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Methylene Blue Mitigated Acetaminophen-Induced Hepatotoxicity in an Experimental Animal Model

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Abstract

Acetaminophen (AMP)-induced hepatotoxicity (AIH) is the critical side effect associated with high-dose exposure. N-acetylcysteine (NAC) is the gold standard to preserve the liver against AMP toxicity, with some drawbacks, leading to the search for alternative therapy. The antioxidant properties of methylene blue (MB), make its application plausible for AIH, as an additive therapy with NAC or alone. Therefore, the present study aimed to define the histological impact of MB on hepatoprotection. A total of 49 rats were enrolled in the present study. Rats were sub-classified into 7 groups: control group, AMP group, MB group, prophylaxis-MB low dose, prophylaxis-MB high dose, treatment-MB low dose, and treatment-MB high dose. Liver histopathological changes were examined under a microscope, and images were taken for all groups. Photomicrograph of a rat's liver section in the control group exposed to normal saline showing intact tissue. Rat liver sections from the AMP-treated group demonstrated central venous dilatation and severe portal inflammation. Rat liver section in the MB-50 prophylaxis group demonstrated mild portal inflammation with an increase in ductular proliferation. Rat liver section in the MB group with portal tracts, with no significant pathology. A rat liver section from the MB-100 prophylaxis group demonstrated a mild increase in portal ductules. The rat liver section in the MB-50 and MB-100 treatment group demonstrated a liver with a normal central vein. The MB intervention demonstrated marked protective effects against AIH in experimental rats, with interventional treatment providing better resolution than prophylaxis; nevertheless, both interventions were effective in blocking hepatotoxicity.

Keywords: Methylene blue, Acetaminophen, Mitochondria, Liver, Hepatotoxicity

Introduction

Acetaminophen (AMP), or paracetamol, is the commonly used over-the-counter analgesic and antipyretic agent globally (Zafeiri *et al*., 2020; Mehrzad *et al*., 2022; Bate *et al*., 2023; Oloyede *et al*., 2023; Nagdalian *et al*., 2024). At usual therapeutic doses, AMP is almost safe, while toxic doses causes liver failure, responsible for thousands of hospital admissions and high annual mortality rate (Rotundo & Pyrsopoulos, 2020; Shoghi & Kian, 2022; Chidiac *et al*., 2023; Huyen *et al*., 2023; Petronis *et al*., 2023; Sakhnenkova *et al*., 2023). The clinical load of AMP-induced hepatotoxicity ranges beyond acute mortality, involving morbidity, extended hospitalizations, and increase healthcare costs (Lancaster *et al*., 2015; Yoon *et al*., 2016; Bessone *et al*., 2019; Cantile *et al*., 2024). This health challenge determines the need for a new treatment modalities to moderate hepatocellular damage and block progression to hepatic failure. The pathogenesis of AMP-induced hepatotoxicity involved metabolic dysregulation, oxidative stress, mitochondrial dysfunction, and inflammatory activation (Jaeschke *et al*., 2019; Nguyen & Hoang, 2022; Trung *et al*., 2022; Ncube *et al*., 2023). At the molecular levels, toxic doses of AMP resulted in the generation of the highly reactive and intermediate metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Lee *et al*., 2015). This overproduction of NAPQI jeopardizes the endogenous antioxidant system, leading to NAPQI-mediated attack on vital biomolecules, including mitochondrial proteins, ultimately resulting in hepatocellular necrosis (Lee *et al*., 2015; Jaeschke *et al*., 2019).

Methylene blue (MB) is a tricyclic compound exemplified by lipophilicity and a low molecular weight, providing fast cellular uptake and superior precipitation within mitochondria due to the organelle's negative membrane potential (Chen *et al*., 2015; Tucker *et al*., 2018; Brekeit *et al*., 2022). The MB demonstrates unique redox cycling potentials, existing in equilibrium between its oxidized (blue, cationic) and reduced (colorless, leuco-methylene blue) forms, a feature that underlies many of its biological activities (Hamed *et al*., 2022; Efati *et al*., 2025). The MB within the mitochondrial matrix works as a substitute for electron carrier, accepting electrons from reduced nicotinamide adenine dinucleotide (NADH) and donating them directly to cytochrome c, thereby bypassing Complex I and Complex III of the electron transport chain—the principal sites of both NAPQI-induced dysfunction and physiological superoxide generation (Lee *et al*., 2015; Jaeschke *et al*., 2019). These actions will protect mitochondrial respiration and ATP production by reducing reactive oxygen species, thereby keeping the antioxidant capacity of endogenous antioxidant biomolecules (superoxide dismutase and catalase) (Chen *et al*., 2015).

The management of AMP poisoning based on administration of the antidote N-acetylcysteine (NAC) (Yoon *et al*., 2016; Jaeschke *et*

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al., 2019; Borgne-Sanchez & Fromenty, 2025). The mechanisms by which NAC provides hepatoprotective effects include operating as a glutathione precursor to replenish exhausted hepatic GSH stores, thereby increasing NAPQI elimination; supplying sulfhydryl groups that can directly detoxify NAPQI and ROS; recovering hepatic capillary bed flow and hence improving oxygen delivery; and possibly imparting anti-inflammatory effects (Rushworth & Megson, 2014; Lancaster *et al*., 2015; Yoon *et al*., 2016). Early administration of NAC within 8 hours of paracetamol poisoning effectively reduces hepatotoxicity; this efficacy decreases as time elapses, alongside other downsides of NAC, including anaphylactoid reactions associated with intravenous administration and nausea or vomiting associated with the loading dose (Borgne-Sanchez & Fromenty, 2025). These negative impacts have led to the investigation of another, more useful compound; therefore, this study was conducted to identify an alternative, more efficacious than NAC that avoids these downside features.

Materials and Methods

Study Settings

The laboratory animals were used in an experimental part of the study conducted in the animal house utility of the College of Veterinary Medicine (University of Mosul, Iraq). A total of 49 rats (10 weeks old; 200 grams) were allocated in the animal house (College of Veterinary Medicine, University of Mosul) for use in this study. These rats were adapted for 2 weeks and handled under standard conditions of light/dark cycle, temperature, and humidity, with free access to food and water.

Experimental Design

The 49 rats were subdivided into 7 groups (7 rats each):

Control Group: received distilled water orally for 13 days, sacrificed at day 14, and tissues were collected for histology.

Acetaminophen Group: received distilled water orally for 13 days, and an oral dose of 2000mg/kg body weight of AMP was administered on day 7, sacrificed at day 14, and tissues were collected for histology.

Methylene Blue Group: received MB orally for 13 days at a dose of 100mg/kg body weight, sacrificed at day 14, and tissues were collected for histology.

Prophylaxis (MB50+AMP) Group: received MB orally for 7 days at a dose of 50mg/kg body weight, and an oral dose of 2000mg/kg body weight of AMP was administered on day 7 (Three days no medication, sacrificed at day 10, and tissues were collected for histology).

Prophylaxis (MB100+AMP) Group: received MB orally for 7 days at a dose of 100mg/kg body weight, and an oral dose of 2000mg/kg body weight of AMP was administered on day 7 (Three days no medication, sacrificed at day 10, and tissues were collected for histology).

Treatment (MB50+AMP+MB50) Group: received MB orally for 13 days at a dose of 50mg/kg body weight, and an oral dose of 2000mg/kg body weight of AMP was administered on day 7 (MB continued until day 13, sacrificed at day 14, and tissues were collected for histology).

Treatment (MB100+AMP+MB100) Group: received MB (orally, 13 days, 100mg/kg body weight), and an oral dose of 2000mg/kg body weight of AMP was administered on day 7 (MB continued until day 13, sacrificed at day 14, and tissues were collected for histology).

Drug Preparation

Drugs have been prepared instantly before administration and given to animals orally by gavage needles. Acetaminophen tablet 500 mg (Troje, Germany), Methylene blue powder (Loba Chemie, India) was diluted for administration using normal saline.

Histological Examination

Rats were sacrificed at the end of the experiment by cervical dislocation under deep anesthesia. The heart tissues were collected, rinsed with phosphate-buffered saline, and fixed in 10% buffered formalin for a few days until ready for further slide preparation. Tissue processing for slide preparation started based on the previous protocol (Abdullah *et al*., 2022). The slices were placed on slides, deparaffinized, and rehydrated, then stained with hematoxylin and eosin (H&E). All slides were examined by light microscopy using a digital camera system.

Results and Discussion

Control Group

The tissue demonstrates normal structure, with preserved hepatic parenchyma; the liver cells are organized into anastomosing structures from the central veins. The cellular density is normal, with no apparent congestion, demonstrating tissue preservation. The liver cells showed clear cellular boundaries, uniform polygonal shapes, and centrally located nuclei. The cytoplasm looks normal with a lightly pale pink color. No inflammatory infiltrates, necrosis, fibrosis, or fatty change was detected, reflecting the healthy hepatic tissue **(Figure 1)**.

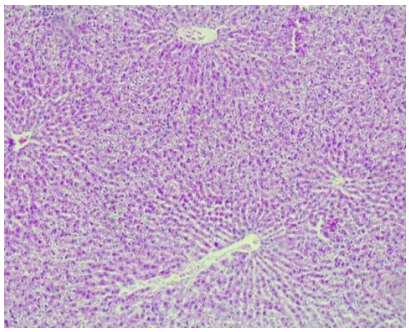
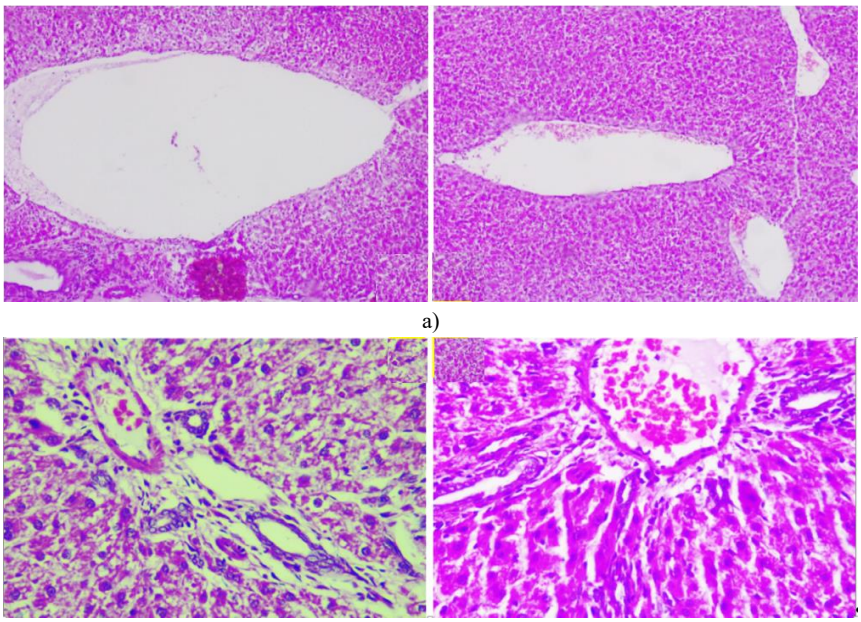


Figure 1. Photomicrograph of a rat's liver section in the normal control group exposed to normal saline (H&E; x100).

Acetaminophen-Alone Group

The rat liver slides from an acetaminophen-treated group showed significant hepatotoxicity of acetaminophen overdose. There is marked dilatation of the central vein with loss of normal hepatic architecture, sinusoidal dilatation, and hemorrhage in some areas. The periphery of the lobules appears congested with vacuolar degeneration **(Figure 2a)**. The liver tissue section shows inflammatory cell infiltration, likely mainly mononuclear cells recruited to areas of hepatocellular injury **(Figure 2b)**.



a)

b)

Figure 2. Photomicrograph of a rat's liver section in the acetaminophen-treated group showing (a) liver sections with dilatation of the central vein. (H&E; x100) (b) liver sections with severe portal inflammation composed of lymphocytes & scattered neutrophils (H&E; x400).

Methylene Blue-Alone Group

The histological section of rat liver tissue demonstrates a preserved hepatic parenchyma with liver cells arranged in patterns with normal morphology, maintaining a characteristic polygonal shape with distinct cell boundaries, without significant pathological changes, alongside no inflammation, fibrosis, or immune cells infiltration **(Figure 3)**.

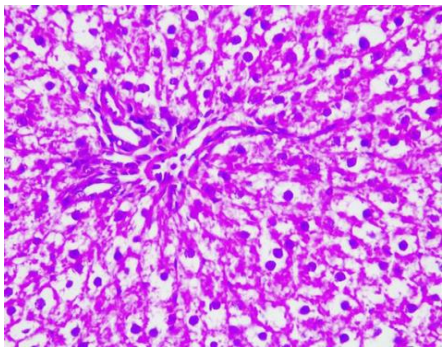


Figure 3. Photomicrograph of a rat's liver section in methylene blue group with portal tracts with no significant pathology (H&E; x400).

Methylene Blue-100 Prophylaxis Group

The histological section of rat liver tissue revealed preserved hepatic parenchyma with hepatocytes organized in their trabecular pattern and sinusoidal spaces remained patent; however, the section also revealed bile duct proliferation reflected by the presence of numerous biliary channels compared to normal liver tissue. No evidence of notable hepatocellular necrosis, degeneration, vacuolation, or marked immune cell infiltration **(Figure 4)**.

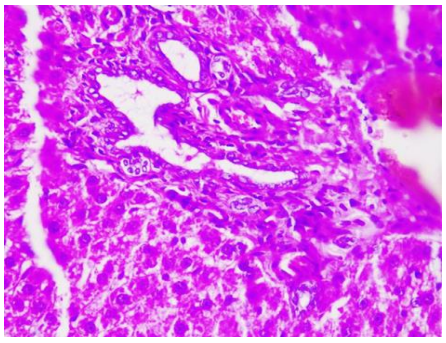


Figure 4. Photomicrograph of a rat's liver section in the methylene blue-100 prophylaxis group showing liver with mild increase of portal ductules (H&E; x400).

Methylene Blue-50 Prophylaxis Group

The histological section of rat liver tissue at portal tracts demonstrated mild infiltration of immune cells (lymphocytes with dispersed neutrophils and eosinophils). The tissue section also indicated an increase in bile ductile proliferation within the portal

zones. The liver parenchyma preserved its architecture, and sinusoidal spaces remain intact and patent **(Figure 5)**.

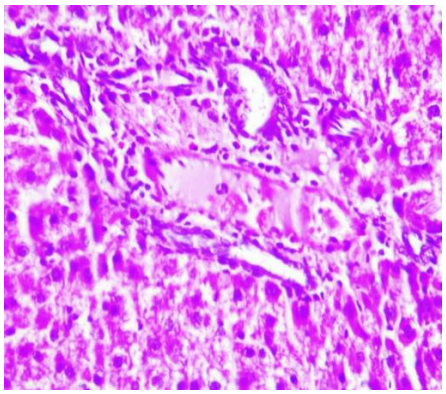


Figure 5. Photomicrograph of a rat's liver section in methylene blue-50 prophylaxis group showing liver with portal mild inflammation composed of lymphocytes & scattered neutrophils & eosinophils, as well as an increase of ductile (H&E; x400)

Methylene Blue-100 Treatment Group

The histological section of rat liver tissue demonstrated that the central vein appears normal with an intact endothelial lining, patent vascular lumen, and no congestion or thrombosis detected. The hepatocytes preserved their typical polygonal shapes. The tissue sections revealed no apparent tissue necrosis, degeneration, or vacuolation **(Figure 6)**.

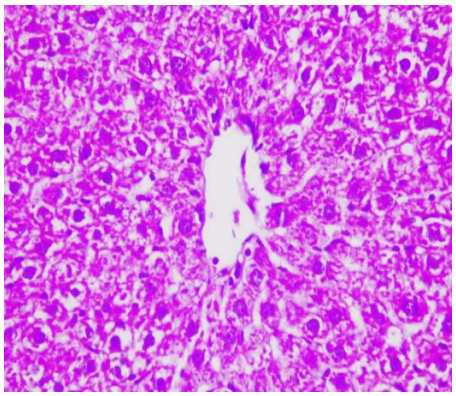


Figure 6. Photomicrograph of a rats liver section in the methylene blue-100 treatment group, showing liver with normal central vein (H&E; x400)

Methylene Blue-50 Treatment Group

The histological section of rat liver tissue revealed preserved liver structure around the normal central vein with minimal inflammatory changes. The vessel remained patent with no congestion, thrombosis, or vascular damage. The liver preserved its normal polygonal shapes with no apparent tissue necrosis, degeneration, or vacuolation **(Figure 7)**.

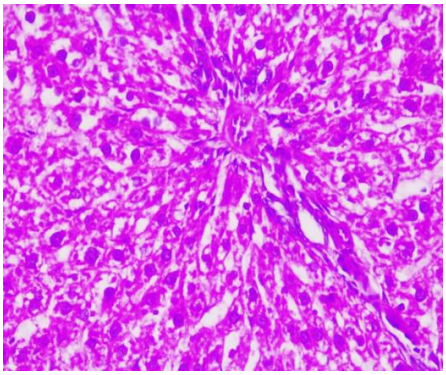


Figure 7. Photomicrograph of a rats liver section in the methylene blue-50 treatment group showing liver with normal central vein with few inflammatory cells (H&E; x400)

The present study findings confirmed that the use of MB as a prophylaxis or treatment against acetaminophen-induced hepatotoxicity in an experimental animal model has resulted in protection against liver injury. The outcomes, confirmed by histological findings, showed no tissue degeneration, vacuolation, or low-grade inflammation, in contrast to high immune cell infiltration, tissue necrosis, or vacuolation, which are representative of the paracetamol liver toxicity group. Additionally, the use of MB has been associated with increased ductile generation, reflecting the healing process and tissue regeneration after paracetamol toxicity.

In the line of the present study, Lee *et al*. (2015) have shown that MB has provided hepatoprotective effects against acetaminophen-induced toxicity and explained the mechanisms based on mitochondrial energy support, antioxidant mechanism, and modulation of autophagy (Lee *et al*., 2015). Methylene blue bypasses mitochondrial complexes I and III, functioning as a substitute for electron transport in the electron transport chain. Acetaminophen extensively jeopardizes hepatocyte mitochondrial function because NAPQI binds to mitochondrial proteins, resulting in oxidative stress damage and ATP depletion. This means that MBpreserves mitochondrial electron flow and hepatocyte viability during the early hours of acetaminophen toxicity (Lee *et al*., 2015; Jaeschke *et al*., 2019; Ramachandran & Jaeschke, 2023). Moreover, MB offered antioxidant action against AMP-induced oxidative stress, preventing methemoglobinemia (Sahu *et al*., 2020; Efati *et al*., 2025). Once glutathione is exhausted, MB can replace glutathione because it scavenges ROS, alleviating cellular damage beyond what NAC alone (Rushworth & Megson, 2014). The mitochondrial damage induced by acetaminophen is associated with increased accumulation of damaged protein and cells overburden localized environment, MB impacts autophagy pathways, potentially aiding cells to clear damaged proteins and organelles overwhelming cellular compensatory mechanisms, enhanced autophagy could accelerate recovery (Zhao *et al*., 2016; Tucker *et al*., 2018; L. Yang *et al*., 2020; Gureev *et al*., 2022).

The MB has shown a great healing impact and regeneration potential, as confirmed by increased ductile formation in the histological section. This regenerative potential has been reported earlier by Chen *et al*. (2015) in a rat model exposed to paraquat,

resulting in liver injury, in which MB enhanced hydroxylase expression, thereby promoting tissue regeneration (Chen *et al*., 2015). In another study, conducted by Boz *et al*. (2020), it was shown that local application of MB solution to surgically repaired tendons in a chicken model reduced tissue adhesion formation postoperatively (Boz *et al*., 2020). The regenerative and healing mechanisms are potentially related to the multifaceted MB roles, including mitigating oxidative stress by competing with molecular oxygen for electron transfer, thereby inhibiting free radical generation (Lee & Boelsterli, 2014; Duicu *et al*., 2017; Aburel *et al*., 2025; González & Jaramillo-Fierro, 2025). Moreover, MB demonstrated anti-inflammatory effects by decreasing levels of proinflammatory cytokines (El Sayed & Sayed, 2019; Nedu *et al*., 2020; Li & Ying, 2023). Alongside, the ability of MB in mitigating fibrotic processes (Xue *et al*., 2021; Seitkazina Assel Yang Jin-Kyoung, 2022; Dibekoğlu *et al*., 2025), and hence enhancing intrinsic healing alongside reducing fibroblastic precipitation that leads to adhesion formation (El-Sayed *et al*., 2016; Yang *et al*., 2020). These mechanisms altogether enhance re-epithelialization and support scarless, more functional tissue healing with lower-grade adhesion (Milyavsky & Dickie, 2017).

The healing of liver tissue promoted by MB in the present study was also reported in previous studies and explained in the context of MB ability to mitigate liver damage due to oxidative stress (Aksu *et al*., 2010; Collange *et al*., 2013; Chen *et al*., 2015; Kim *et al*., 2020). Methylene blue reduced lipid peroxidation and malondialdehyde formation, restored superoxide dismutase activity (a primary antioxidant defense pathway), mitigated nitric oxide formation, blocked neutrophil infiltration at the site of injury, and promoted regeneration and healing (Yildiz *et al*., 2011; Ayvaz *et al*., 2014). Alongside reduced collagen deposition and fibroblast precipitation indicated by reduced tissue hydroxyproline marker, perhaps related to the capacity of MB in blocking oxidative stress and lipid peroxidation, which in turn stimulates stellate cell activation and extracellular matrix production, and hence blocking the liver damage of cholestatic injury to cirrhosis (Aksu *et al*., 2010; Collange *et al*., 2013). These antioxidant properties are indirectly related to the capacity of MB to compete with oxygen molecules at the iron-sulfur centers of xanthine oxidase, jolting electron flow away from pathways that generate superoxide radicals and instead redirecting them to form leukomethylene blue (Tucker *et al*., 2018).

Conclusion

Methylene blue provided protective effects against acetaminophen-induced hepatotoxicity in experimental rats, with interventional treatment providing better resolution than prophylaxis. Histopathological sections improved in the treatment group and, to a lesser extent, in the prophylaxis group compared with the acetaminophen-alone group.

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Conflict of interest: None

Financial support: None

Ethics statement: The present study has been registered for approval at the College of Medicine (University of Mosul, Iraq), Approval letter number CM/UoM30 on 06 October 2025.

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