



Original Research Paper

NEUROPROTECTIVE EFFECTS OF COMBINED LYCOPENE AND PRIMIDONE IN A WEIGHT-DROP MOUSE MODEL OF TRAUMATIC BRAIN INJURY: MODULATION OF OXIDATIVE STRESS AND NEUROTRANSMITTER HOMEOSTASIS

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ABSTRACT

Background: Traumatic brain injury triggers a multifaceted secondary damage cascade characterized by oxidative/nitrosative stress, excitotoxicity, and neurotransmitter dysregulation, resulting in enduring neurological impairments. Few effective therapies address these interrelated processes. Lycopene, a powerful antioxidant, and primidone, an anticonvulsant with neurostabilizing properties, present a rational combinatorial strategy.

Objective: To assess the individual and combined neuroprotective effects of Lycopene and repurposed Primidone on behavioral, biochemical, and neurotransmitter changes generated by TBI.

Methods: Traumatic brain injury was generated in mice utilizing a weight-drop technique. Animals received lycopene, Primidone, or a combination 30 minutes after injury. Behavioral evaluations were performed at 24 hours and on day 7, followed by biochemical tests of antioxidant enzymes (SOD, CAT, GSH), lipid peroxidation, nitrosative stress, cerebral edema, blood-brain barrier integrity, and neurotransmitters (GABA, glutamate, dopamine, serotonin, AChE).

Results: Traumatic brain injury induced significant behavioral impairments, oxidative and nitrosative stress, neurotransmitter dysregulation, and blood-brain barrier disruption ($p < 0.001$ compared to control). Lycopene and Primidone monotherapy resulted in partial enhancement of these measures ($p < 0.05$ compared to vehicle). Combination treatment yielded markedly enhanced neuroprotection ($p < 0.01$ compared to monotherapy), reinstating antioxidant defenses, diminishing lipid peroxidation and nitrosative stress, resetting neurotransmitter equilibrium, and lowering cerebral edema. These effects continued or were enhanced on day 7.

Conclusion: The combination of Lycopene and Primidone treatment offers improved and prolonged neuroprotection in traumatic brain injury by addressing redox imbalance and neurotransmitter dysregulation, hence endorsing a multi-target therapeutic approach with translational promise.

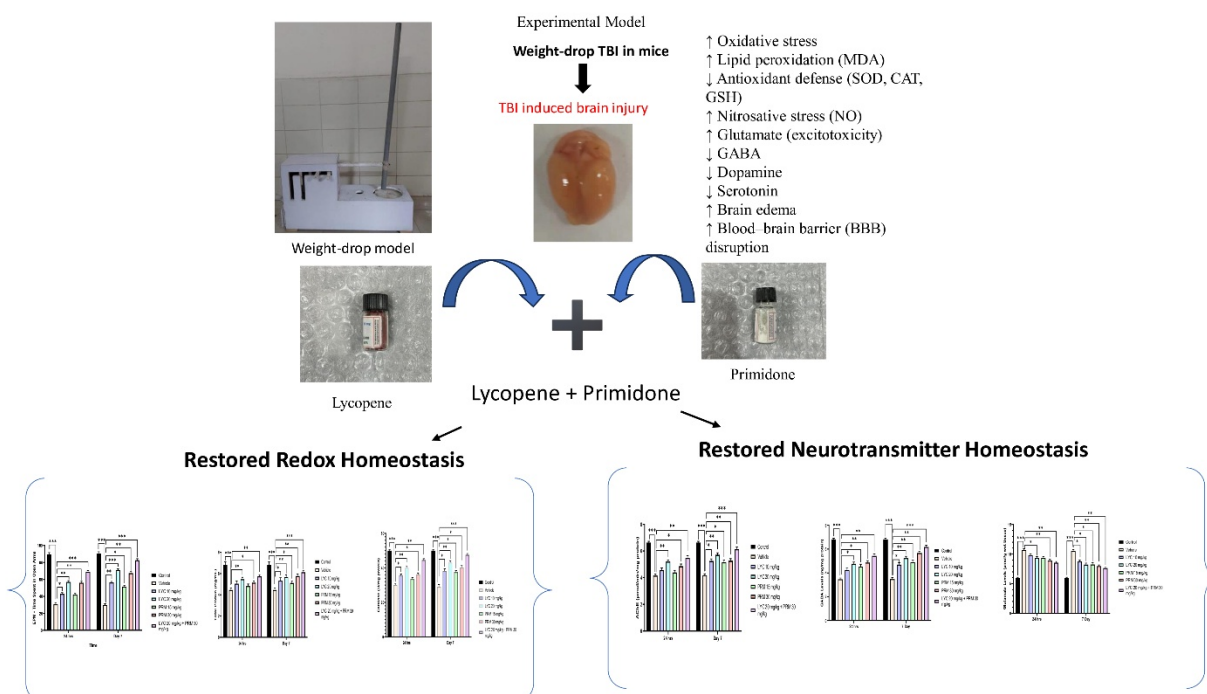
Keywords: Traumatic brain injury; Lycopene; Primidone; Oxidative stress; Neurotransmission; Neuroprotection

GRAPHICAL ABSTRACT

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1. INTRODUCTION

Traumatic brain injury (TBI) is a significant global health issue and a primary cause of mortality and enduring neurological disability, characterized by persistent motor, cognitive, and behavioral deficits [1]. The initial mechanical injury transpires instantaneously; however, the progression of neurological damage is chiefly governed by a multifaceted cascade of secondary injury mechanisms, encompassing oxidative and nitrosative stress, excitotoxicity, mitochondrial dysfunction, and neuroinflammation [2–4]. Among these, oxidative stress constitutes a principal pathological phenomenon, facilitating lipid peroxidation, protein oxidation, neuronal apoptosis, and the disruption of redox homeostasis and synaptic function [5, 6]. Despite extensive preclinical and clinical investigations, the current management of traumatic brain injury (TBI) remains primarily supportive, lacking approved pharmacological interventions that effectively address the secondary injury cascade [7–9]. This unmet therapeutic requirement underscores the imperative to formulate multi-target strategies capable of modulating both initial and advancing pathological processes subsequent to injury. In this context, drug repurposing has surfaced as a promising translational strategy, facilitating swift clinical application through the use of agents with well-established safety and pharmacokinetic profiles [10].

Lycopene (LYC), a naturally occurring carotenoid, demonstrates significant antioxidant and anti-inflammatory effects. It removes reactive oxygen species (ROS), stops lipid peroxidation, and changes redox-sensitive signaling pathways like Nrf2 and NF- κ B [11, 12]. Preclinical studies have shown that it protects neurons in models of neurodegenerative diseases like Parkinson's, Alzheimer's, and epilepsy by lowering

oxidative stress, mitochondrial dysfunction, and neuronal loss [13, 14]. Nevertheless, the combined potential of LYC in conjunction with agents that inhibit excitotoxic neuronal signaling in traumatic brain injury has not been comprehensively examined.

Primidone (PRM), a clinically sanctioned anticonvulsant, primarily functions by augmenting GABAergic neurotransmission and stabilizing neuronal excitability. PRM not only works as an anticonvulsant, but it has also been shown to lessen excitotoxicity caused by glutamate and may lower oxidative stress indirectly by restoring the balance between excitatory and inhibitory signals [15, 16]. These characteristics indicate that PRM may confer neuroprotection in scenarios marked by heightened excitatory signaling, such as traumatic brain injury (TBI).

To date, no research has comprehensively investigated a combinatorial strategy that simultaneously targets redox imbalance and neurotransmitter dysregulation in traumatic brain injury (TBI). Because LYC and PRM work in different ways to reduce oxidative stress and excitotoxic neurotransmission, we thought that giving them both at the same time would make them work better together to protect the brain by modulating both pathways. This combinatorial strategy is especially pertinent in traumatic brain injury (TBI), where interconnected redox and neurotransmitter disruptions lead to secondary neuronal damage. Consequently, this study aimed to assess the individual and combined effects of LYC and PRM in a weight-drop model of experimental traumatic brain injury in mice. Neuroprotective efficacy was evaluated during both acute (24 hours) and subacute (day 7) phases to elucidate initial injury mechanisms and prolonged recovery. Outcomes encompassed behavioral performance, oxidative stress

indicators, cerebral edema, and neurotransmitter equilibrium.

We suggest that simultaneously addressing redox imbalance and neurotransmitter dysregulation with LYC

and PRM will improve neuroprotection, offering a mechanistically sound and clinically applicable therapeutic approach for reducing secondary brain injury after TBI.

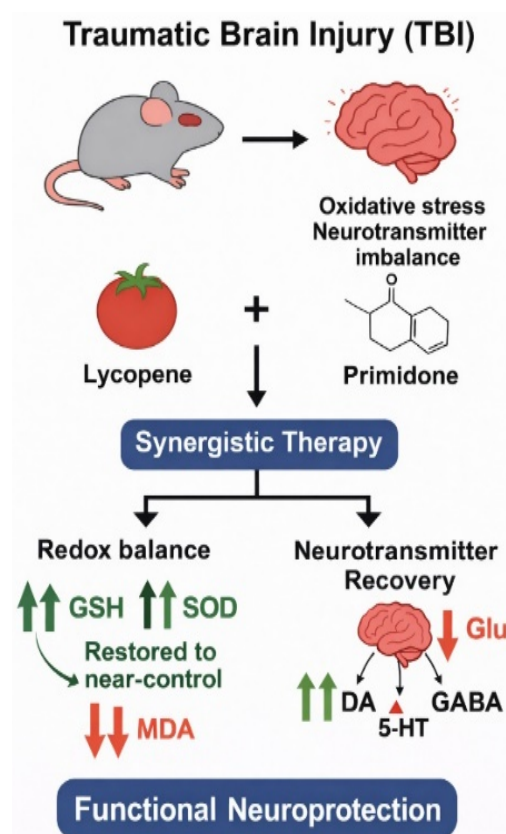


Figure 1. Experimental design and outcome assessment following TBI

Traumatic brain injury induces oxidative stress and neurotransmitter imbalance, contributing to secondary neuronal injury. Combined administration of LYC and PRM restores redox homeostasis by increasing antioxidant defenses (GSH and SOD) and reducing lipid peroxidation (MDA), while normalizing neurotransmitter levels by increasing dopamine (DA), serotonin (5-HT), and GABA and decreasing glutamate (Glu). These complementary effects collectively promote functional neuroprotection.

Graphical depiction of the experimental procedures for inducing traumatic brain injury in a mouse model via the weight drop system and giving LYC and/or PRM to the animals after being injured. The animals were subjected to a series of behavioral tests for anxiety, coordination, cognitive skills, and neurological impairments 24 hours following the injury. Once the test procedures were completed, brain and cerebrospinal fluids were acquired for biochemical, enzymatic, and neurotransmitter assessments (including markers of oxidative stress, including SOD, CAT, GSH, MDA, NO), total protein, brain water content, acetylcholinesterase, and levels of

the primary neurotransmitters (GABA, glutamate, dopamine, and serotonin).

2. MATERIALS AND METHODS

2.1 Animal Study

This study utilized male Swiss albino mice (25–30 g), maintained in polypropylene cages within standard laboratory conditions, characterized by a temperature of $25 \pm 2^\circ\text{C}$, relative humidity of 60–65%, and a 12-hour light/dark cycle, with unimpeded access to standard pellet food and water. Before the experiment started, the animals were given seven days to get used to their new environment. The Institutional Animal Ethics Committee (IAEC) gave the experimental protocols the green light, and they followed the rules set by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India, approval number RVNI/IAEC/23-24/20.

2.2 Drugs and Chemicals

All chemicals/reagents were of analytical grade and obtained from commercial suppliers (Sigma-Aldrich, Merck, SRL, Loba Chemie, BDH & Thomas Baker).

Lycopene (LYC 98% purity) and primidone (PRM $\geq 99\%$ purity) were purchased from Sigma-Aldrich USA.

2.3 Experimental Design

2.3.1 Induction of TBI

A model of TBI was developed that induced an impact-acceleration mechanism (weight drop impact) using a weight drop from 1 m onto a 5 mm diameter circular disk or to an area on the top of the head of a mouse. The mechanism used to induce impact in each case was based on previously described methods except for a few small changes [17]. Isoflurane (2-3%) was used as an inhalant for anesthesia (2-3% induction/resources) to cause loss of the righting reflex. Each mouse was placed on a rounded foam or sponge pad to reduce injury from rebound forces. A midline scalp incision was made to expose the area of impact on the skull. A 5 mm diameter disc was glued to the top of the skull to increase surface area for producing a larger area for distributing force during impact; a 5 mm diameter disc would provide approximately 25 mm of surface area if the impact zone were a circle. Moderate injuries were created by dropping a 30 g object from 1 m through a vertical 2 cm diameter tube onto the disc attached to the skull of the mouse. The incision was sutured, and Neosporin® was used as a topical antibiotic to reduce the chance of infection after a mouse was impacted. After impact, the mouse was not moved into another cage until it recovered completely from anesthesia. Each of the above parameters created a reproducible moderate TBI in a mass group of mice and was consistent with the weight drop described in a previous study of TBI [17].

2.3.2 Drug Treatment and Dosing Schedule

Swiss albino mice were assigned randomly to 7 experimental groups (n=6 animals/group). Test Compounds were given in a single dose 30min after induction of TBI. The doses of both LYC and PRM were chosen based upon prior studies which have demonstrated neuroprotective effects and safety in rodent models [18]. LYC was freshly prepared and suspended in 0.5% carboxymethylcellulose (CMC) immediately before administration, while PRM was freshly dissolved in normal saline. All treatments were administered orally (p.o.).

The definitions of the experimental groups were:

1. Group 1: Sham-operated control
2. Group 2: TBI + vehicle
3. Group 3: TBI + lycopene (LYC) (10 mg/kg, p.o.)
4. Group 4: TBI + lycopene (LYC) (20 mg/kg, p.o.)

5. Group 5: TBI + primidone (PRM) (15 mg/kg, p.o.)
6. Group 6: TBI + primidone (PRM) (30 mg/kg, p.o.)
7. Group 7: TBI + lycopene (LYC) (20 mg/kg, p.o.) + primidone (PRM) (30 mg/kg, p.o.)

2.3.3 Post-Injury Observation and Neurobehavioral Evaluation

Following TBI induction, animals were constantly watched to see how they reacted right after the impact and how they recovered from anesthesia. Neurobehavioral evaluations were conducted at 24 hours and on day 7 post-injury to assess sensorimotor and cognitive impairments. The Neurological Severity Score (NSS) was the tool used to figure out how bad the person's neurological problems were. It takes into account motor, sensory, reflex, and balance factors.

The weight-drop injury paradigm utilized in this study exemplifies a non-penetrating impact-acceleration model that induces reproducible cortical injury of moderate severity [17, 18].

2.4 Behavioural Assessments

Behavioral evaluations were conducted 24 h and day 7 following TBI induction in a standardized sequence to minimize task-related stress and interference. The following tests were employed:

- **Elevated Plus Maze (EPM):** Assessment of anxiety-like and exploratory behavior [19].
- **Novel Object Recognition (NOR):** Evaluation of recognition memory and cognitive performance [20].
- **Neurological Severity Score (NSS):** Quantification of neurological and motor deficits [21].
- **Rotarod Test:** Assessment of motor coordination, balance, and muscle strength [22].
- **Y-Maze Test:** Evaluation of spatial working memory and spontaneous alternation behavior [23].

2.5 Biochemical Estimations

Following completion of behavioral experiments, animals were humanely sacrificed, and brain tissues were rapidly removed, rinsed with ice-cold saline to remove blood residues, gently blotted dry, and homogenized in ice-cold phosphate buffer (0.1 M, pH 7.3). The homogenates were centrifuged at $10,000 \times g$ for 15 min at 4 °C, and the resulting supernatants were subjected to spectrophotometric estimation of oxidative stress parameters and enzymatic biomarkers using standard assay procedures, as outlined in Table 1.

Table 1: UV–Vis spectrophotometric principles and methods for biochemical assays.

Parameters	UV–Vis method principle
Acetylcholinesterase (AChE)	Hydrolysis of acetylthiocholine iodide with formation of yellow 5-thio-2-nitrobenzoate; absorbance measured at 412 nm [24]
Catalase (CAT)	Rate of hydrogen peroxide (H ₂ O ₂) decomposition monitored by

	decrease in absorbance at 240 nm [25]
Superoxide dismutase (SOD)	Inhibition of pyrogallol autoxidation measured at 420 nm [26]
Reduced glutathione (GSH)	Reaction with DTNB forming yellow 5-thio-2-nitrobenzoate; absorbance measured at 412 nm [27]
Malondialdehyde (MDA)	Thiobarbituric acid reactive substances (TBARS) assay; pink MDA-TBA adduct measured at 532 nm [28]
Nitric oxide (NO)	Griess reaction producing azo dye; absorbance measured at 540 nm [29]
Total protein	Folin-Ciocalteu (Lowry) method; absorbance measured at 750 nm [30]

AChE: acetylcholinesterase, *CAT*: catalase, *SOD*: superoxide dismutase, *GSH*: reduced glutathione, *MDA*: malondialdehyde, *NO*: nitric oxide.

2.5.1 Assessment of Brain Edema

The brain's water content was measured using the wet and dry weight technique to evaluate cerebral edema, as

previously outlined [31]. In summary, newly removed brain tissue was weighed to determine its wet weight and subsequently dried in a hot-air oven at 100–105°C until a consistent dry weight was achieved. The water content of the brain was determined as follows (Equation 1):

$$\text{Water content (\%)} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100 \dots\dots\dots \text{(Equation 1)}$$

2.6 Neurotransmitter Analysis

The levels of γ -aminobutyric acid (GABA), dopamine, glutamate, and serotonin (5-HT) were quantified using established fluorometric techniques in cortical tissue. Brain samples were homogenized in ice-cold 0.1 M perchloric acid with 0.05% EDTA to precipitate proteins and stabilize neurotransmitters. The homogenates were subjected to centrifugation at $15,000 \times g$ for 15 minutes at 4 °C, after which the supernatants were quantitatively tested for neurotransmitters.

Dopamine was quantified with an oxidative fluorometric technique (excitation 330 nm, emission 380 nm). Serotonin levels were quantified via the o-phthalaldehyde (OPA) condensation technique (excitation 360 nm, emission 475 nm) [32]. The concentration of GABA was assessed utilizing the ninhydrin-sulfite fluorometric method (excitation at 390 nm, emission at 475 nm). Glutamate concentrations were quantified enzymatically by NADH-based fluorometric detection (excitation at 340 nm, emission at 460 nm). Neurotransmitter concentrations were determined using external standards, adjusted to total protein content, and represented as ng/mg protein [33].

2.7 Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical comparisons were conducted utilizing one-way analysis of variance (ANOVA) accompanied by Tukey's post hoc test. Analyses were performed utilizing GraphPad software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was established at $p < 0.05$.

3. RESULTS

3.1 Impact of LYC and PRM on Post-Traumatic Anxiety-Like Behaviour (Elevated Plus Maze)

TBI significantly reduced time spent in the open arms of the elevated plus maze at both 24 h and day 7 compared

with controls ($p < 0.0001$), indicating increased anxiety-like behavior. At 24 hours, vehicle-treated animals showed reduced open-arm exploration. The LYC and PRM treatments increased open-arm time in a dose-dependent manner ($p < 0.05$ vs. vehicle), with greater effects observed at higher doses. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) produced a statistically significant improvement compared with both monotherapy groups, significantly exceeding both vehicle and monotherapy groups ($p < 0.01$). On day 7, anxiety-like behavior persisted in vehicle-treated animals ($p < 0.0001$ vs. control), while all treatment groups showed further improvement compared with 24 hours. LYC and PRM significantly increased open-arm exploration ($p < 0.05$ vs. vehicle), with higher doses showing greater effects. The combination group exhibited and produced a statistically significant improvement compared with both monotherapy groups, with values approaching control levels and remaining significantly higher than monotherapy groups ($p < 0.01$; Figure 2A).

3.2 Effects of LYC and PRM on Motor Coordination and Balance Assessed by the Rotarod Test

TBI significantly reduced rotarod latency at both 24 h and day 7 compared with sham controls ($p < 0.0001$). At 24 h, vehicle-treated animals showed decreased latency to fall. LYC increased rotarod performance in a dose-dependent manner ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) produced the greatest improvement, significantly exceeding both vehicle and monotherapy groups ($p < 0.01$). At day 7, motor impairment persisted in vehicle-treated animals ($p < 0.0001$ vs. control), while all treatment groups showed further improvement compared with 24 h (Figure 2B). LYC and PRM increased rotarod latency at both doses ($p < 0.05$ vs. vehicle), with greater

effects at higher doses. The combination group remained significantly improved compared with the vehicle ($p < 0.01$), although differences between the combination and high-dose monotherapy groups were not significant at this time point.

3.3 Effects of LYC and PRM on Neurological Recovery Assessed by the Neurological Severity Score (NSS)

TBI significantly increased NSS at both 24 h and day 7 compared with sham controls ($p < 0.0001$). At 24 hours, vehicle-treated animals showed elevated NSS values. LYC decreased NSS in a dose-dependent fashion ($p < 0.05$ vs. vehicle), whereas PRM exhibited a significant effect solely at 30 mg/kg. The combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) led to the most significant decrease in NSS, which was much lower than the vehicle and monotherapy groups ($p < 0.01$). At day 7, NSS remained elevated in vehicle-treated animals ($p < 0.0001$ vs. control), while all treatment groups showed further improvement compared with 24 h. LYC reduced NSS at both doses ($p < 0.05$ vs. vehicle), whereas PRM was effective at 30 mg/kg only. The combination group showed the lowest NSS values, significantly lower than the vehicle and monotherapy groups ($p < 0.01$; Figure 2C). NSS values in the combination group remained higher than control levels.

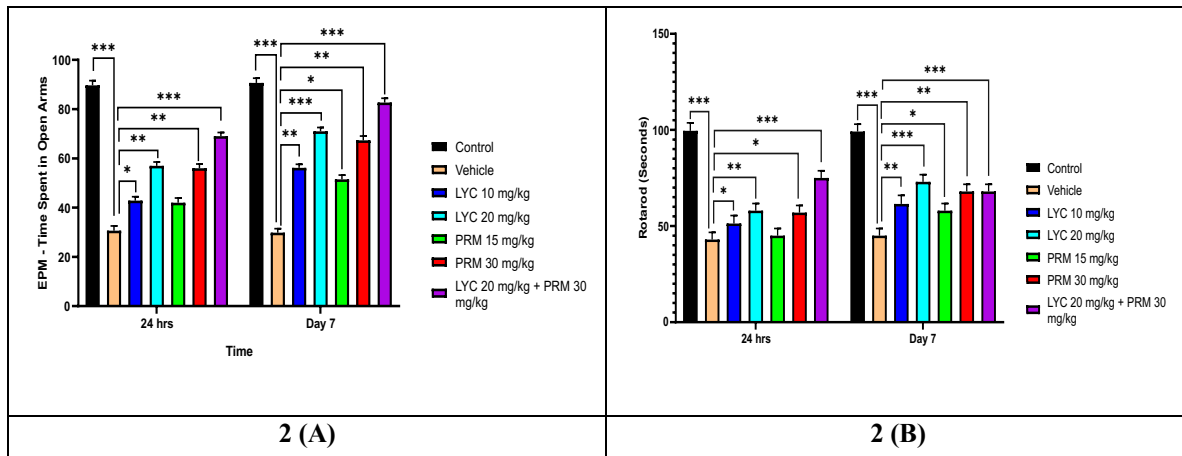
3.4 Effects of LYC and PRM on Recognition Memory in the Novel Object Recognition (NOR) Test

TBI significantly reduced the discrimination index at both 24 h and day 7 compared with sham controls ($p < 0.001$). At 24 h, vehicle-treated animals showed a decreased discrimination index. LYC improved recognition memory in a dose-dependent manner ($p <$

0.05 vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) produced the demonstrated superior efficacy relative to individual treatments, significantly exceeding both vehicle and monotherapy groups ($p < 0.01$). At day 7, impairment persisted in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed further improvement compared with 24 h. LYC and PRM increased discrimination index at both doses ($p < 0.05$ vs. vehicle), with greater effects at higher doses. The combination group showed the highest discrimination index, significantly greater than vehicle and monotherapy groups ($p < 0.01$; Figure 2D). Discrimination index values in the combination group were not significantly different from control.

3.5 Effects of LYC and PRM on Spatial Working Memory Assessed by the Y-Maze Test

TBI significantly reduced spontaneous alternation behavior at both 24 h and day 7 compared with sham controls ($p < 0.001$). At 24 h, vehicle-treated animals showed reduced alternation performance. LYC improved spontaneous alternation, with a significant effect at 20 mg/kg only ($p < 0.05$ vs. vehicle). PRM did not produce a significant effect at either dose. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly improved alternation behavior compared with vehicle ($p < 0.01$). At day 7, impairment persisted in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 h. LYC and PRM increased spontaneous alternation at both doses ($p < 0.05$ vs. vehicle). The combination group showed the highest alternation performance, significantly greater than vehicle ($p < 0.01$; Figure 2E), and values were not significantly different from control.



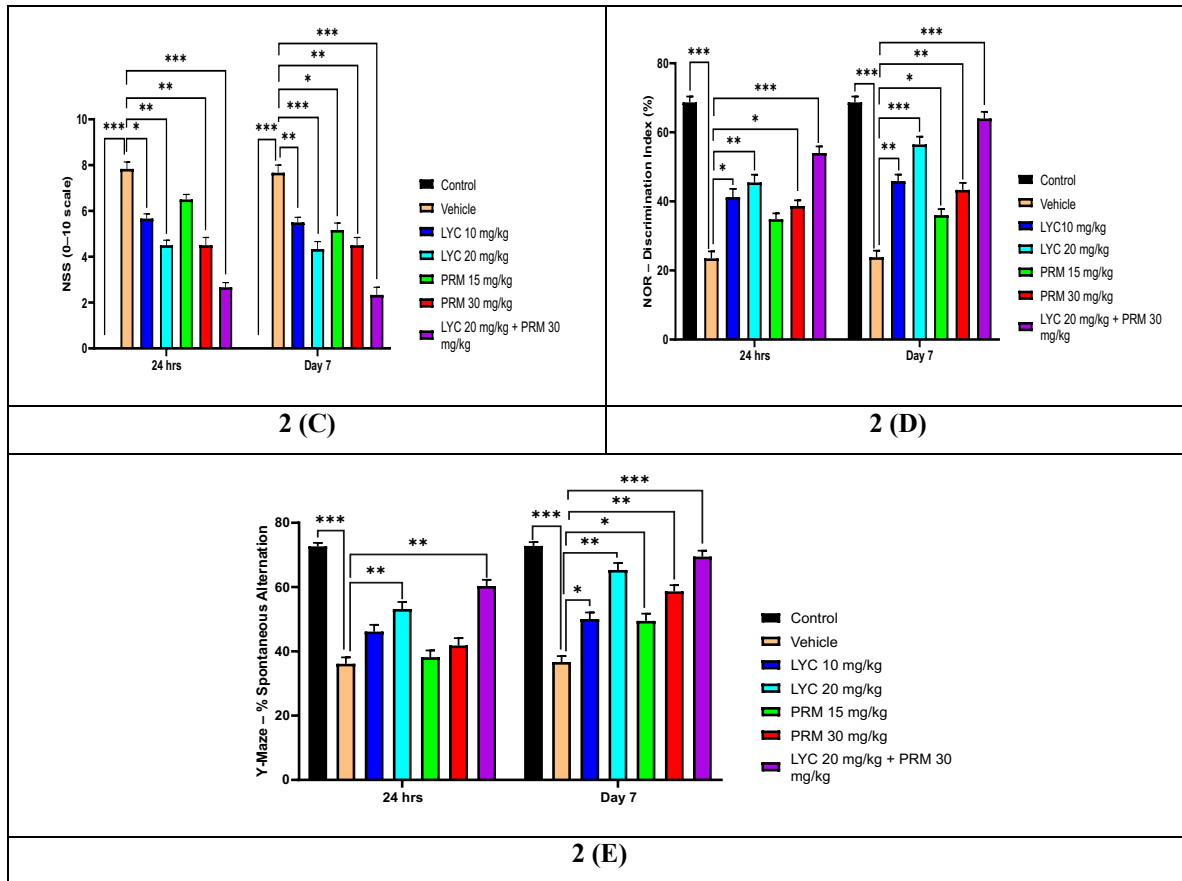


Figure 2: Summary of Behavioral Performance Across EPM, Rotarod, NSS, NOR, and Y-Maze Tests.

(A) Anxiety-like behavior assessed by the Elevated Plus Maze (EPM). (B) Motor coordination and balance assessed by the Rotarod test. (C) Neurological recovery assessed using the Neurological Severity Score (NSS). (D) Recognition memory assessed by the Novel Object Recognition (NOR) test. (E) Spatial working memory assessed by the Y-Maze test. Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test.

3.6 Effects of LYC and PRM on Superoxide Dismutase (SOD) Activity

TBI significantly reduced cortical SOD activity at both 24 hours and day 7 compared with sham controls ($p < 0.001$). At 24 hours, vehicle-treated animals showed decreased SOD activity. LYC increased SOD levels with a significant effect at only 20 mg/kg ($p < 0.05$ vs. vehicle), while PRM did not produce a significant effect at either dose. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased SOD activity compared with vehicle ($p < 0.01$; Figure 3A). On day 7, SOD activity remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 hours. LYC increased SOD activity at both doses ($p < 0.05$ vs. vehicle), whereas PRM was effective at 30 mg/kg only. The combination group showed the greatest increase in SOD activity, significantly higher than

vehicle ($p < 0.01$). SOD levels in the combination group remained lower than control values.

3.7 Effects of LYC and PRM on Catalase (CAT) Activity

TBI significantly reduced cortical CAT activity at both 24 hours and day 7 compared with sham controls ($p < 0.001$). At 24 hours, vehicle-treated animals showed decreased CAT activity. LYC increased CAT levels in a dose-dependent manner ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased CAT activity compared with vehicle ($p < 0.01$). On day 7, CAT activity remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 hours. LYC and PRM increased CAT activity at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest increase in CAT activity, significantly higher than vehicle ($p < 0.01$; Figure 3B). CAT levels in the combination group remained lower than control values.

3.8 Effects of LYC and PRM on Reduced Glutathione (GSH) Levels

TBI significantly reduced GSH levels at both 24 hours and day 7 compared with sham controls ($p < 0.001$). At 24 hours, our vehicle-treated animals showed decreased GSH levels. LYC increased GSH levels with a

significant effect at only 20 mg/kg ($p < 0.05$ vs. vehicle), while PRM did not produce a significant effect at either dose. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased GSH levels compared with vehicle ($p < 0.01$). At day 7, GSH levels remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 hours. LYC and PRM increased GSH levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest increase in GSH levels, significantly higher than vehicle ($p < 0.01$; Figure 3C), and values were not significantly different from control.

3.9 Effects of LYC and PRM on Lipid Peroxidation (MDA Levels)

TBI significantly increased cortical MDA levels at both 24 hours and day 7 compared with sham controls ($p < 0.001$). At 24 hours, vehicle-treated animals showed elevated MDA levels. LYC reduced MDA levels in a dose-dependent manner ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly reduced MDA levels compared with vehicle ($p < 0.01$). At day 7, MDA levels remained elevated in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed further reduction compared with 24 h. LYC and PRM decreased MDA levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest reduction in MDA levels, significantly lower than vehicle ($p < 0.01$; Figure 3D), and values were not significantly different from control.

3.10 Effects of LYC and PRM on Cortical Nitrite Levels

TBI significantly increased cortical nitrite levels at both 24 hours and day 7 compared with sham controls ($p < 0.001$). At 24 hours, vehicle-treated animals showed elevated nitrite levels. LYC decreased nitrite levels in a dose-dependent manner ($p < 0.05$ vs. vehicle), whereas PRM exhibited a significant effect solely at 30 mg/kg. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly reduced nitrite levels compared with vehicle ($p < 0.01$). On day 7, nitrite levels were still high in animals that had been given the vehicle ($p < 0.001$ vs. control), but all treatment groups had lower levels than they did 24 hours earlier. LYC and PRM decreased nitrite levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest reduction in nitrite levels, significantly lower than vehicle ($p < 0.01$; Figure 3E). Nitrite levels in the combination group remained higher than control values.

3.11 Effects of LYC and PRM on Cortical Total Protein Content

TBI significantly reduced cortical total protein levels at both 24 h and day 7 compared with sham controls ($p <$

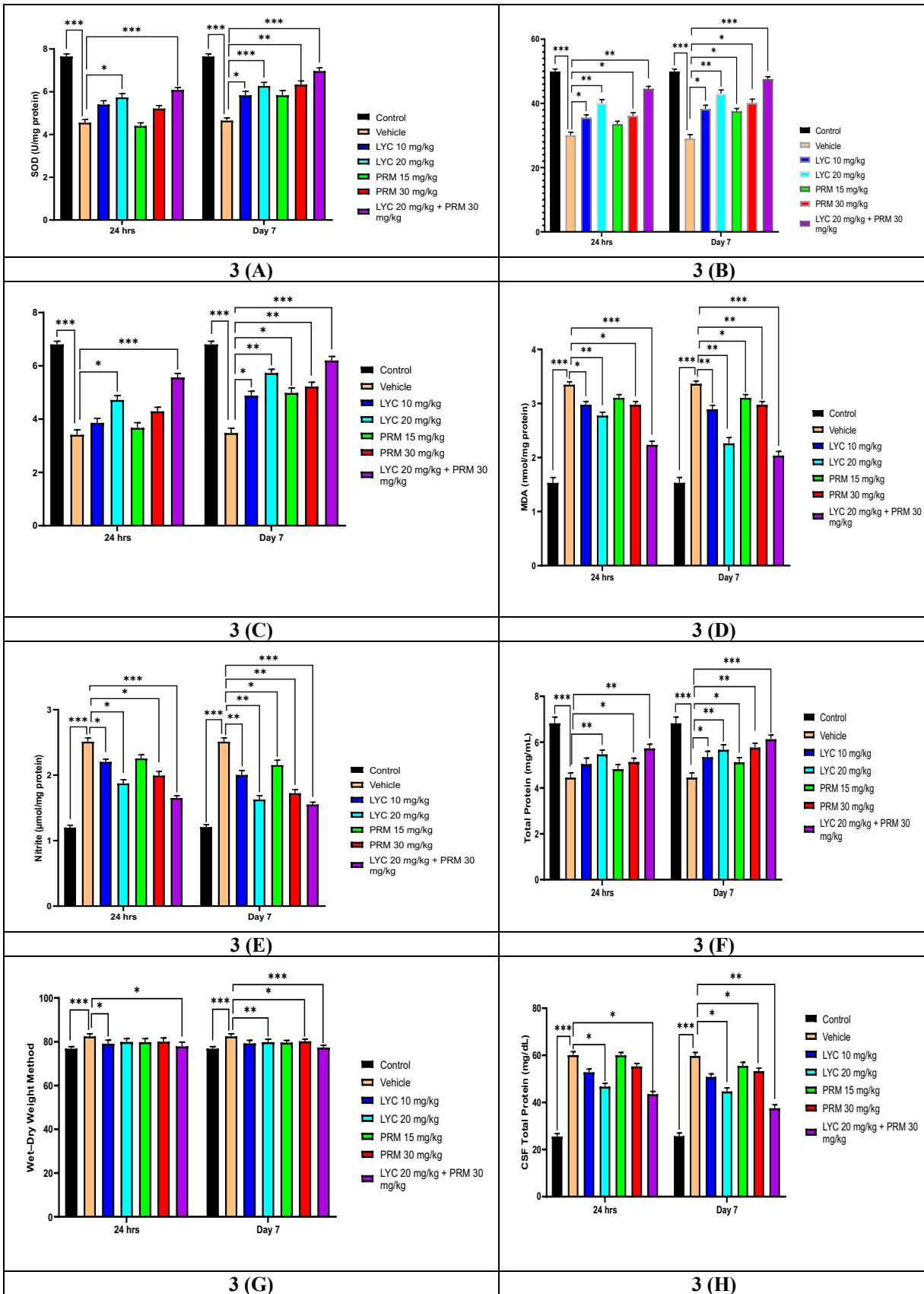
0.001). At 24 hours, vehicle-treated animals showed decreased protein content. LYC increased protein levels, with a significant effect at 20 mg/kg only ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased protein levels compared with vehicle ($p < 0.01$). At day 7, protein levels remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 hours. LYC and PRM increased protein levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest increase in protein levels, significantly higher than vehicle ($p < 0.01$; Figure 3F), and values approached control levels.

3.12 Effects of LYC and PRM on Cerebral Edema Assessed by Brain Water Content

TBI significantly increased brain water content at both 24 hours and day 7 compared with sham controls ($p < 0.001$). At 24 hours, vehicle-treated animals showed elevated brain water content. LYC and PRM did not produce significant reductions at either dose. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly reduced brain water content compared with vehicle ($p < 0.05$). On day 7, brain water content remained elevated in vehicle-treated animals ($p < 0.001$ vs. control), while treatment groups showed improvement compared with 24 hours. LYC reduced brain water content at 20 mg/kg only ($p < 0.05$ vs. vehicle), while PRM was effective at 30 mg/kg only. The combination group showed the greatest reduction in brain water content, significantly lower than vehicle ($p < 0.01$; Figure 3G), and values were not significantly different from control.

3.13 Effects of LYC and PRM on Cerebrospinal Fluid Total Protein Levels

TBI significantly increased CSF total protein levels at both 24 h and day 7 compared with sham controls ($p < 0.001$). At 24 hours, vehicle-treated animals showed elevated CSF protein levels. LYC reduced CSF protein levels with a significant effect at only 20 mg/kg ($p < 0.05$ vs. vehicle), while PRM did not produce a significant effect at either dose. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly reduced CSF protein levels compared with vehicle ($p < 0.01$). On day 7, CSF protein levels remained elevated in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 hours. Both doses were effective ($p < 0.05$ vs. vehicle), while PRM was effective only at 30 mg/kg. The combination group showed the greatest reduction in CSF protein levels, significantly lower than vehicle ($p < 0.01$; Figure 3H). CSF protein levels in the combination group remained higher than control values.

**Figure 3: Biochemical Parameters Reflecting Oxidative Stress and Neuroinflammation**

(A) Superoxide dismutase (SOD) activity. (B) Catalase (CAT) activity. (C) Reduced glutathione (GSH) levels.

(D) Malondialdehyde (MDA) levels. (E) Nitrite (NO) levels. (F) Total protein content. (G) Brain water content.

(H) Cerebrospinal fluid (CSF) total protein levels. Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test.

3.14 Effects of LYC and PRM on Acetylcholinesterase (AChE) Activity

TBI significantly reduced AChE activity at both 24 h and day 7 compared with sham controls ($p < 0.001$). At 24 h, vehicle-treated animals showed decreased AChE activity. LYC increased AChE activity at both doses ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased AChE activity compared with vehicle ($p < 0.01$; Figure 4A). At day 7, AChE activity remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 h. LYC and PRM increased AChE activity at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest increase in AChE activity, significantly higher than vehicle ($p < 0.01$), and values were not significantly different from control.

3.15 Effects of LYC and PRM on Cortical GABA Levels

TBI significantly reduced cortical GABA levels at both 24 h and day 7 compared with controls ($p < 0.001$). At 24 h, vehicle-treated animals showed decreased GABA levels. LYC increased GABA levels, with a significant effect at 20 mg/kg only ($p < 0.05$ vs. vehicle), while PRM increased GABA levels at both doses ($p < 0.05$ vs. vehicle). Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased GABA levels compared with vehicle ($p < 0.01$). At day 7, GABA levels remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 h. LYC and PRM increased GABA levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the highest GABA levels, significantly higher than vehicle ($p < 0.01$; Figure 4B), and values were not significantly different from control.

3.16 Effects of LYC and PRM on Cortical Glutamate Levels

TBI significantly increased cortical glutamate levels at both 24 h and day 7 compared with controls ($p < 0.001$). At 24 h, vehicle-treated animals showed elevated glutamate levels. LYC reduced glutamate levels, with a significant effect at 20 mg/kg only ($p < 0.05$ vs. vehicle),

while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly reduced glutamate levels compared with vehicle ($p < 0.01$). At day 7, glutamate levels remained elevated in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed a reduction compared with 24 h. LYC and PRM decreased glutamate levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest reduction in glutamate levels, significantly lower than vehicle ($p < 0.01$; Figure 4C). Glutamate levels in the combination group remained higher than control values.

3.17 Effects of LYC and PRM on Cortical Dopamine Levels

TBI significantly reduced cortical dopamine levels at both 24 h and day 7 compared with controls ($p < 0.001$). At 24 h, vehicle-treated animals showed decreased dopamine levels. LYC increased dopamine levels, with a significant effect at 20 mg/kg only ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased dopamine levels compared with vehicle ($p < 0.01$). At day 7, dopamine levels remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 h. LYC and PRM increased dopamine levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the greatest increase in dopamine levels, significantly higher than vehicle ($p < 0.01$; Figure 4D), and values were not significantly different from control.

3.18 Effects of LYC and PRM on Cortical Serotonin Levels

TBI significantly reduced cortical serotonin levels at both 24 h and day 7 compared with controls ($p < 0.001$). At 24 h, vehicle-treated animals showed decreased serotonin levels. LYC increased serotonin levels at both doses ($p < 0.05$ vs. vehicle), while PRM produced a significant effect at 30 mg/kg only. Combination treatment (LYC 20 mg/kg + PRM 30 mg/kg) significantly increased serotonin levels compared with vehicle ($p < 0.01$). At day 7, serotonin levels remained reduced in vehicle-treated animals ($p < 0.001$ vs. control), while all treatment groups showed improvement compared with 24 h. LYC and PRM increased serotonin levels at both doses ($p < 0.05$ vs. vehicle). The combination group showed the highest serotonin levels, significantly higher than vehicle ($p < 0.01$; Figure 4E), and values were not significantly different from control.

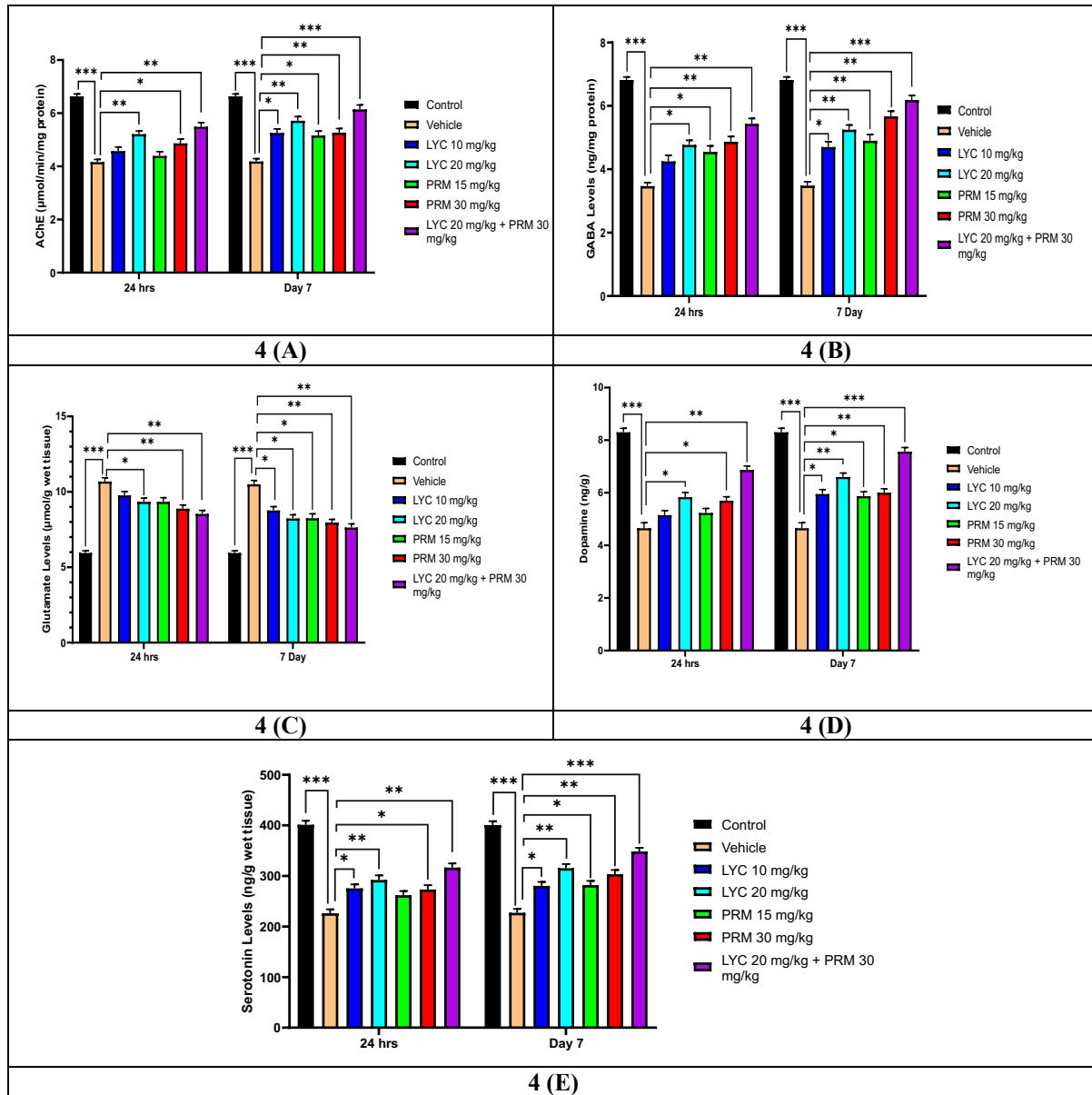


Figure 4: Summary of Neurochemical Parameters: Acetylcholinesterase Activity, GABA, Glutamate, Dopamine, and Serotonin Levels

(A) Acetylcholinesterase (AChE) activity. (B) γ -Aminobutyric acid (GABA) levels. (C) Glutamate levels. (D) Dopamine levels. (E) Serotonin (5-hydroxytryptamine; 5-HT) levels. Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test.

4. DISCUSSION

In the present study, we demonstrate that post-traumatic administration of LYC and PRM, alone or in combination, confers robust neuroprotection in a mouse model of TBI. At both acute (24 h) and subacute (day 7) time points, TBI induced pronounced behavioral impairments, including anxiety-like behavior, cognitive deficits, motor dysfunction, and increased neurological severity. These functional deficits were accompanied by marked biochemical and neurochemical disturbances, characterized by elevated oxidative and nitrosative stress (increased MDA and nitrite; reduced SOD, CAT, and GSH); cholinergic

dysfunction (reduced AChE activity); neurovascular disruption (increased brain water content and CSF protein); and a shift toward excitatory neurotransmission (reduced dopamine, serotonin, and GABA with elevated glutamate) [34, 35]. Despite the clinical use of antiepileptic drugs such as phenytoin and levetiracetam for seizure prophylaxis following TBI, their therapeutic utility is largely limited to seizure prevention, with minimal impact on oxidative stress and neurochemical restoration. PRM, a barbiturate derivative metabolized to phenobarbital and phenylethylmalonamide, enhances inhibitory GABAergic transmission and suppresses glutamate-mediated

excitotoxicity, thereby indirectly reducing oxidative injury. Consistent with previous reports, PRM has been shown to restore GABAergic tone and attenuate glutamate accumulation; in agreement with these findings, the present study demonstrated increased GABA levels alongside reduced glutamate levels following PRM treatment, supporting its role in correcting excitatory–inhibitory imbalance induced by TBI. Although PRM is not routinely employed for TBI management, these pharmacological properties make it an attractive repurposable candidate for neuroprotection. When LYC (20 mg/kg) was co-administered with PRM (30 mg/kg), the combination consistently produced the highest degree of functional recovery across behavioral, biochemical, and neurochemical parameters [36]. There were no explicit formal assessments via isobolographic plots or combination indices for this class of compounds, but the data suggest that the use of the two drugs together (LYC + PRM) produces a much stronger brain-protective effect than the two drugs administered separately. Thus, PRM is worth examining as a protective agent for TBI in conjunction with another agent like LYC, both of which exhibit antioxidant properties and prevent or ameliorate complications resulting from TBI [37].

This study's findings support previous studies that have examined the protective effect of LYC against brain injury as a potent antioxidant and cytoprotective agent by regulating the cellular membrane structure. LYC reduced lipid peroxidation and decreased the accumulation of oxidants that cause nitrosative stress and also increased the body's production of its endogenous antioxidant enzymes (SOD, CAT, and GSH). In this study, LYC was shown to have an effect on the return of SOD, CAT, and GSH levels to normal and to reduce malondialdehyde (MDA) and nitrite levels in rats; thus, it appears that LYC may restore the redox balance in TBI and/or exert protective effects against damage caused by harmful environmental substances. Restoration of the redox balance is most likely important for the stabilization of cell membranes, preservation of enzyme function, and prevention of protein breakdown, which was evident in the increased levels of total protein and AChE activity. These biochemical improvements were linked to better behavior and strengthen the idea that quickly stopping oxidative injury is essential for brain cells to remain as they should and for function to return to normal after TBI [38].

PRM had complementary neuroprotective effects mainly by changing how neurons react. The PRM treatment brought back inhibitory neurotransmission. This was shown by rising GABA levels and by lowering harmful glutamate levels. PRM also brought improvement to monoaminergic signaling, especially dopamine. This effect is important for motor and cognitive processing. Although PRM monotherapy caused positive change, it was less than combination therapy. This shows that

PRM's positive motor effects and PRM's brain effects are not as positive as brain and motor combination therapy. PRM's positive effects are probably because of brain effects alone, and PRM's positive effects are not as positive as motor combination therapy after post-TBI secondary injuries (Yi & Hazell, 2006).

PRM and LYC together showed better neuroprotective effects than each alone. This co-treatment caused an almost complete return of antioxidant enzyme changes after treatment and caused positive changes in the harmful levels of the chemicals. This treatment caused positive changes in monoaminergic and cholinergic neurotransmission, stopped glutamate excitotoxicity, and lessened cerebral edema [40]. This co-treatment caused improvement in an all-encompassing way in several brain functions of anxiety, memory, movement, coordination, and brain health. PRM and LYC have the best neuroprotective effects. The positive neuroprotective effects of LYC and PRM are probably because of the combination of both of their effects on the post-TBI secondary injuries. LYC primarily attenuates oxidative and nitrosative stress by scavenging ROS and activating antioxidant pathways, including Nrf2-mediated transcription of cytoprotective enzymes, while PRM stabilizes neuronal excitability by enhancing GABAergic neurotransmission and suppressing glutamate-driven excitotoxicity. The simultaneous engagement of excitotoxic signaling and oxidative stress might short-circuit the feed-forward loop of neurotransmitter imbalances and mitochondrial dysfunction. This ultimately helps mitigate further neuronal injury. The protective mechanism may benefit the preservation of synaptic function and explains the excellent biochemical and functional recovery seen with the combination therapy[41].

Targeting more than one pathway of secondary injury seems more effective than a single-pathway injury solution approach. Combination therapy may be more effective than previous therapies when protecting the brain from damage due to energy imbalance and excitotoxic injury following TBI. The 24-hour window is vital, as many injuries occur during this time. These include oxidative stress, excitotoxic damage, degradation of the blood-brain barrier, and irregularities occurring with neurotransmitters following TBI. The results from this study indicate that the neurostabilizer and antioxidant combination may halt irreversible changes that lead to neurodegeneration and loss of function that are chronic in nature. The normalization of brain water content and protein content in the CSF suggests that combination therapy protected neurovascular structures, which may determine the neurological outcome after the injury [42]. PRM and LYC are safe and have predictable behavior in humans. This may allow us to use this approach clinically soon. LYC is an antioxidant and is taken as an ordinary dietary supplement, while PRM is an anticonvulsant that has been used clinically for a long

time. The findings support the possibility of using existing compounds to create neuroprotective interventions that target multiple secondary injury pathways in TBI during the early phases of development. Nonetheless, certain limitations need to be addressed in future studies. Initially, this study only assessed acute neuroprotective effects after a single administration of the drugs; therefore, it could not evaluate chronic neuroprotective properties, repeated dosing regimens, or delayed treatment time windows. Secondly, the use of male Swiss albino mice exclusively; since there are known sex-specific differences in TBI pathophysiology and therapeutic response, future studies should test both males and females to increase translational potential. Thirdly, while this study does provide thorough behavioral and biochemical characterization of LYC–PRM-treated animals, no histopathological analyses were conducted. Analyses such as histopathological scoring and assessments of key molecular pathways (Nrf2, NF- κ B, apoptotic signaling, etc.) would provide more mechanistic insights. Finally, pharmacokinetic and pharmacodynamic interactions between LYC and PRM were not assessed. Fourthly, future studies should assess chronic neurobehavioral and neurological deficits, dose optimization, different treatment time windows, sex-dependent differences, and variations in injury severity. Inclusion of histological, molecular, and imaging-based endpoints will be vital to elucidate mechanisms and bring LYC–PRM combination therapy one step closer to clinical translation [43, 44].

Overall, our study provides robust preclinical evidence that co-administration of LYC and PRM produces a combined neuroprotective effect during the acute stage of TBI. This combination therapy works by targeting two major aspects of the secondary injury cascade: oxidative/nitrosative stress and excitatory/inhibitory neurotransmission derangement. Together, they limit neurovascular damage and provide a favorable environment for functional recovery. Combination treatment was superior to single-agent treatment, indicating the benefits of polypharmacy in the treatment of neurological disease [41]. This finding warrants further basic and translational study of LYC–PRM combination therapy for TBI.

CONCLUSION

The current results indicate that combined targeting of oxidative stress and neurotransmitter dysregulation with LYC and repurposed PRM offers significant neuroprotection in an experimental model of TBI. Both therapies successfully reinstated endogenous antioxidant defenses, mitigated oxidative and nitrosative stress, rectified neurotransmitter imbalances, and diminished cerebral edema and cerebrospinal fluid protein leakage, signifying enhanced neurovascular integrity. The combination of LYC and PRM demonstrated clearer evidence of neuroprotection than each drug alone via their effect on behavioral, biochemical, and

neurochemical measurements, which demonstrates both greater neuroprotective efficacy and enhanced resilience of cells with respect to reducing the degree of secondary neuronal cell damage. In conclusion, the combination of LYC+PRM treatment provides more effective neuroprotection than monotherapy through the concurrent restoration of redox balance and neurotransmitter homeostasis, supporting the dual-target method as a valid translational strategy for the prevention of secondary injury after TBI.

FUTURE PERSPECTIVES

Future research is needed to propose a therapeutic strategy for acute and subacute neuroprotection using LYC–PRM combination therapy. Key future research priorities include the following:

- [1] **Determining Long-Term Efficacy:** The effects of LYC–PRM on neurobehavioral, cognitive, and histopathological outcomes should be evaluated beyond the acute phase to assess the durability of neuroprotective effects on the central nervous system.
- [2] **Determining the Optimal Dosage and Timing of LYC–PRM Combination Therapy:** The therapeutic window, dose regimen, and timing of repeated or delayed administration of LYC–PRM should be defined to allow for optimal application of therapy in the clinical setting.
- [3] **Determining the Mechanism of Action of LYC–PRM Combination Therapy:** Detailed characterization of the biological mechanisms of action that include mitochondrial function, neuroinflammatory response, cell death, and reactive oxygen species.
- [4] **Integrating Advanced Biomarkers:** Future studies will incorporate proteomic, metabolomic, and imaging-based biomarker validation and quantitation to support and confirm neuroprotective efficacy.
- [5] **Clinical Translation of LYC–PRM Combination Therapy:** LYC–PRM combination therapy should be validated through the use of higher-order animal models, and Phase I clinical trials should be initiated to evaluate safety and tolerability. Subsequent studies should explore dose translation from mg/kg to a human equivalent dose, interactions between LYC and PRM, and evaluate the combined safety profile of LYC and PRM in humans.

These future objectives will enable the refinement of the therapeutic approach and support the clinical advancement of the LYC/PRM combination therapy for use in patients with TBI.

ABBREVIATIONS

AChE – acetylcholinesterase enzyme activity

ANOVA – analysis of variance statistical method
 BBB – blood–brain barrier
 BWC – brain water content (indicator of cerebral edema)
 CAT – catalase antioxidant enzyme
 CMC – carboxymethylcellulose (vehicle/suspending agent)
 CSF – cerebrospinal fluid
 DA – dopamine neurotransmitter
 EPM – elevated plus maze test
 GLU – glutamate (excitatory neurotransmitter)
 GABA – gamma-aminobutyric acid (inhibitory neurotransmitter)
 GSH – reduced glutathione antioxidant
 LYC – lycopene
 MDA – malondialdehyde (lipid peroxidation marker)
 NO – nitric oxide
 NOR – novel object recognition task
 NSS – neurological severity score
 PRM – primidone
 ROS – reactive oxygen species
 RNS – reactive nitrogen species
 SD – standard deviation
 SEM – standard error of the mean
 SOD – superoxide dismutase enzyme
 TBI – traumatic brain injury
 5-HT – 5-hydroxytryptamine (serotonin)

DECLARATIONS

Ethics approval and consent to participate: All experimental procedures involving animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) under the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Approval No. RVNI/IAEC/23-24/20). All animal experiments were performed in accordance with CPCSEA guidelines and institutional regulations for the care and use of laboratory animals.

Consent for publication: Not applicable. This manuscript does not contain any identifiable person's data, images, or clinical information requiring consent for publication.

Availability of data and materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no conflict of interest.

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Author Contributions

- **SG:** Conceptualization, methodology, investigation, data curation, formal analysis, and writing—original draft preparation.
- **SK:** Conceptualization, supervision, project administration, and writing—review and editing.
- **KM:** Methodology, validation, supervision, and writing—review and editing.
- **MT:** Methodology, validation, supervision, and writing—review and editing.
- **AL:** Supervision, resource provision, and critical review of the manuscript.
- **DK:** Methodology and language editing.

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