

## Research Article



# Evaluation of Leucomethylene Blue as a Protective Agent Against Acetaminophen-Induced Acute Lung Injury

Majid efati<sup>1</sup>, Sahar Ghoflchi<sup>1</sup>, Khaterreh Kharazmi<sup>2</sup>, Soodeh Alidadi<sup>3</sup>, Daryoush Hamidi-alamdari<sup>1,4\*</sup>, Hossein Hosseini<sup>\*</sup>

<sup>1</sup> Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>2</sup> Department of Physiology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>3</sup> Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

<sup>4</sup> Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

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## \* Corresponding Author:

Hossein Hosseini; Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Email: [Hoseinihs@mums.ac.ir](mailto:Hoseinihs@mums.ac.ir)

Daryoush Hamidi Alamdari; Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Email: [HamidiAD@mums.ac.ir](mailto:HamidiAD@mums.ac.ir)

## ABSTRACT

**Objectives:** Acetaminophen overdose may lead to acute pulmonary complications, such as acute lung injury, due to its harmful effects on cellular systems caused by oxidative stress. Leucomethylene blue (LMB) may have beneficial effects by improving hemodynamic stability and reducing oxidative damage through its nitric oxide synthase inhibitory and antioxidant activities. This study aimed to evaluate the effect of LMB on acetaminophen-induced pulmonary injury in rats.

**Methods:** Lung samples were collected from 30 male Wistar rats, which were randomly divided into five groups and frozen for later analysis. The groups included control, acetaminophen, N-acetylcysteine (NAC)-treated, LMB-treated, and NAC+LMB combination-treated. We evaluated total antioxidant capacity (TAC), glutathione reductase (GR), TNF- $\alpha$  and IL-6 levels, histopathology, and relevant tissue remodeling changes.

**Results:** Our results demonstrated that the administration of LMB significantly diminished the oxidative and inflammatory damage caused by APAP toxicity in the lungs. LMB restored TAC and GR activity, which were significantly reduced by APAP toxicity. Additionally, LMB restricted the overproduction of pro-inflammatory cytokines released from lung tissue. Moreover, LMB substantially counteracted the pulmonary lesions caused by APAP, including edema, hemorrhage, and infiltration of inflammatory cells, as confirmed by histopathological analysis.

**Conclusion:** The results of this study show that LMB can effectively reduce lung damage caused by acetaminophen poisoning.

**Keywords:** LeucoMethylene Blue, lung injury, Acetaminophen, Inflammation, Oxidative stress

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## Introduction

**A**

cetaminophen (N-acetyl-p-aminophenol, APAP), a widely used analgesic and antipyretic agent, is generally considered safe at therapeutic doses (1).

However, some studies have reported that high-dose or chronic APAP exposure can result in severe pulmonary complications, including acute lung injury (ALI) (2, 3). Emerging evidence suggests that chronic or high-dose use of APAP may contribute to the development of lung disease, primarily through oxidative stress, glutathione depletion, and inflammatory pathways (4). APAP metabolism involves the production of a reactive intermediate, N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses, NAPQI is detoxified by conjugation with glutathione (5). However, excessive or prolonged use of APAP depletes glutathione stores. Since glutathione is critical for neutralizing reactive oxygen species (ROS) in the lungs, its depletion increases oxidative stress and damages lung tissue (6). Research has shown that elevated oxidative stress can disrupt the integrity of the alveolar-capillary barrier and induce apoptosis of lung epithelial cells (7, 8). Studies have linked prenatal exposure to APAP with an increased risk of asthma and wheezing, suggesting that it may interfere with normal lung development by inducing oxidative stress and immune dysregulation (9, 10).

Leucomethylene blue (LMB) represents the reduced, colorless form of methylene blue (MB), which can be reversibly oxidized back to MB under physiological conditions. This redox interconversion plays a critical role in the pharmacodynamics and bioactivity of MB, especially in contexts involving oxidative stress and mitochondrial dysfunction (11, 12). Functionally, LMB retains the capacity to penetrate biological membranes more readily than its oxidized counterpart due to its uncharged state, enabling intracellular accumulation and targeted action within mitochondria (13). Once within the mitochondria, LMB is reoxidized to MB, thereby facilitating electron transfer across the mitochondrial electron transport chain (ETC), particularly between complexes I and III, where MB substitutes for impaired or damaged components (11). This electron shuttling enhances ATP production while simultaneously reducing the generation of reactive oxygen species (ROS), thereby contributing to the compound's cytoprotective effects during inflammatory and hypoxic insults (11, 14, 15). Given the well-established antioxidant and anti-inflammatory properties of LMB (16, 17), this study was undertaken to evaluate its potential protective effects against acetaminophen-induced acute lung injury. Specifically, we aimed to assess its impact on oxidative stress markers, inflammatory cytokines, and histopathological changes in a rat model.

## Material

### Study population

In the present study, 30 male Wistar rats weighing

180–200 grams were obtained from the Animal Center of Mashhad University of Medical Sciences (MUMS). The animals were randomly assigned to experimental groups and maintained under controlled conditions with a 12-hour light/dark cycle and a constant temperature of  $23 \pm 1$  °C. Throughout the study, all groups had free access to food and water. The experimental protocol was approved by the Animal Ethics Committee of the Faculty of Medicine at MUMS (Approval code: IR.MUMS.AEC.1402.105). It is important to note that the animals used in this study were the same rats previously included in our earlier published research on acetaminophen-induced hepatotoxicity (16). However, the present study specifically investigates pulmonary outcomes.

### Experiment design

A total of 30 rats were randomly allocated into five experimental groups, including a control group, APAP group, N-acetylcysteine (NAC) group, LMB group, and NAC+LMB group. All groups, except the control group, received an intraperitoneal injection of 1500 mg/kg APAP (Aria Pharmaceutical Company). Three hours later, the NAC group received NAC (100 mg/kg), the LMB group received LMB (5 mg/kg), and the NAC+LMB group received identical doses of both NAC and LMB. Rats were euthanized 24 hours after APAP administration. All rats were sedated via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Lung samples were frozen in liquid nitrogen and stored at  $-80$  °C for subsequent analysis of lung tissue. For histopathological examination, a portion of lung tissue was fixed in 10% neutral-buffered formalin. Whole blood was collected from the heart, and serum was obtained by centrifugation at  $1000 \times g$  for 20 minutes at 4 °C.

### Serum lung markers Levels

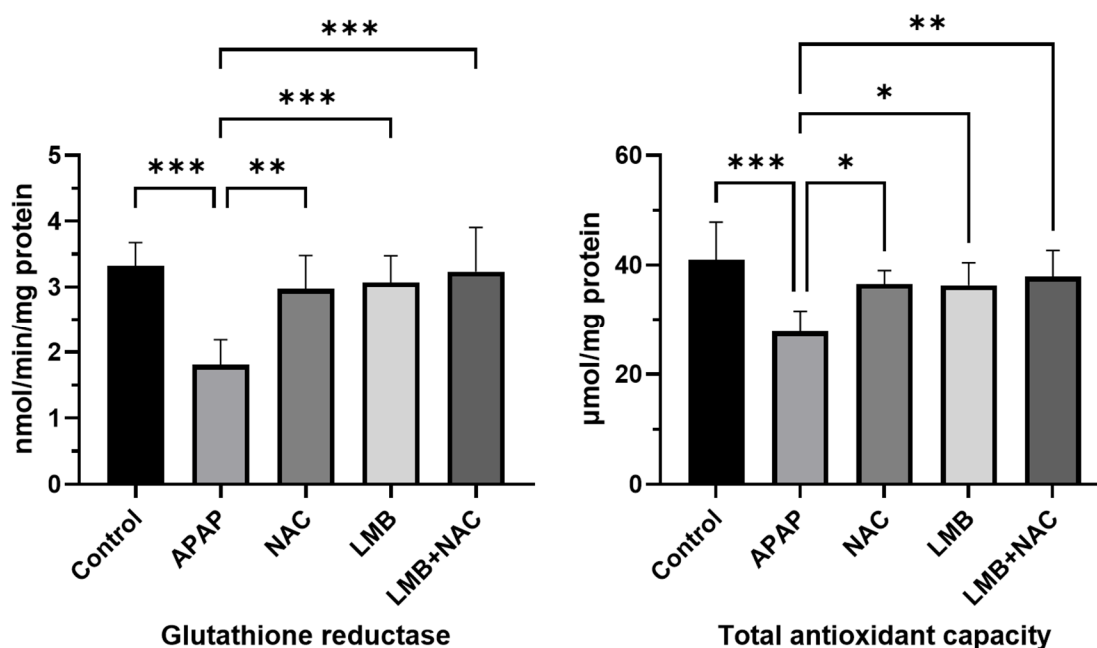
Serum levels of total antioxidant capacity (TAC) and glutathione reductase (GR) were measured using a colorimetric assay kit (Naxifer™, Navandsalamat Co., Iran). In addition, the levels of TNF- $\alpha$  and IL-6 in lung tissue were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Karmania Pars Gene Co., Kerman, Iran).

### Histopathological analysis

Lung tissue samples were collected, rinsed with cold isotonic saline, and sectioned into small pieces. These pieces were fixed in 10% neutral-buffered formalin for preservation until further processing. The fixed tissues were then embedded in paraffin blocks. Sections of 5  $\mu$ m thickness were cut from the paraffin-embedded blocks and stained with Hematoxylin and Eosin (H&E) for histological analysis under a light microscope.

### Statistical analyses

Statistical analyses were conducted using GraphPad Prism 10 software, employing one-way ANOVA



**Figure 1.** Effect of LMB and NAC on TAC and GR as antioxidant markers in lung tissue. Data are expressed as mean  $\pm$  SD. (\*  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ). TAC: total antioxidant capacity, GR: glutathione reductase

followed by Tukey's post-hoc test. The statistical significance of data distribution was assessed using the Kolmogorov–Smirnov test. Data are expressed as mean  $\pm$  standard deviation (SD). Results with a p-value of less than 0.05 were considered statistically significant.

## Results

### Effects of LMB on oxidative stress markers

The impact of APAP toxicity on oxidative stress was evaluated by measuring serum levels of TAC and GR activity. Our results indicated that APAP significantly reduced TAC levels compared to the control group ( $27.80 \pm 3.7$   $\mu\text{mol/mg protein}$  vs.  $40.95 \pm 6.8$   $\mu\text{mol/mg protein}$ ). Additionally, LMB significantly elevated TAC levels compared to the APAP group ( $36.31 \pm 4.08$   $\mu\text{mol/mg protein}$  vs.  $27.80 \pm 3.7$   $\mu\text{mol/mg protein}$ ). GR activity was also significantly reduced in the APAP group compared to the control group ( $1.81 \pm 0.3$   $\text{nmol/min/mg protein}$  vs.  $3.31 \pm 0.3$   $\text{nmol/min/mg protein}$ ). Furthermore, our findings indicate that LMB markedly elevated GR activity compared to the APAP group ( $3.07 \pm 0.4$   $\text{nmol/mg protein}$  vs.  $1.81 \pm 0.3$   $\text{nmol/mg protein}$ ) (Fig. 1).

### Effects of LMB on Inflammatory Markers

The effect of APAP toxicity on inflammation was assessed by quantifying inflammatory markers in lung tissue. APAP administration led to substantial lung injury and significantly increased IL-6 ( $1.01 \pm 0.17$   $\text{ng/mg protein}$  vs.  $0.52 \pm 0.17$   $\text{ng/mg protein}$ ) and TNF- $\alpha$  ( $6.65 \pm 1.4$   $\text{ng/mg protein}$  vs.  $3.58 \pm 0.48$   $\text{ng/mg protein}$ ) levels compared to the control group. Treatment with

LMB significantly reduced this inflammatory response, as evidenced by the marked decrease in IL-6 and TNF- $\alpha$  levels relative to the APAP group, indicating its anti-inflammatory effects (Fig. 2).

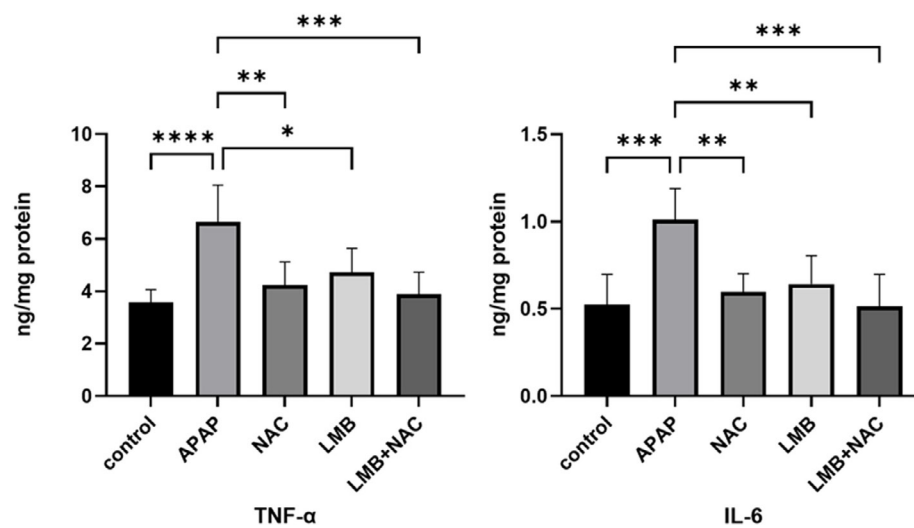
### Histopathological findings

Compared to the control group (Fig. 3A), acetaminophen administration induced several histopathological lesions in the lungs. These included pulmonary edema, characterized by the presence of homogenous eosinophilic material within the alveoli, along with congestion and hemorrhage. Additionally, necrosis of bronchiolar epithelial cells and sloughing of necrotic cells into the lumen, abnormal enlargement of alveolar air spaces (emphysema), incomplete alveolar expansion (atelectasis), and infiltration of mononuclear inflammatory cells into the alveoli and interstitial spaces were observed in the APAP group (Fig. 3B and 3C). Treatment with NAC (Fig. 3D), LMB (Fig. 3E), and the NAC+LMB combination (Fig. 3F) considerably mitigated the lesions caused by APAP exposure, reducing the severity of edema and hemorrhage. However, moderate emphysema remained in all three treatment groups, as well as persistent inflammation in the NAC group and atelectasis in the NAC+LMB group (Fig. 3).

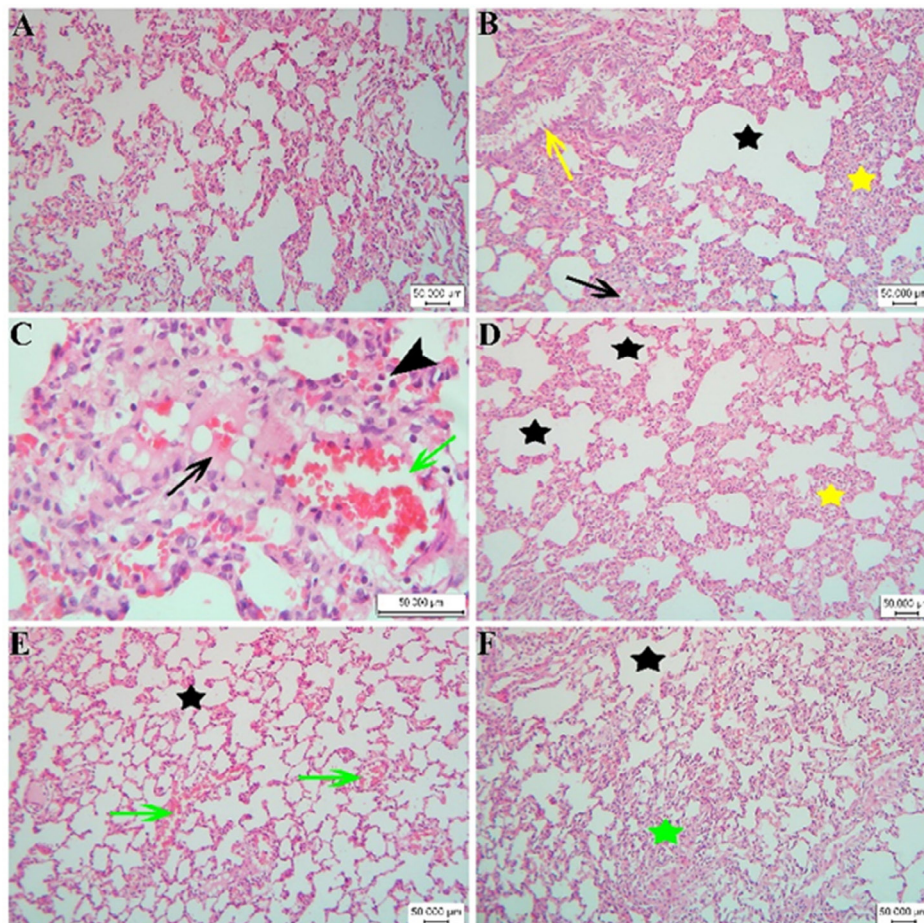
## Discussion

This study provides compelling evidence that LMB significantly attenuates APAP-induced pulmonary injury in rats by reducing oxidative stress and inflammation. Although APAP is commonly associated with hepatotoxicity, some studies suggest its toxic effects may extend to the lungs, particularly in cases of overdose or chronic use (18). Several mechanisms underlie this pulmonary toxicity, including glutathione depletion,





**Figure 2.** Effect of LMB and NAC on IL6 and TNF- $\alpha$  levels in lung tissue. Data are expressed as mean  $\pm$  SD. (\*  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ). IL6: interleukin 6, TNF- $\alpha$ : tumor necrosis factor  $\alpha$



**Figure 3.** Photomicrographs showing the H&E-stained lung sections. (A) The control group, exhibiting nearly typical lung microstructure ( $\times 100$  magnification). (B) The APAP group, showing emphysema (black star), infiltration of mononuclear inflammatory cells (yellow star), edema with hemorrhage (black arrow), and sloughing of necrotic bronchiolar epithelial cells into the lumen (yellow arrow), is seen ( $\times 100$  magnification). (C) Higher magnification ( $\times 400$ ) of B, revealing congestion (green arrow), mononuclear inflammatory cell infiltration (arrowhead), and hemorrhage along with edema (black arrow). (D) Emphysema (black star) and inflammation (yellow star) are still evident in the NAC group. (E) Emphysema (black star) is present in the LMB group. (F) NAC+LMB group, showing emphysema (black star) and atelectasis (green star) ( $\times 100$  magnification for D-F). Hematoxylin and eosin stain; scale bar = 50  $\mu$ m for all.

ROS generation, and cytokine-mediated inflammation (18, 19). In this context, McKeever et al. reported that glutathione depletion is a central mechanism contributing to APAP-induced tissue damage, including in lung tissue (20). In agreement with these findings, our study demonstrated that APAP administration led to marked reductions in TAC and GR activity, both of which are key defenses against oxidative lung injury.

MB is a recognized treatment for methemoglobinemia, an oxidative condition, and its *in vivo* conversion to LMB enhances its therapeutic potential in APAP-induced oxidative injury. MB is a potent antioxidant with the ability to scavenge ROS and reduce oxidative stress. Additionally, MB modulates mitochondrial function, which is crucial for cellular energy production and survival (16, 21–23). These results suggest that MB may exert its protective effects on lung function by augmenting the antioxidant defense system and preventing oxidative damage to cellular components. Consistent with these findings, our study demonstrated that acetaminophen administration significantly increased oxidative stress, as evidenced by decreased TAC and GR activity, while treatment with LMB effectively counteracted this effect by restoring antioxidant levels and reducing oxidative damage.

Our results showed that treatment with LMB significantly suppressed lung inflammation, as evidenced by the reduction of TNF- $\alpha$  and IL-6. These effects are likely due to the anti-inflammatory properties of LMB, a reduced form of MB that inhibits the NF- $\kappa$ B signaling pathway, thereby curbing excessive cytokine production and immune cell recruitment to lung tissue (16). Previous studies have also shown that MB and its reduced form, LMB, exert broad anti-inflammatory effects via similar mechanisms, further supporting this mode of action (16, 24).

Additionally, in our study, histopathological evaluation revealed structural damage such as alveolar hemorrhage, edema, emphysema, and inflammatory cell infiltration—findings consistent with acute lung injury pathology. The histological improvements observed in the LMB and NAC+LMB groups underscore the protective effect of LMB in preserving lung architecture. Notably, while NAC also mitigated APAP-induced damage, the combination therapy did not produce substantially greater improvements than LMB alone, suggesting that LMB may have a more prominent role in addressing oxidative lung damage. Furthermore, our findings are supported by previous animal models. For instance, Dobrinskikh et al. demonstrated that APAP exposure resulted in inflammatory gene expression and histological lung changes in mice (18).

NAC is widely used to treat acetaminophen overdose by replenishing glutathione stores, a key component in APAP detoxification. However, its intravenous administration is often associated with adverse reactions, including anaphylaxis—particularly in asthmatic individuals—as well as gastrointestinal symptoms and skin rashes (25–27). The narrow therapeutic window of NAC further limits its clinical applicability. In contrast, MB offers broader cytoprotective effects through multiple mechanisms, including direct antioxidant action, mitochondrial support, and modulation of

signaling pathways (22, 23). Additionally, the reduced form of MB, LMB, is more lipophilic, allowing better cellular penetration and potentially enhancing its therapeutic efficacy (21). Given these attributes, MB may represent a safer and more versatile alternative to NAC in oxidative stress-related conditions. Overall, this study highlights LMB as a promising adjunct treatment to counteract APAP-induced pulmonary injury through modulation of oxidative stress and inflammatory signaling. Future research should focus on the clinical translation of these findings and investigate the potential benefits of LMB in human models of drug-induced lung injury.

In summary, the results of this study showed that LMB can effectively reduce lung damage caused by acetaminophen poisoning. LMB administration improved antioxidant status by increasing TAC and GR activity, both of which were significantly decreased by acetaminophen toxicity. LMB also significantly reduced the levels of inflammatory cytokines in lung tissue, indicating its anti-inflammatory role. In addition, histopathological analysis corroborated these biochemical findings, revealing that LMB lessened the severity of lung lesions such as edema, hemorrhage, and inflammatory cell infiltration. These protective effects may be attributed to the ability of LMB to reduce oxidative stress, inhibit the NF- $\kappa$ B pathway, and support mitochondrial function. Compared with NAC, LMB alone showed a greater effect in improving lung injury, and its combination with NAC did not produce greater improvement than either agent alone.

Overall, the findings of this study suggest that LMB could be used as an effective adjunctive therapeutic agent to reduce lung injury induced by acetaminophen overdose. Given the properties of this compound, further investigation in human clinical models is recommended.

### Author Contribution

All authors contributed to the study design, conception, and manuscript creation, and have reviewed and approved the final version of the manuscript. Language editing and clarity improvements in this manuscript were assisted by OpenAI's ChatGPT. The tool was employed strictly for language refinement and did not contribute to the scientific content, data interpretation, or conclusions of the study.

### Conflict of Interest

The authors declared that they have no conflict of interest.

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