

Study of Hematological Alterations and Oxidative Stress in *Labeo rohita* Exposed to Sub-lethal Methylene Blue

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ABSTRACT

Methylene Blue (MB), a synthetic dye widely used in aquaculture for its antifungal properties, poses significant ecological risks due to its sub-lethal toxicity in aquatic organisms. This study investigates the hematological and oxidative stress responses in *Labeo rohita* (rohu) exposed to sub-lethal concentrations of MB (1/5th and 1/20th of the 96-hour LC50 of 8.5 mg/L) over 7, 14, and 28 days. Hematological parameters, including red blood cell (RBC) morphology, hemoglobin (Hb%) levels, total leukocyte count (TLC), and differential leukocyte profiles, were analyzed alongside lipid peroxidation (LPO) to assess oxidative damage. Results revealed significant dose- and time-dependent disruptions: RBCs exhibited crenation and membrane shrinkage, Hb% declined by 51% ($4.8 \pm 0.3\%$) in the 1/5th LC50 group by day 28, and TLC increased by 116% ($15.8 \pm 0.7 \times 10^4$ cells/mm³), accompanied by neutrophilia ($37.5 \pm 1.8\%$) and lymphocytopenia ($58.0 \pm 1.8\%$). Oxidative stress markers, quantified as malondialdehyde (MDA), surged to 7.8 ± 0.6 nmol/g in high-dose groups, correlating strongly with Hb% reduction ($r = -0.89$, $p < 0.01$), underscoring ROS-mediated erythrocyte damage. Statistical analysis (ANOVA, $p < 0.001$) confirmed the significance of these alterations. The study identifies MB-induced anemia, immunosuppression, and oxidative stress as critical threats to fish health, validating hematological biomarkers as reliable indicators of sub-lethal toxicity. These findings emphasize the need for regulatory limits on MB discharge and advocate for eco-friendly alternatives in aquaculture. Future research should explore molecular mechanisms of MB toxicity and long-term ecological impacts to inform sustainable practices and mitigate risks to aquatic biodiversity.

Keywords- Methylene Blue Toxicity, Hematological Biomarkers, Oxidative Stress, Aquatic Toxicology.

I. INTRODUCTION

Methylene Blue (MB), a synthetic thiazine dye, is widely utilized in aquaculture, medicine, and industrial processes due to its antifungal, antiparasitic, and staining properties (Srivastava et al., 2004). In aquaculture, it is frequently applied to treat parasitic infections (e.g., *Ichthyophthirius multifiliis*) and fungal outbreaks in fish (Noga, 2010). However, its indiscriminate use and improper disposal have led to its accumulation in aquatic ecosystems, raising concerns about its chronic toxicity to non-target organisms, particularly fish (Das & Mukherjee, 2003). While acute toxicity studies of MB are well-documented, its sub-lethal effects—those occurring at concentrations below

the lethal threshold—remain understudied, despite their potential to disrupt physiological homeostasis and compromise long-term survival (Velisek et al., 2009).

Hematological parameters serve as critical biomarkers for evaluating the physiological stress and sub-lethal effects of pollutants in fish (Fazio, 2019). Red blood cells (RBCs), hemoglobin (Hb%), and white blood cells (WBCs) are particularly sensitive to chemical stressors, as they reflect the organism's oxygen transport efficiency, immune response, and metabolic health. For instance, reduced RBC counts and hemoglobin levels are classic indicators of anemia, often linked to impaired erythropoiesis or oxidative damage to erythrocytes (Adhikari et al., 2004). Similarly,

alterations in leukocyte profiles, such as neutrophilia or lymphocytopenia, signal immune activation or immunosuppression under chemical stress (Sharma & Jindal, 2020). In *Labeo rohita* (rohu), a commercially significant freshwater fish in South Asia, hematological disruptions have been correlated with exposure to pesticides and heavy metals, underscoring the utility of these parameters in ecotoxicological assessments (Das & Mukherjee, 2003).

Oxidative stress, a key mechanism of dye toxicity, arises from the imbalance between reactive oxygen species (ROS) production and antioxidant defenses. MB is known to generate ROS through redox cycling, leading to lipid peroxidation (LPO), protein denaturation, and DNA damage (Srivastava et al., 2004). In fish, ROS-induced membrane degradation in RBCs can manifest as morphological abnormalities (e.g., crenation, poikilocytosis) and reduced oxygen-carrying capacity (Pandey et al., 2008). Chronic exposure to sub-lethal MB concentrations may also suppress antioxidant enzymes like glutathione (GSH), exacerbating cellular damage (Olojo et al., 2005). Despite these risks, few studies have investigated the temporal progression of oxidative stress and hematological alterations in fish exposed to MB, particularly at environmentally relevant concentrations.

This study focuses on *Labeo rohita*, to evaluate the hematological and oxidative stress responses induced by sub-lethal MB exposure. The objectives are threefold:

1. To assess dose- and time-dependent changes in RBC and WBC morphology, hemoglobin levels, and leukocyte counts.
2. To quantify oxidative stress through lipid peroxidation (LPO) and correlate it with hematological disruptions.
3. To establish the sub-lethal toxicity thresholds of MB for *Labeo rohita*, providing data to inform regulatory guidelines.

By analyzing these parameters over 7, 14, and 28 days of exposure to 1/5th and 1/20th LC50 concentrations of MB, this research bridges a critical gap in understanding the chronic effects of MB on fish physiology. The findings have implications for aquaculture practices and environmental management, particularly in regions where MB contamination threatens aquatic biodiversity and food security.

II. REVIEW OF LITERATURE

Hematological parameters are pivotal biomarkers in aquatic toxicology, offering insights into the physiological and immunological status of fish exposed to environmental stressors. These parameters, including red blood cell (RBC) count,

hemoglobin (Hb%) levels, and leukocyte profiles, are highly sensitive to xenobiotics such as heavy metals, pesticides, and synthetic dyes (Fazio, 2019). For instance, RBC count and Hb% are directly linked to oxygen transport efficiency, and their decline often signals anemia or oxidative damage to erythrocytes. Conversely, leukocyte dynamics, such as total leukocyte count (TLC) and differential leukocyte count (DLC), reflect immune responses, with neutrophilia indicating inflammation or stress and lymphocytopenia suggesting immunosuppression (Sharma & Jindal, 2020).

The vulnerability of these hematological indices to pollutants is well-documented. In *Cyprinus carpio* (common carp), exposure to industrial effluents containing heavy metals like lead and cadmium resulted in a 40% reduction in RBC count and a 35% decline in Hb%, impairing oxygen delivery and metabolic functions (Velisek et al., 2009).

Similarly, *Oreochromis niloticus* (Nile tilapia) exposed to sub-lethal concentrations of the pesticide chlorpyrifos exhibited neutrophilia (up to 45% neutrophils) and lymphocytopenia (55% lymphocytes), indicative of stress-induced immune activation (Shalaby et al., 2016). These studies underscore the role of hematological parameters as early warning systems for chemical toxicity, even at sub-lethal concentrations.

Methylene Blue (MB), though widely used in aquaculture for its antifungal properties, is a potent oxidative stressor. MB generates reactive oxygen species (ROS) through redox cycling, disrupting cellular membranes and metabolic pathways (Srivastava et al., 2004). In fish, ROS overproduction damages erythrocyte membranes, leading to morphological anomalies such as crenation, poikilocytosis, and cell shrinkage. For example, *Clarias gariepinus* (African catfish) exposed to MB exhibited a 30% increase in lipid peroxidation (LPO) and a 25% reduction in glutathione (GSH) levels, confirming ROS-mediated oxidative damage (Olojo et al., 2005). Chronic MB exposure also suppresses antioxidant enzymes like catalase and superoxide dismutase, exacerbating cellular injury (Das & Mukherjee, 2003).

Despite these findings, most studies on MB toxicity focus on acute exposure or high concentrations, leaving a gap in understanding its sub-lethal, chronic effects. For instance, Das and Mukherjee (2003) demonstrated that *Labeo rohita* exposed to 2 mg/L MB for 96 hours showed significant hematological disruptions, including a 20% drop in RBC count. However, the long-term impacts of lower, environmentally relevant concentrations (e.g., 1/5th or 1/20th LC50) remain unexplored. Such concentrations are critical to

study, as they mimic real-world scenarios where MB residues persist in water bodies due to continuous discharge from aquaculture or textile industries.

The interplay between oxidative stress and hematological dysfunction further underscores the need for prolonged exposure studies. In *Channa punctata* (spotted snakehead), sub-chronic exposure to the textile dye methyl orange caused a 50% increase in LPO and a 30% decline in Hb%, correlating strongly with RBC membrane damage (Pandey et al., 2008). Similarly, *Wallago attu* exposed to industrial dyes exhibited dose-dependent neutrophilia and eryptosis (premature RBC death), highlighting the cascading effects of oxidative stress on hematopoiesis (Pandey et al., 2008). These findings suggest that even low-level MB exposure could trigger similar pathways in *Labeo rohita*, compromising its survival in polluted habitats.

III. METHODOLOGY

Experimental Design

Adult *Labeo rohita* (rohu) specimens, with a mean weight of 250 ± 30 g, were procured from a local aquaculture farm and acclimatized for 15 days in 500-L fiberglass tanks containing dechlorinated water. The acclimatization environment was maintained at a pH of 7.2, temperature of $25 \pm 2^\circ\text{C}$, dissolved oxygen level of 6.5–7.0 mg/L, and a 12-hour light/dark photoperiod. Fish were fed a commercial pellet diet (30% protein) at 3% body weight daily, with fasting 24 hours prior to experiments to minimize metabolic interference. Water quality parameters (ammonia, nitrite, nitrate) were monitored daily and kept within safe limits (<0.02 mg/L, <0.5 mg/L, and <5 mg/L, respectively).

Methylene Blue (MB) stock solutions were prepared using analytical-grade dye (Sigma-Aldrich) dissolved in distilled water. Sub-lethal concentrations (1/5th and 1/20th LC50) were derived from 96-hour acute toxicity tests using Probit analysis (Finney, 1971). The LC50 value for MB was calculated as 8.5 mg/L, yielding sub-lethal concentrations of 1.7 mg/L (1/5th LC50) and 0.425 mg/L (1/20th LC50). Fish were distributed into triplicate tanks ($n = 10$ fish/tank) for each concentration and exposure duration (7, 14, and 28 days). Control groups were maintained in MB-free water under identical conditions.

Hematological Analysis

Blood samples were collected from the caudal vein using a 23-gauge heparinized syringe after euthanizing fish with clove oil (50 mg/L). Approximately 1 mL of blood was drawn per fish and divided into two aliquots: one for immediate analysis and another for smear preparation.

- i. **RBC and WBC Morphology:** Blood smears were prepared by spreading 5 μL of blood on glass slides, air-dried, and fixed with absolute methanol for 5 minutes. Slides were stained with Giemsa stain (1:10 dilution in phosphate buffer, pH 6.8) for 15 minutes (Bancroft & Gamble, 2008). Cellular abnormalities (e.g., crenation, poikilocytosis) were assessed under a light microscope (1000 $\times$ magnification, oil immersion).
- ii. **Hemoglobin (Hb%):** The cyanmethemoglobin method was employed (Drabkin, 1946). Blood (20 μL) was mixed with 5 mL Drabkin's reagent (0.61 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.77 mM KCN, 1.03 mM KH_2PO_4 , 0.1% Triton X-100). Absorbance was measured at 540 nm using a spectrophotometer, and Hb% was calculated against a standard curve.
- iii. **Total Leukocyte (TLC) and RBC Counts:** Blood was diluted 1:20 in Hayem's fluid (RBCs) and 1:10 in Türk's solution (WBCs). Cells were counted manually using an improved Neubauer hemocytometer under 400 $\times$ magnifications. Results were expressed as cells/ mm^3 (Jain, 1986).
- iv. **Differential Leukocyte Count (DLC):** Stained smears were scanned systematically to classify 200 leukocytes into neutrophils, lymphocytes, and monocytes based on nuclear morphology and cytoplasmic granularity (Campbell & Ellis, 2007).

Oxidative Stress Assessment

Lipid peroxidation (LPO) was quantified as malondialdehyde (MDA) levels using the thiobarbituric acid (TBA) method (Ohkawa et al., 1979). Liver tissue (100 mg) was homogenized in 1.15% KCl, centrifuged at 10,000 $\times g$ for 15 minutes, and the supernatant reacted with 0.8% TBA in 20% acetic acid (pH 3.5) at 95°C for 1 hour. Absorbance was measured at 532 nm, and MDA concentration (nmol/g tissue) was calculated using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

Statistical Analysis

Data were analyzed using SPSS v26. Normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were confirmed. One-way ANOVA compared means across control and treated groups, with Tukey's post-hoc test for pairwise comparisons ($p < 0.05$). Probit analysis (Finney, 1971) modeled concentration-mortality relationships to determine LC50. Descriptive statistics (mean $\pm$ SD) were tabulated, and Pearson's correlation assessed relationships between LPO and hematological parameters.

IV. RESULTS AND DISCUSSIONS

Hematological Alterations

Exposure to sub-lethal concentrations of Methylene Blue (MB) induced significant dose- and time-dependent disruptions in the hematological profile of *Labeo rohita*.

- i. **RBC Morphology:** Erythrocytes in exposed fish exhibited crenation and membrane shrinkage (Figure 1), indicative of oxidative damage to the cell membrane. These morphological anomalies align with findings in *Channa punctata* exposed to textile dyes, where reactive oxygen species (ROS)-mediated lipid peroxidation destabilized RBC membranes (Pandey et al., 2008). Such deformities impair oxygen transport efficiency, exacerbating physiological stress in polluted environments.
- ii. **Hemoglobin Decline:** Hemoglobin (Hb%) levels decreased progressively with exposure duration and concentration (Table 1). By day 28, the 1/5th LC50 group showed a 51% reduction in Hb% ($4.8 \pm 0.3\%$) compared to controls ($9.6 \pm 0.3\%$), signaling severe anemia. This mirrors observations in *Clarias gariepinus* exposed to lead, where Hb% declines were linked to ROS-induced eryptosis (premature RBC death) and suppressed erythropoiesis (Olojo et al., 2005). The hypoxia caused by reduced Hb% compromises metabolic functions, affecting growth and survival.
- iii. **Leukocyte Response:** Total leukocyte count (TLC) increased by 116% in the 1/5th LC50 group ($15.8 \pm 0.7 \times 10^4$ cells/mm³) by day 28 (Table 2). Concurrently, differential leukocyte count (DLC) revealed neutrophilia ($37.5 \pm 1.8\%$) and lymphocytopenia ($58.0 \pm 1.8\%$), indicative of stress-induced immune activation. Similar leukocyte shifts were reported in *Oreochromis niloticus* exposed to pesticides, where elevated neutrophils signaled inflammation, while reduced lymphocytes reflected immunosuppression (Shalaby et al., 2016). This immune dysregulation heightens susceptibility to secondary infections in contaminated habitats.

Oxidative Stress Correlation

Lipid peroxidation (LPO) levels surged to 7.8 ± 0.6 nmol MDA/g in the 1/5th LC50 group by day 28 (Table 3), confirming ROS-mediated cellular damage. The strong negative correlation between Hb% and LPO ($r = -0.89$, $p < 0.01$) underscores oxidative stress as a primary driver of anemia. MB generates ROS through redox cycling, which oxidizes polyunsaturated fatty acids in RBC membranes, leading to hemolysis and hemoglobin degradation (Srivastava et al., 2004). These findings

align with studies on *Wallago attu*, where industrial dyes induced similar oxidative hematotoxicity (Pandey et al., 2008).

Statistical Validity

One-way ANOVA revealed significant intergroup differences ($p < 0.001$) for all parameters (Table 4), confirming the dose- and time-dependent effects of MB. The robustness of the LC50 value ($R^2 = 0.96$) derived via Probit analysis (Finney, 1971) further validated the experimental design. The consistency of these results with prior studies on fish exposed to xenobiotics (Das & Mukherjee, 2003; Sharma & Jindal, 2020) reinforces the reliability of hematological biomarkers in ecotoxicological assessments.

Table 1: Hemoglobin (Hb%) levels in *Labeo rohita* exposed to sub-lethal MB concentrations.

Exposure Duration (days)	Group	Hemoglobin (%)
7	Control	9.8 ± 0.3
	1/20th LC50	8.5 ± 0.4
	1/5th LC50	7.0 ± 0.3
14	Control	9.7 ± 0.3
	1/20th LC50	7.8 ± 0.3
	1/5th LC50	6.0 ± 0.4
28	Control	9.6 ± 0.3
	1/20th LC50	6.5 ± 0.4
	1/5th LC50	4.8 ± 0.3

Table 2: Total Leukocyte Count (TLC) in *Labeo rohita* exposed to MB.

Exposure Duration (days)	Group	TLC ($\times 10^4$ cells/mm ³)
7	Control	7.5 ± 0.3
	1/20th LC50	9.0 ± 0.4
	1/5th LC50	12.5 ± 0.5
14	Control	7.4 ± 0.3
	1/20th LC50	10.0 ± 0.5
	1/5th LC50	14.0 ± 0.6
28	Control	7.3 ± 0.3
	1/20th LC50	11.5 ± 0.6
	1/5th LC50	15.8 ± 0.7

Table 3: Lipid Peroxidation (LPO) levels in *Labeo rohita* exposed to MB.

Exposure Duration (days)	Group	LPO (nmol MDA/g)
7	Control	1.8 ± 0.2
	1/20th LC50	3.2 ± 0.3
	1/5th LC50	4.5 ± 0.4
14	Control	1.7 ± 0.1
	1/20th LC50	4.0 ± 0.3
	1/5th LC50	6.2 ± 0.5

28	Control	1.7 ± 0.1
	1/20th LC50	4.8 ± 0.4
	1/5th LC50	7.8 ± 0.6

Table 4: ANOVA results for hematological and oxidative stress parameters.

Parameter	F-Value	p-Value	Significance
Hemoglobin (Hb%)	27.89	<0.001	Significant
TLC	36.54	<0.001	Significant
LPO	48.65	<0.001	Significant
RBC Count	25.76	<0.001	Significant

V. CONCLUSION

The study conclusively demonstrates that sub-lethal exposure to Methylene Blue (MB) induces significant hematological and oxidative stress disruptions in *Labeo rohita*, compromising their physiological resilience. Dose- and time-dependent reductions in hemoglobin levels (51% decline at 1/5th LC50 by day 28) and RBC counts, coupled with morphological erythrocyte abnormalities (crenation, membrane shrinkage), confirm MB-induced anemia. Concurrently, elevated total leukocyte counts (116% increases) and neutrophilia (37.5%) reflect immune activation and stress, while lipid peroxidation (7.8 ± 0.6 nmol MDA/g) underscores ROS-mediated cellular damage. These findings align with prior studies on fish exposed to xenobiotics, validating hematological parameters as reliable biomarkers for assessing sub-lethal toxicity (Fazio, 2019; Sharma & Jindal, 2020). The strong correlation between oxidative stress and hematological dysfunction ($r = -0.89$) highlights MB's role in disrupting erythrocyte integrity and immune homeostasis. Such physiological impairments threaten fish survival in polluted habitats, emphasizing the ecological risks of unchecked MB discharge.

FUTURE RECOMMENDATIONS

To mitigate the ecological and physiological risks posed by Methylene Blue (MB), regulatory agencies should establish permissible discharge limits based on sub-lethal toxicity thresholds identified in this study, ensuring aquaculture and industrial effluents are monitored for MB residues. Furthermore, replacing MB with eco-friendly alternatives such as natural dyes (e.g., turmeric) or non-toxic antifungal agents (e.g., chitosan) could reduce dependency on synthetic dyes. Mechanistic studies are needed to elucidate molecular pathways linking MB exposure to eryptosis and immunosuppression, particularly focusing on ROS-mediated apoptosis and cytokine dysregulation. Chronic exposure research should evaluate multi-generational impacts on fish reproduction, growth, and genetic integrity to assess long-term ecological consequences.

Advanced remediation strategies, including biochar adsorption and photocatalytic degradation, must be developed to remove MB from contaminated water bodies. Finally, community awareness programs should educate aquaculture stakeholders about MB's risks and sustainable alternatives through workshops and policy advocacy to foster environmentally responsible practices.

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