (Cho et al., 2013; Duran et al., 2013; Taha et al., 2010). With the application of montelukast, apoptosis was reduced in tissue and the expression of caspases-8 and -9 decreased, indicating that montelukast inhibits caspase-8 and -9 mediated pathways of apoptosis in hepatic I/R injury.

In conclusion, the present study demonstrated that montelukast, a CysLT1 receptor antagonist, reduces I/R-induced neutrophil accumulation, oxidative injury and liver dysfunction, which suggests that CysLTs is one of the mediators leading to tis-

sue damage following a hepatic I/R. These therapeutic effects of montelukast on reperfusion-induced injury can be attributed to its ability to inhibit neutrophil infiltration, to balance oxidant-antioxidant status, and to regulate the generation of inflammatory mediators, which suggests a future role in the treatment of liver failure due to IRI.

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