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PAPER

Neuroprotective effect of nerolidol in traumatic brain injury associated behavioural comorbidities in rats

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Abstract

Traumatic brain injury (TBI) is an insult to the brain from an external mechanical force, leading to temporary/permanent secondary injuries, i.e. impairment of cognitive, physical, and psycho-social functions with altered consciousness. The leading mechanism responsible for neuronal damage following TBI is an increase in oxidative reactions initiated by free radicals generated by the injury along with various other mechanisms. Nerolidol is reported to have potent antioxidant and anti-neuroinflammatory properties. The present study was designed to explore the neuroprotective effect of nerolidol in weight-drop-induced TBI in rats. Animals were injured on the 1st day by dropping a free-falling weight of 200 gm from a height of 1 m through a guide pipe onto the exposed skull. After 14 days of injury, nerolidol (25, 50, and 100 mg/kg, *i.p.*) treatment was given for the next 14 days. Locomotor activity and motor coordination were evaluated using an actophotometer and rotarod, respectively. Cognitive impairment was observed through the Morris Water Maze and Object Recognition Test. On the 29th day, animals were sacrificed, and their brains were collected for the biochemical estimation. The weight drop model significantly decreased locomotor activity, motor coordination, increased Acetylcholinesterase (AChE) activity, oxidative stress, and induced cognitive deficits in TBI rats. Nerolidol significantly improved locomotor activity, reversed motor incoordination and cognitive impairment, and reduced the AChE activity and oxidative/nitrosative stress. The present study demonstrates the promising neuroprotective effects of nerolidol, which might improve the quality of life of TBI patients.

Key words: traumatic brain injury, weight drop model, nerolidol, behavioural analysis, oxidative stress, biochemical estimation, neuroprotective effect

INTRODUCTION

Traumatic Brain Injury (TBI) is the damage to the brain caused by an external mechanical force, which leads to the short term or permanent impairment of brain function. The secondary injury involves a cascade of various biochemical, cellular, and molecular events that are mainly triggered by the primary insult and several interconnected pathways, leading to deterioration [1].

There are notable alterations of cerebral metabolism and blood flow that results in cellular dysfunction and leads to secondary injuries [2]. Secondary or delayed neuronal damage develops over hours, days, weeks, or months, including biochemical and molecular changes [3,4]. Patients often have short- and long-term cognitive, behavioural, and emotional impairments. Experimentation on TBI models has unveiled that various mechanisms

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associated with ischemia, excitotoxicity, oxidative stress, energy failure, resultant cell death, inflammation, etc., are involved in generating secondary brain injury [5,6].

The "Marmarou" weight drop model is widely used to mimic diffused TBI in humans, typically caused by falls or motor vehicle accidents. Studies reported that it significantly induced memory impairment in rats [7]. It is a surgical model of contusion consisting of a 1- or 2-m guide pipe, which allows a direct impact to the head of the immobilized animal [8].

Nerolidol (NRD) [3,7,11-trimethyl-1,6,10-dodecatrien-3-ol] is sesquiterpene alcohol, which is the main component of essential oils from plants such as *Baccharis dracunculifolia*, *Amaranthus retroflexus*, and *Canarium schweinfurthii* [4,9–11]. It is commonly used in cosmetics, perfumes, shampoos, soaps, and also in non-cosmetic products (cleansers and detergents). Oxidative stress is involved in TBI's pathogenesis, and NRD has reported exhibiting antioxidant property, anti-neuroinflammatory, and anxiolytic effects [11–13]. It has also shown its activity linked with the GABAergic system [14,15]. Moreover, NRD has already been reported as neuroprotective in Parkinson's disease (PD) and epilepsy [4]. With this background, the present study was planned to examine the beneficial effect of NRD against weight-drop-induced TBI in rats.

MATERIAL AND METHODS

Wistar rats (either sex) weighed 200–300 gm were obtained from the Central Animal House facility of Maharaja Ranjit Singh Punjab Technical University, Bathinda, Punjab (India). The animals were kept in polyacrylic cages and maintained under standard laboratory conditions such as room temperature 22 ± 2 ˚C and relative humidity of 55–60% with 12 hours light/dark cycles. The food and water made available *ad libitum*. Behaviour assessments were carried out between 9:00 am and 5:00 pm. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) with protocol no. MRSPTU/IAEC/2018/05. All the experiments were carried out following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

In an attempt to avoid variability and discrepancy between the experimental groups, age-matched animals were used to carry out all the experiments for a given treatment. The animals were randomly divided into five experimental groups, with six [6] animals in each group.

Group 1:- Sham control.
Group 2:- TBI control.
Group 3:- TBI + NRD (25 mg/kg, *i.p.*)
Group 4:- TBI + NRD (50 mg/kg, *i.p.*)
Group 5:- TBI + NRD (100 mg/kg, *i.p.*)

The injury was done on the 1st day of the study using Marmarou's weight drop model after anaesthetizing the animals. Then, the animals were kept under the rehabilitation period for 14 days. A mixture of Tween 80 and normal saline was used as a solvent to dissolve NRD. Drug treatment was started on the 15th day (i.e. 14 days after induction of injury) and continued until the 28th day of the study. On the 29th day, animals were sacrificed, and brain samples were collected for various biochemical estimations (Fig. 1).

Behavioural assessment

Actophotometer. The locomotor activity (horizontal and vertical activity) can be easily measured using an actophotometer (IMCORP, Ambala, India), which operates on photoelectric cells connected in a circuit with a counter [16]. When the animal cuts off the beam of light falling on the photocell, a count is recorded. In our study, animals were placed in the apparatus one by one and allowed to explore the device for 1 minute. After that, animals were allowed to remain inside for 5 minutes, and readings were noted down in the form of total no. of counts in both the upper and lower beams. Initial readings were recorded on the 0th day, followed by observation on the 7th, 14th, 21st, and 28th days of the study.

Rotarod. The rotarod test is generally practised to investigate rodents' motor coordination and stability on a rotating rod. It is motorized and does not require any specialized training for the conductance of this test. The latency of rodents to fall from the rotating rod is designated as the test's endpoint measure. The cutoff time for the experiment was 180 seconds [17]. Before the commencement of the study, animals were trained for the 4 consecutive days on the rotarod apparatus. Initial readings (fall-off time from the accelerating rod for each animal) were recorded on the 0th day, followed by observation on the 7th, 14th, 21st, and 28th days of the study.

Morris water maze. The Morris water maze (MWM) task is extensively used to investigate spatial navigation plus memory in rodents and is based on distal leads to navigate from starting positions throughout the boundary of an open swimming field to discover an underwater escape platform. Richard G Morris described it in 1981. It consists of a round pool having 180 cm in diameter and 45 cm in height [18].

Hidden platform. Spatial learning and memory were analysed by using this apparatus on days 21–24 after injury. In the hidden platform task, the round pool was filled with water maintained at the temperature between 25 and 28°C. A platform of 15-cm diameter was installed 1 cm below the surface of the water, and the test aimed to explore the submerged platform. The position of the platform was fixed throughout all the trials. Various dark-coloured cues extraneous to the maze were detectable from the pool and could be used by the animal for spatial orientation. During the whole duration of the study, the position of the cues was kept alike to assist the animals in identifying the hidden platform. Rats were trained for 4 days, and training was remade four times per day with an interval of 15 minutes between two consecutive trials. If the animal failed to locate the platform within 90 seconds, then they were gently guided to it by the experimenter. All the animals were made to remain on the platform for 30 seconds before withdrawn from the tank. Escape latency, i.e. time taken by the animal to reach the platform, was recorded.

Probe trial. A probe trial was conducted 24 hours after the last day of the hidden platform testing, i.e. on the 25th day. Each animal was permitted to swim for 90 seconds in the pool with the platform removed to determine the time spent in the target quadrant (TSTQ) (where the platform was installed earlier) [18,19].

Novel object recognition task. An object recognition task (ORT) is used to assess recognition and is based on the natural tendency of rodents to spend more time traversing an unknown object than a familiar one [20]. Animals were tested for their memory in the novel ORT on the 27th and 28th days of the study. Rats were individually habituated to open field arena (70 × 70 × 25) for 5 minutes, 24 hours before the test. During the acquisition

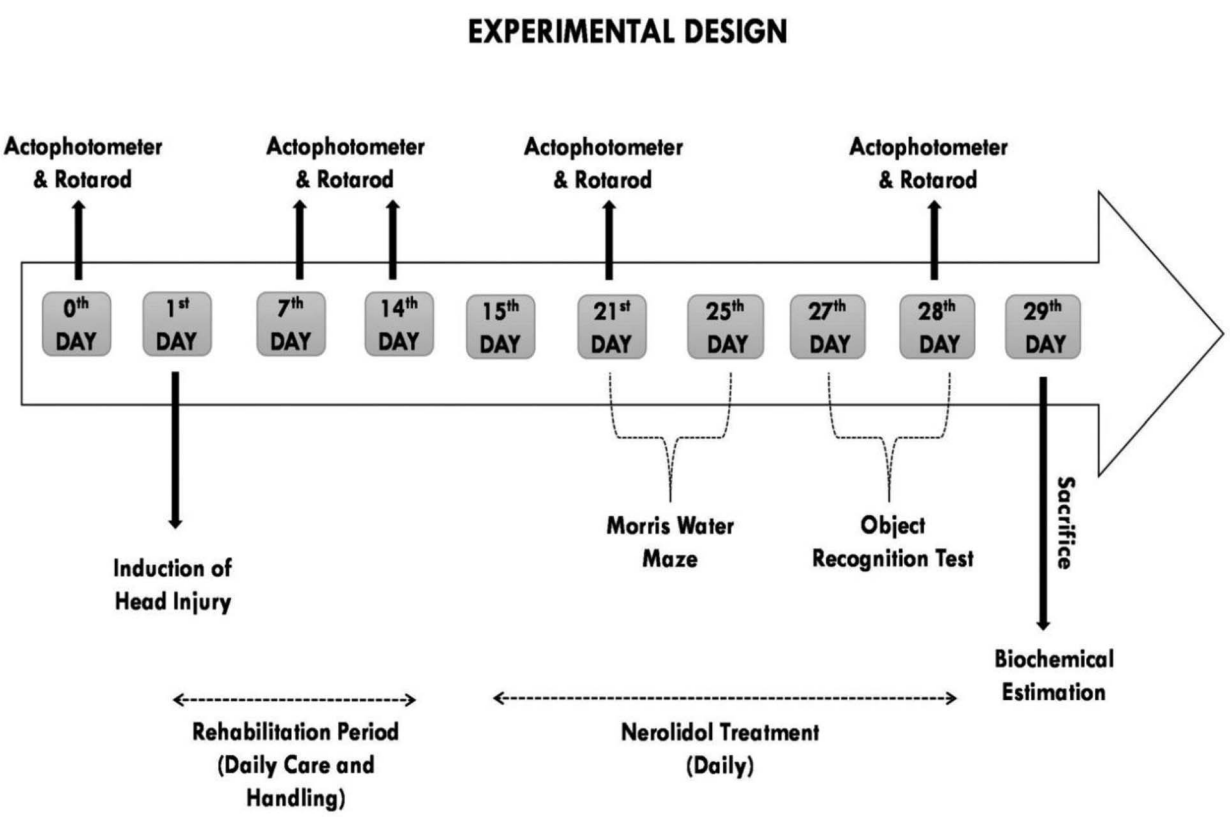

Figure 1: Experimental Design

phase, two indistinguishable objects (A and B), sufficiently heavy and high to make sure that rats could neither move nor climb over them, were placed in the uniform position within the field. Each animal was then placed in the arena and permitted to inspect the object for 10 minutes. After 24 hours of the acquisition phase, one object (A or B randomly) was exchanged with a novel one (C), and exploratory behaviour was again analysed for 10 minutes. The open-field arena and all objects were thoroughly cleansed using 70% ethanol between continuous sessions to prohibit odour recognition. The examination of an object was signalized as rearing on it or sniffing it at a distance of less than 2 cm or touching it with the nose. The preferential exploration of novel objects revealed successful recognition. Discrimination of visual novelty was assessed by preference index [21–23].

$$\text{Preference index} = \frac{\text{Time spent near new object} - \text{Time spent near old object}}{\text{Time spent near new object} + \text{Time spent near old object}}$$

Biochemical evaluation

Estimation of lipid peroxidation. The degree of lipid peroxidation (LPO) was measured quantitatively by performing the method as described by Wills *et al*., 1966. The amount of malondialdehyde (MDA), a measure of LPO, was measured by reaction with thiobarbituric acid at 532 nm using a spectrophotometer [24].

Estimation of nitrite. The build-up of nitrite in the supernatant, an indicator of the production of nitric oxide (NO) was determined by a colourimetric assay with Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% phosphoric acid). Absorbance was measured at 546 nm, and the values of nitrite concentration were obtained from a sodium nitrite standard curve and were expressed in µg/ml [25].

Estimation of reduced glutathione. Reduced glutathione (GSH) was evaluated according to the method described by Ellman, 1959. The yellow colour developed was read immediately at 412 nm using a spectrophotometer. The results were expressed as µmol of reduced GSH per mg protein [26].

Estimation of superoxide dismutase. Superoxide dismutase (SOD) was assayed according to the method described by Kono *et al*., wherein the reduction of nitazobluetetrazolium (NBT) was inhibited by the SOD, measured at 560 nm for 2 min at 30/60 seconds intervals using a spectrophotometer. The results were expressed as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50% [27].

Estimation of protein. The protein content was estimated by using Biuret reagent. The peptide group of proteins forms a purple complex with copper ions in an alkaline medium. The OD of the samples was measured at 540 nm using a spectrophotometer.

Estimation of acetylcholinesterase (AChE) Activity. Brain acetylcholinesterase was evaluated using the method of Ellman *et al*. The change in OD was measured immediately at 412 nm for 2 minutes, and the change in absorbance per min was calculated [28].

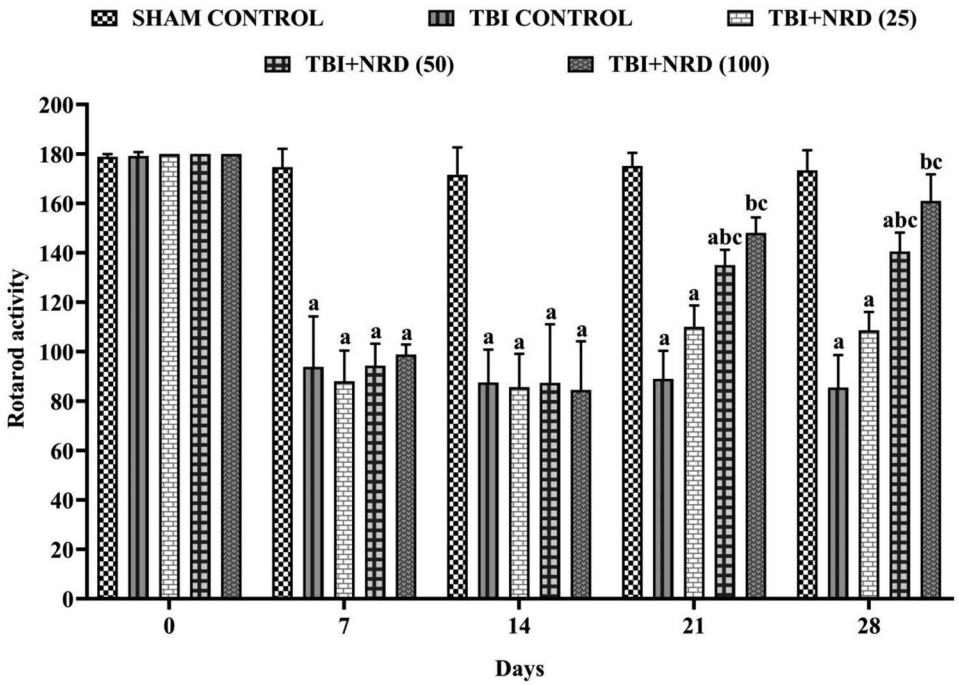

Figure 2: Effect of nerolidol on changes in rotarod performance in TBI-treated rats. Mean + SD. [a]$p < 0.0001$ v/s sham control, [b]$p < 0.0001$ v/s TBI control, [c]$p < 0.001$ v/s TBI + NRD (25 mg).

Statistical analysis. The results are expressed as Mean + SD. The behavioural parameters were analysed using a two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. While biochemical parameters were analysed using a one-way analysis of variance (ANOVA) followed by Bonferroni's Post hoc test for comparison. $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of nerolidol on rotarod and locomotor activity in TBI induced rats

TBI produced a significant ($p < 0.0001$) gradual decrease in rotarod activity and locomotor activity as compared to the sham control group. Treatment with nerolidol at the moderate and high dose (50 & 100 mg/kg, *i.p.*) significantly ($p < 0.0001$) improved motor coordination and locomotor activity as compared to the TBI group. However, NRD at the lower dose (25 mg/kg, *i.p.*) has failed to show any significant result as compared to the TBI group (Figs 2 and 3).

Effect of nerolidol on ELT and TSTQ in MWM in TBI-induced rats

The TBI control rats manifested a substantial delay in ELT to reach the hidden platform as compared to the sham group, clearly indicating an unsatisfactory learning performance ($P < 0.0001$). Nerolidol (50 and 100 mg/kg, *i.p.*) treatment significantly compressed the ELT as compared with the TBI control group ($P < 0.0001$). However, a lower dose of NRD (25 mg/kg, *i.p.*) has failed to show any significant result in contrast to the TBI group. Also, during retention trial, when the platform was removed, the TBI control group remained unsuccessful to remember the platform's exact position, thus, spending remarkably less time in the target quadrant than the sham animals ($P < 0.0001$). Again, treatment with a lower dose of nerolidol (25 mg/kg, *i.p.*) has failed to exhibit any significant effect. However, moderate and higher doses of nerolidol (50 and 100 mg/kg, *i.p.*) treatment notably intensified the TSTQ as compared to the TBI control group, indicating retention in memory performance ($P < 0.0001$) (Figs 4 and 5).

Effect of nerolidol on novel ORT in TBI-induced rats

TBI control group showed a statistically significant deficit in the discriminatory index as compared to the sham control group ($P < 0.0001$). While the moderate and higher doses of nerolidol (50 and 100 mg/kg, *i.p.*) showed significant improvement in the discriminatory index ($P < 0.0001$). However, treatment with the lower dose of nerolidol (25 mg/kg, *i.p.*) has failed to generate any significant effect on the discriminatory index as compared to the TBI control group (Figs 6–8).

Effect of nerolidol on LPO and nitrite levels in TBI-induced rats

TBI in rats induced remarkable increment in oxidative and nitrosative damage as confirmed by the elevated LPO and nitrite levels as compared to the sham group ($P < 0.0001$). Nerolidol (50 and 100 mg/kg, *i.p.*) significantly reduced the LPO ($P < 0.0001$) and nitrite ($P < 0.0005$) levels as compared with TBI control. However, the lower dose of nerolidol (25 mg/kg, *i.p.*) has failed to confer any significant improvement in LPO and nitrite levels as compared to the TBI control group (Figs 9 and 10).

Effect of nerolidol on reduced glutathione and SOD levels in TBI-induced rats

TBI group exhibited a reduction in GSH and SOD levels as compared to the sham group ($P < 0.0001$). Nerolidol (50 & 100 mg/kg,

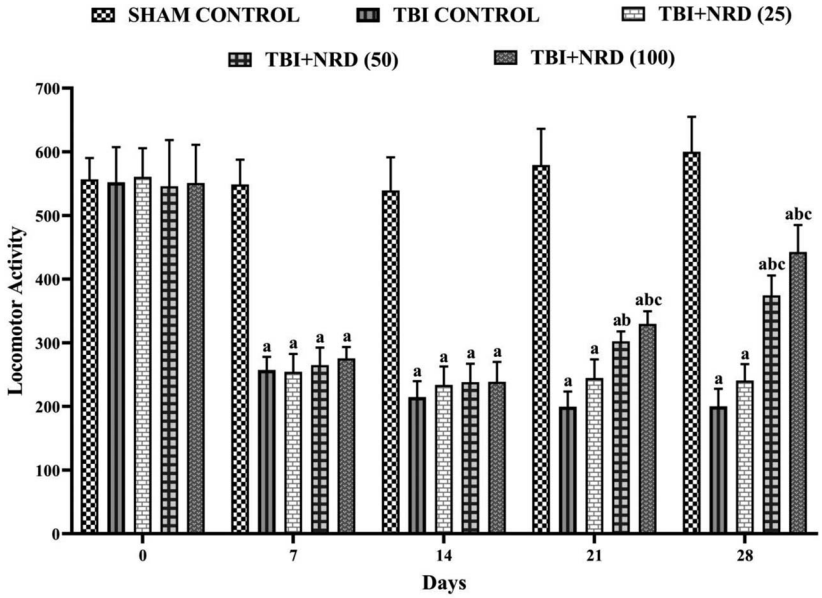

Figure 3: Effect of nerolidol on TBI-induced changes in locomotor activity. Mean + SD. [a]$p < 0.0001$ v/s sham control, [b]$p < 0.0001$ v/s TBI control, [c]$p < 0.005$ v/s TBI + NRD (25 mg)

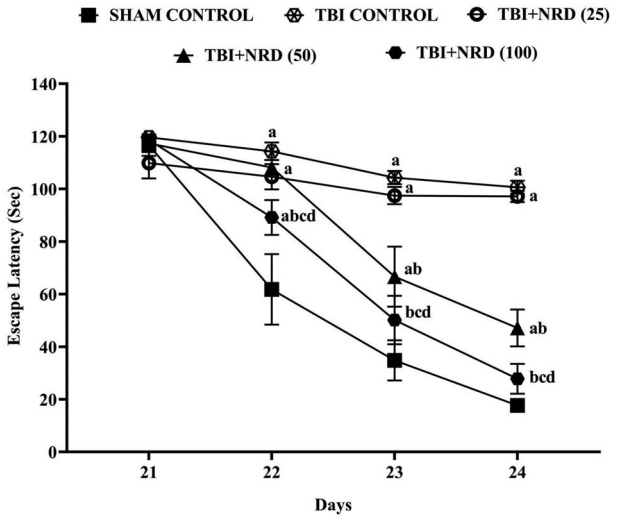

Figure 4: Effect of nerolidol on escape latency time in MWM. Mean + SD. [a]$p < 0.0001$ v/s sham control, [b]$p < 0.0001$ v/s TBI control, [c]$p < 0.0005$ v/s TBI + NRD (25 mg), [d]$p < 0.0001$ v/s TBI + NRD (50 mg)

i.p.) significantly increased the GSH and SOD levels as compared to the TBI control ($P < 0.0001$). However, the lower dose of nerolidol (25 mg/kg, *i.p.*) again failed to show substantial results contrasted to the TBI group (Figs 11 and 12).

Effect of nerolidol on brain AChE levels in TBI-induced rats

The TBI control group showed a significant increase in the AChE enzyme levels as compared to the sham group ($P < 0.0001$). Nerolidol (50 and 100 mg/kg, *i.p.*) treatment significantly attenuated the increased AChE activity as compared to the TBI control animals ($P < 0.0001$). Though the treatment with lower dose of nerolidol (25 mg/kg, *i.p.*) has failed to confer a significant upswing as compared to the TBI and thus presented non-significant results (Figure 13).

DISCUSSION

The present study mainly focused on investigating the neuroprotective potential of NRD against TBI-induced neurotoxicity and comorbidities, like cognitive impairment in rats. The weight drop model was used as an animal model of TBI to study the various aspects of the neurodegeneration process and cognitive deficits. In the present study, grip strength and locomotor activity were assessed using rotarod and OFT, respectively. The impairment in grip strength, locomotor activity, and motor functions was observed in the TBI control group when compared with the sham group. Treatment with NRD significantly prevented the alteration in motor coordination and locomotor activity in the TBI control animals. Memory performance/cognitive power were evaluated using MWM and ORT. In the MWM test, escape latency, i.e. time taken to reach the hidden platform and probe trial, which was performed after 4 days of training, was significantly increased; however, TSTQ (retrieval trial) was significantly decreased in the case of traumatized rats clearly indicating the impairment of learning and memory. Another test, novel ORT which is based on the natural/inborn tendency of rodents to investigate novel objects over familiar ones, was performed differentially. The results we obtained from the novel ORT further support our MWM findings. These findings are in conformity with the recent report, which showed that memory impairment is linked with brain trauma and NRD administration-enhanced cognitive power in animals [8,29–31]. In the present study, persistent treatment with NRD significantly improved cognitive behavioural deficits observed in traumatically injured rats.

A recent study revealed the neuroprotective effects of nerolidol against rotenone-induced neuroinflammation and oxidative

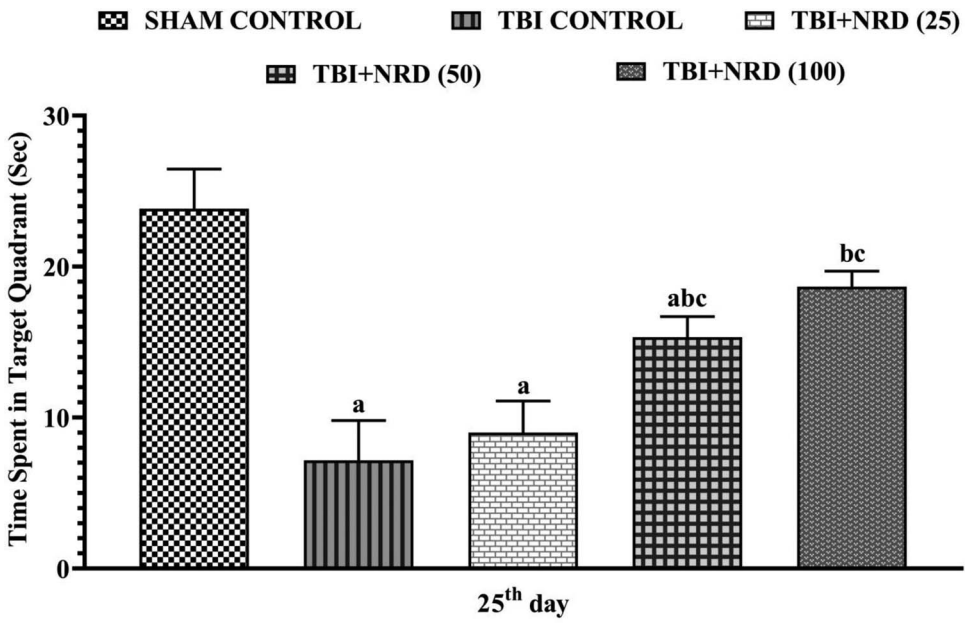

Figure 5: Effect of nerolidol on TSTQ in MWM. Mean + SD. [a]$p < 0.0001$ v/s sham control, [b]$p < 0.0001$ v/s TBI, [c]$p < 0.0001$ v/s TBI + NRD (25 mg)

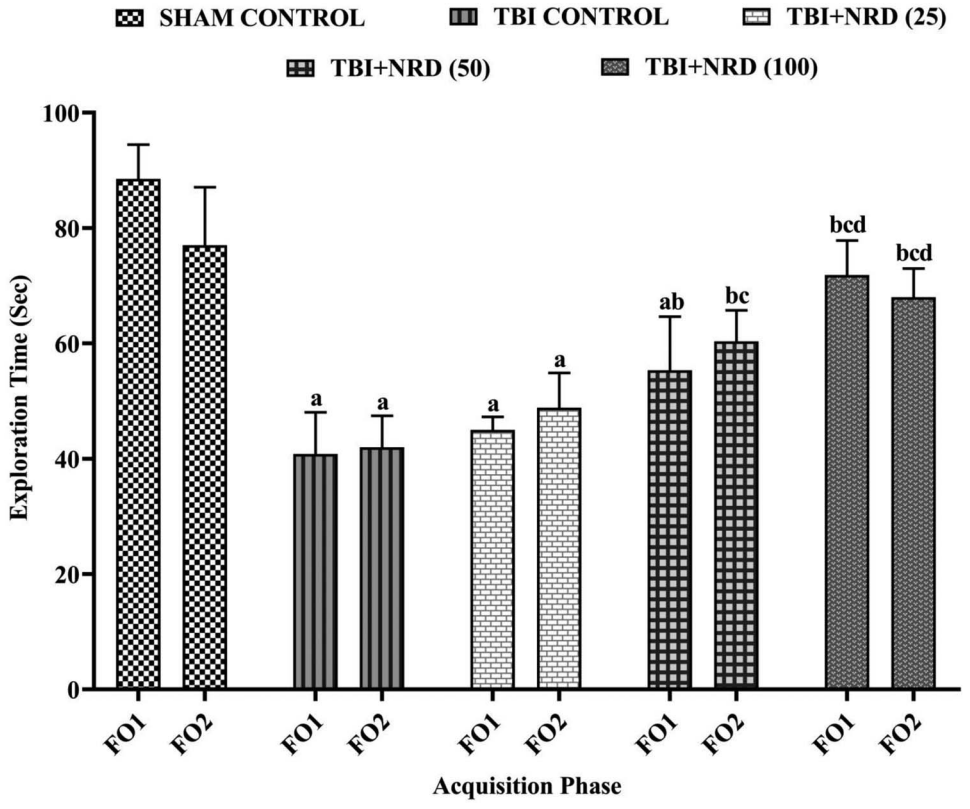

Figure 6: Effect of nerolidol on exploration time in novel object recognition task (FO1: Familiar Object 1; FO2: Familiar Object 2). Mean + SD. [a]$p < 0.0001$ v/s sham control, [b]$p < 0.005$ v/s TBI control, [c]$p < 0.005$ v/s TBI + NRD (25 mg), [d]$p < 0.05$ v/s TBI + NRD (50 mg)

stress through its anti-inflammatory and antioxidant properties [9]. There is an association between the progression of neurodegenerative diseases and cognitive impairment with hiked levels of oxidative stress markers and decreased levels of antioxidant enzymes within the brain. Oxidative stress is known to be one of the leading factors in the pathogenesis of traumatic head injury [16]. Generation of superoxide, hydroxyl ions, and other free radicals, after traumatic injury, are the major contributors in the pathogenesis of secondary injury cascade [32]. Oxidative stress is a common factor for BBB disruption in blunt TBI as well as in other neurological disorders, including AD and PD [33]. Experimental and clinical studies have found that extracellular

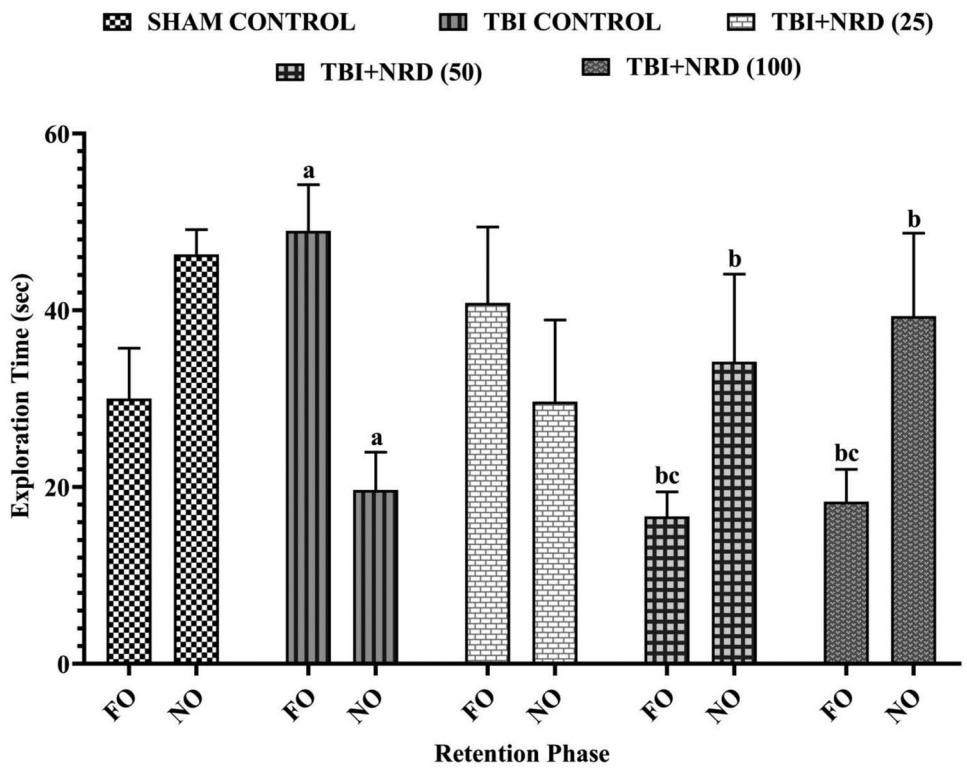

Figure 7: Effect of nerolidol on exploration time of familiar (FO) and novel (NO) objects. Mean + SD. $^{a}p < 0.001$ v/s sham control, $^{b}p < 0.005$ v/s TBI control, $^{c}p < 0.0001$ v/s TBI + NRD (25 mg)

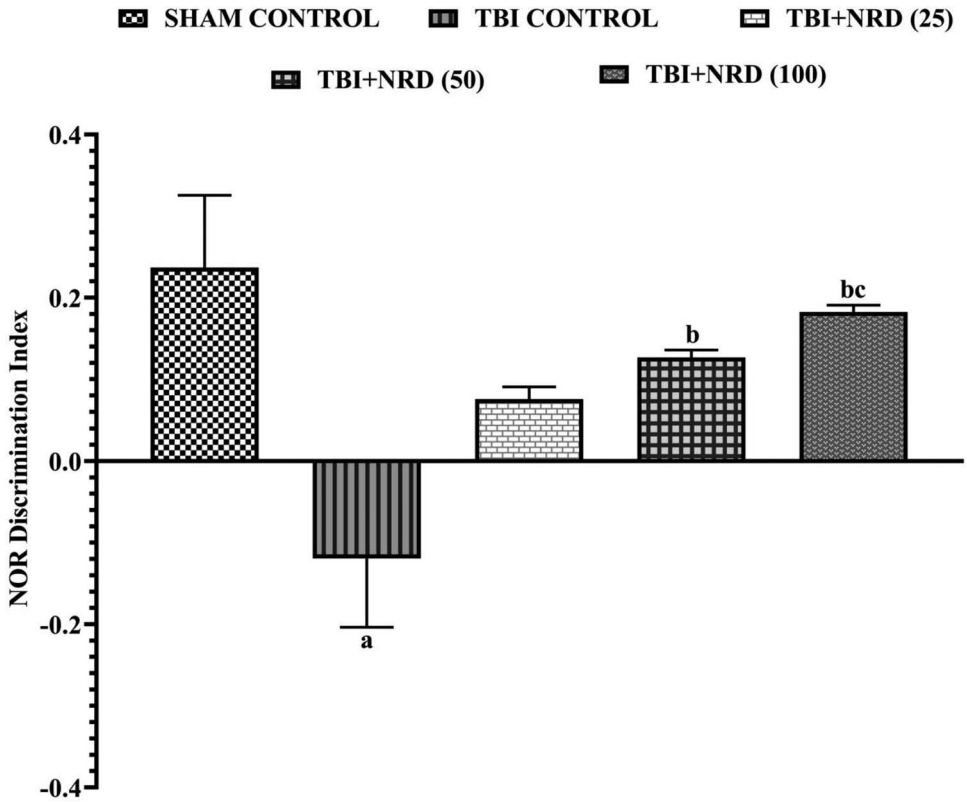

Figure 8: NOR discrimination index in novel object recognition task. Mean + SD. $^{a}p < 0.0001$ v/s sham control, $^{b}p < 0.0001$ v/s TBI control, $^{c}p < 0.05$ v/s TBI + NRD (25 mg)

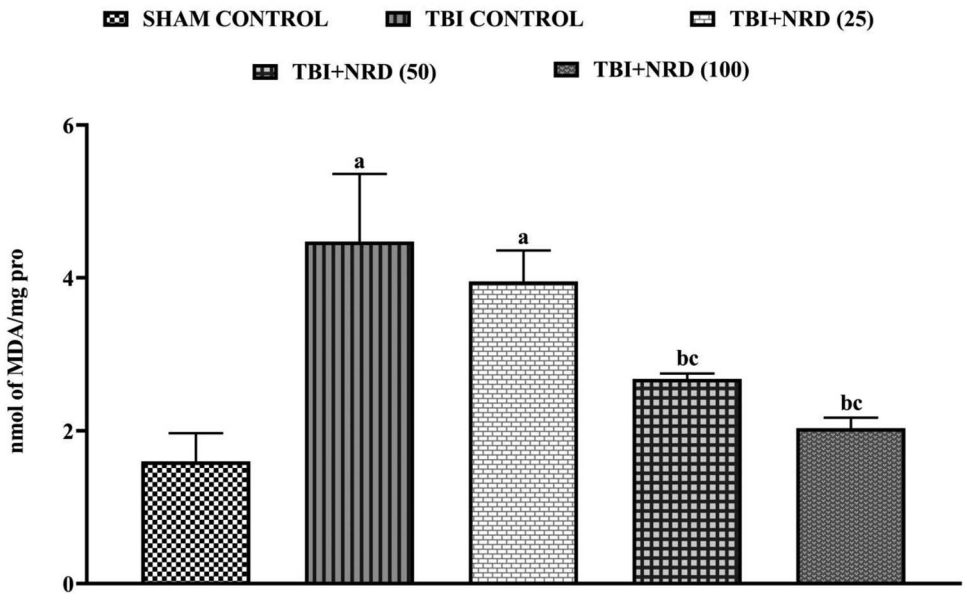

Figure 9: Effect of nerolidol on lipid peroxidation (LPO) levels in TBI rats. Mean + SD. $^{a}p < 0.0001$ v/s sham control, $^{b}p < 0.0001$ v/s TBI control, $^{c}p < 0.005$ v/s TBI + NRD (25 mg)

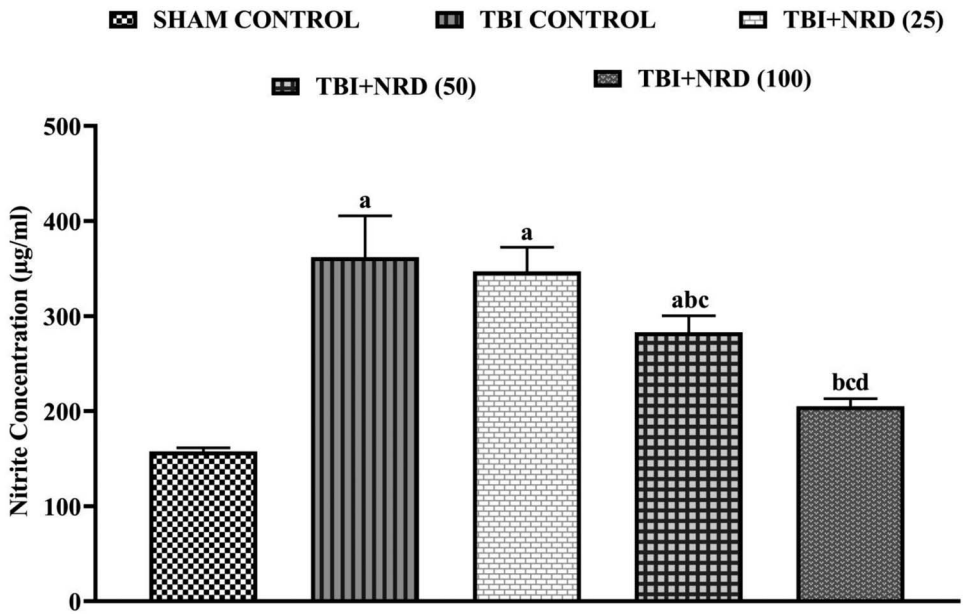

Figure 10: Effect of nerolidol on nitrite levels in TBI rats. Mean + SD. $^{a}p < 0.0001$ v/s sham control, $^{b}p < 0.0005$ v/s TBI control, $^{c}p < 0.005$ v/s TBI + NRD (25 mg), $^{d}p < 0.001$ v/s TBI + NRD (50 mg)

glutamate levels increase sharply within a short while after TBI [34]. NRD showed its antioxidant effects in the mice hippocampus [12]. Free radicals or ROS are well known to be responsible for oxidative stress, and when the accumulation of ROS exceeds the antioxidant potential of the cell, pathological/neurological processes such as cognitive dysfunction, epilepsy, and depression take place [8,35]. Similarly, the present study also showed the elevated levels of LPO, nitrite, and a pronounced reduction in GSH, ACh (increased levels of AChE), and SOD levels after trauma. However, the long-term treatment with nerolidol remarkably declined head trauma mediated alterations in the biological antioxidant enzyme levels, which is in the complete favour of the previous study stated that NRD notably declines the LPO level and nitrite content in the mice hippocampus, which protects against oxidative stress [12].

The cholinergic function is vital and is actively involved in the process of learning and memory, and its alteration directly leads to the development of cognitive impairment. It has been reported in previous studies that acetylcholine levels got declined in the brains of AD patients, and further AChE enzyme activity leads to the quick depletion of the rest little acetylcholine which got produced, thus leads to the memory loss and other cognitive dysfunctions [36]. Previous research has also proved that AChE encourages the amyloid-β-peptide's assembly into amyloid fibrils of a neurotoxic kind that is toxic for retinal neuronal cultures/neuroblastoma cells of chicken [37].

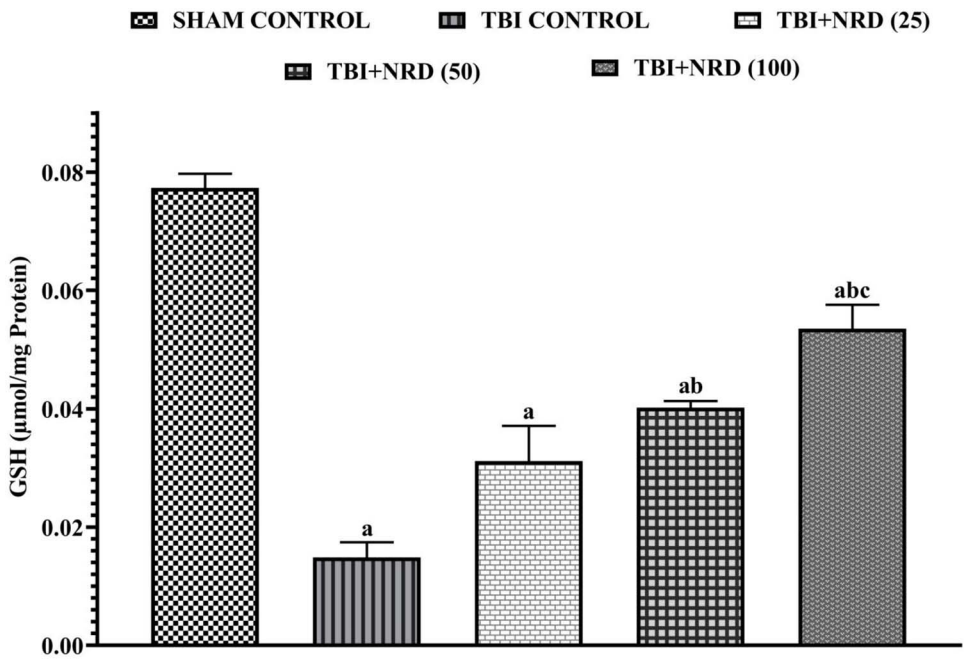

Figure 11: Effect of nerolidol on reduced glutathione (GSH) levels in TBI rats. Mean + SD. [a]$p < 0.0001$ v/s sham control, [b]$p < 0.0001$ v/s TBI control, [c]$p < 0.01$ v/s TBI + NRD (25 mg), [d]$p < 0.005$ v/s TBI + NRD (50 mg)

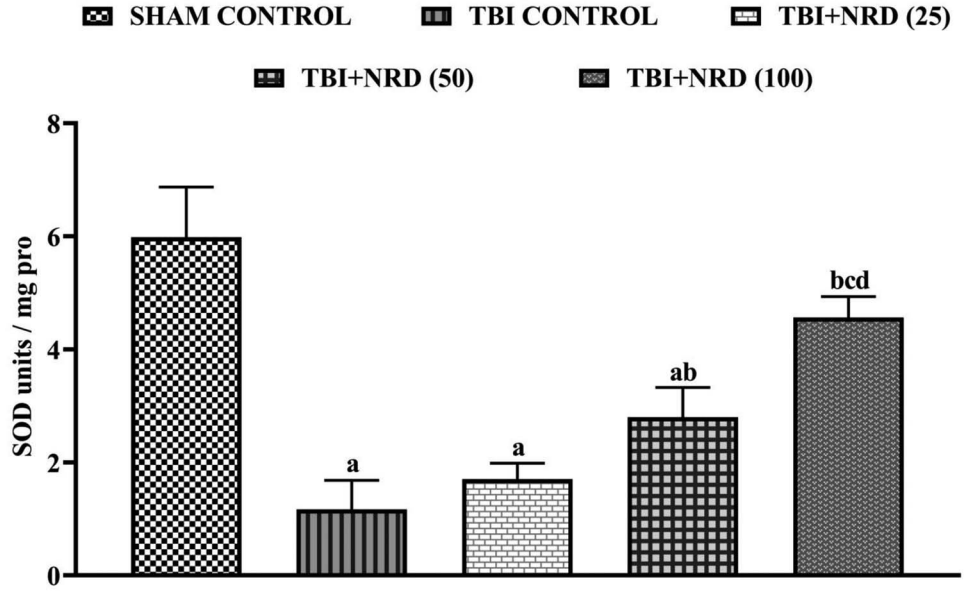

Figure 12: Effect of nerolidol on SOD levels in TBI rats. Mean + SD [a]$p < 0.0001$ v/s sham control, [b]$p < 0.005$ v/s TBI control, [c]$p < 0.0001$ v/s TBI + NRD (25 mg), [d]$p < 0.005$ v/s TBI + NRD (50 mg)

Treatments for human-related memory-deficit problems often involve the enhancement of the retention of acetylcholine in brain synapses [38]. In the experimental model of brain trauma, it has been proved that the loss of cholinergic neurons and alteration of the ACh neurotransmission occurred [39]. Similarly, we found a significant increase in the levels of AChE in different brain regions of traumatized rats, that is further significantly attenuated on chronic treatment with NRD. Treatment with NRD markedly reversed behaviour and biochemical alterations. According to a published report, NRD treatment has been found to prevent rotenone-induced glial cell activation and nerve fibres/dopaminergic neuronal loss that ultimately attenuated rotenone-induced dopaminergic neurodegeneration [4]. Besides this, NRD also exerts antinociceptive property with possible involvement of the GABAergic system and anti-inflammatory activity, ascribed to the suppression of TNF-α and IL-1β proinflammatory cytokines [13]. Previous studies reported that NRD produced protective effects against PTZ-induced kindling

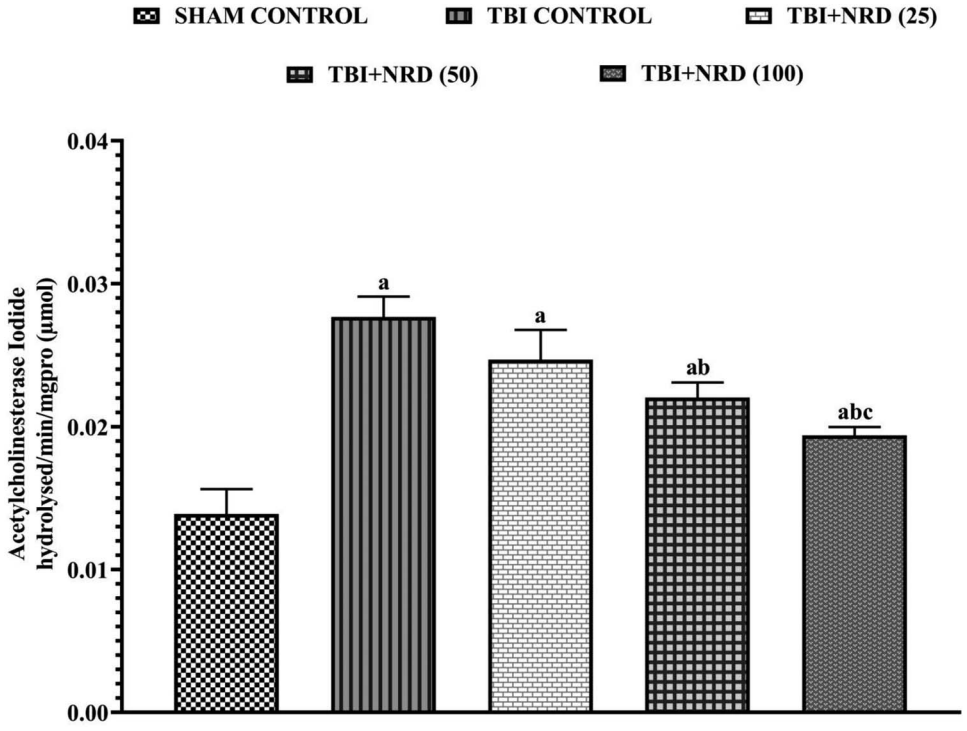

Figure 13: Effect of nerolidol on brain AChE levels in TBI rats. Mean + SD. $^{a}p < 0.0001$ v/s sham control, $^{b}p < 0.0001$ v/s TBI control, $^{c}p < 0.0005$ v/s TBI + NRD (25 mg)

and associated oxidative stress and behavioural comorbidities [40]. One more research showed that nerolidol exerts an anxiolytic effect without altering the motor coordination [11]. NRD showed meaningful activity against short- and long-term inflammation, used for the management of pain and inflammation because of showing central and peripheral antinociceptive effects [41].

CONCLUSION

In conclusion, the present study reveals that NRD effectively ameliorates TBI-induced motor deficits, cognitive impairment, and oxidative stress through its various neuroprotective mechanisms. Thus, it might prove as a boon to improve the quality of life of TBI patients. Based on the reported considerations mentioned above, it is expected to investigate the possible roles of NRD in other neurodegenerative diseases. Further molecular and cellular studies are recommended to explore the neuroprotective mechanism of NRD.

Compliance with ethical standards

The authors certify that formal approval to conduct the experiments described has been obtained from the animal subjects review board of their institution and could be provided upon request. The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

Authors' contribution

All authors equally contributed to the completion of this work. All authors read and approve the manuscript.

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Conflict of interest statement

There are no competing interests.

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