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Nerolidol ameliorates cyclophosphamide-induced oxidative stress, neuroinflammation and cognitive dysfunction: Plausible role of Nrf2 and NF- κB

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ABSTRACT

Aim: Cyclophosphamide (CP) is a potent anticancer and immunosuppressant drug. Studies have shown significant oxidative stress and cognitive impairment but neuroinflammatory and histological aberrations with its administration is underexplored. Nerolidol (NER) is a lipophilic bioactive molecule with antioxidant and anti-inflammatory properties but it has not been explored for neuroprotective potential in CP-induced neurotoxic manifestations. Therefore, in the present study, we aimed to evaluate the neuroprotective potential of NER in CP-induced neuroinflammation and associated comorbid conditions like depression and cognitive dysfunctions.

Materials and method: *In-silico* study using Schrödinger software was used to assess the binding affinity of NER with Nrf2. In the In vivo study, NER 200 and 400 mg/kg p.o. were given from 1st day to 14th day. CP 200 mg/kg, i.p., was administered on the 7th day. After 24 h of the last dosing, neurobehavioral tests like spontaneous body alternation, passive avoidance and forced swim test were performed. On completion of study, mice were sacrificed, hippocampus and cortex were removed for biochemical estimations, histopathology and immunohistochemistry of p65 NF- κB and Nrf2.

Key findings: *In-silico* study showed significant binding of NER into the pocket domain of Nrf2. In-vivo study showed protective effect of NER against CP-induced neuroinflammation, oxidative stress, cognitive impairment and structural abnormalities in the hippocampus and cortex regions.

Significance: Findings of the study suggested that NER is a potential therapeutic molecule which can mitigate CP-induced neurotoxic manifestations via Nrf2 and NF-κB pathway. However, more detailed studies are needed to explicate the mechanism underlying its neuroprotective effect.

1. Introduction

Neurological disorder is one of the major complications seen with the patients undergoing chemotherapy. It has been reported that 15–80% of the patients suffer from cognitive dysfunction and depression when exposed to chemotherapy [1,2]. The significant neurotoxic manifestations were reported as seizure, encephalopathy, cerebrovascular disorders, ataxia, locomotive disorder, cranial neuropathy, myelopathy, plexopathy, peripheral neuropathy and myalgia [3]. 'Chemo brain' is a term often used to represent chemotherapy-induced cognitive impairment (CICI) [4]. CICI and associated co-morbid conditions are manifested, only when the anticancer drug or its metabolite disrupt and cross the blood-brain barrier (BBB). A study conducted by Edward et al. in 1983 showed BBB disruption by 5-FU, doxorubicin, cyclophosphamide (CP) and cisplatin at the therapeutic doses cause hemorrhagic necrosis, pyknosis of neurons with vacuolization, swelling and softening of cerebral arteries, infarction in the basal ganglia and neuronophagia in the temporal lobe [5]. However, till date the exact mechanism of neurotoxicity by anticancer drugs is not clear but according to the literature, mechanisms like direct damage to the neurons, alteration in the neurotransmitter level, oxidative and nitrative stress, immunological dysfunction, cerebral thrombosis and hypoxia probably play role in this toxicity [6].

Cyclophosphamide is an immunosuppressant and anticancer drug

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which is routinely used in case of solid tumors, hematological malignancies and in organ transplantation to prevent graft rejection [7]. It is a prodrug which gets metabolized into phosphoramide mustard (PM) and acrolein. PM is an anticancer moiety and acrolein is a neurotoxic metabolite that crosses the BBB [8]. CP, when administered alone or in combination with other anticancer drugs like doxorubicin, causes neuronal oxidative stress and cognitive dysfunction [9,10]. Oxidative stress causes reduction in Nrf2 expression and increases NF-κB expression in the brain (hippocampus and frontal cortex) that leads to neuroinflammation and further predispose into cognitive dysfunction and depression [11,12]. It is well established that the hippocampus and cortex regions of brain are the focal points for learning, memory and aptitude. Hence, damage to the hippocampus and cortex region directly translate into CICI or neurobehavioral abnormalities [13]. Despite of higher prevalence rate of CICI, there is no therapy available to take care of this problem. Hence, there exist an utmost need for certain adjuvant therapy that can mitigate CICI.

Nerolidol is a natural bioactive molecule which possesses antioxidant, anti-inflammatory and anti-apoptotic properties that could be of therapeutic interest [14]. NER is a sesquiterpene and reported to exert anti-ulcer, anti-leishmanial, anti-schistosomal, anti-malarial, antinociceptive and anti-tumor activity [15–19]. The rationale of selection of nerolidol includes its log *P* value which is 4.55–5.31, signifying it as highly lipophilic (log $P > 2.5$ is required to cross BBB) and its previously reported role in reducing lipid peroxidation and nitrite content in brain against PTZ-induced seizure, which also signify its permeability against BBB [14,20–23]. Therefore, looking into the neurotoxic manifestations of cyclophosphamide and previously reported neuroprotective potential of nerolidol in different experimental models, we came across the two gray areas that need further attention. Firstly, cyclophosphamide has not been studied for its inflammatory and histological effect in the brain [8,9,24–26]. Secondly, nerolidol has not been explored for its neuroprotective potential in anticancer drug-induced neurotoxicity. Additionally, the reported neuroprotective potential of nerolidol in different experimental models is only the preliminary findings and mechanism underlying its neuroprotective potential is not explored. Therefore, objective of the present study was to assess the neuroprotective role of NER in CP-induced neuroinflammation, oxidative stress and cognitive dysfunction via NF-κB p65 and Nrf2 expression in the hippocampus and frontal cortex by correlating the docking studies, biochemical estimations and histological findings (H & E staining and cresyl violet staining).

2. Materials and methods

2.1. Drugs and chemicals

Cyclophosphamide (Endoxan®, Batch No AEU1040) was obtained from Baxter Oncology GmbH, Frankfurt Germany. Nerolidol CAS No 7212-44-4, Lot# STBG8020 was purchased from Sigma Aldrich (St. Louis, Missouri, United States). Antibodies, NF-κB p65 catalog No C-20 (SC-372), Lot# D2310 was procured from Santa Cruz Biotechnology Dallas, Texas, United States and Nrf2, catalog No PA1–38312, Lot# QA1961701 was procured from Thermo Fisher Scientific Waltham, Massachusetts, United States. ELISA kits for interleukins and cytokines (IL-6, catalog No KB2068; IL-10 catalog No KB2072; IL-1β catalog No KB2063; and TNF-α catalog No KB2145) were procured from KRISHGEN Biosystems, Worli, Mumbai, India.

2.2. Molecular docking simulations

Molecular docking study of nerolidol was performed for Nrf2 receptor catalytic ligand binding site using Glide module of Maestro version 9.4 software, Schrödinger. The 3-Dimensional crystallographic X-ray protein structure of Nrf2 was downloaded from the RCSB protein data bank in the .pdb file format. A crystallographic water molecule, exhibiting less than three hydrogen bonds, were deleted and hydrogen bonds corresponding to pH 7 were added considering the appropriate ionization states for both acidic and basic amino acid residues. The best-docked structure was identified using Glide score function, Glide energy, and Glide E model energy. The molecular interaction of ligands with the receptor was analyzed and a map of hydrophobic and hydrophilic interaction of the constituents at active ligand binding site of Nrf2 receptor was recorded through a ligand interaction diagram in 2-D and 3-D format.

2.3. Experimental animals

Male Swiss albino mice (35–40 g) were obtained from the Central Animal House facility of Jamia Hamdard. The experimental protocol was approved by the Institutional Animal Ethics Committee of Jamia Hamdard (IAEC/JH-1484). Experiment was conducted according to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and all efforts were made to minimize animal suffering. Animals were allowed to acclimatize for 1 week and housed in standard polypropylene cages and had access to commercial standard pellet diet. Animals were maintained under controlled room temperature ($23 \pm 2^{\circ}C$) and relative humidity ($60 \pm 5\%$) with 12 h light/12 h dark cycle in the central animal house facility, Jamia Hamdard, New Delhi, India.

2.4. Treatment protocol

Doses of nerolidol 200 mg/kg, p.o., 400 mg/kg, p.o. and cyclophosphamide 200 mg/kg i.p., as well as their dosing schedule were selected from the published literature [15,25,27,28]. Animals were divided into 5 groups ($n = 6$). The schematic representation of the treatment schedule is shown in Table 1.

After 24 h of CP administration on the 7th day, animals were subjected to forced swim test (FST) on alternate day (i.e., on 8th 10th 12th and 14th day) to monitor the extent of gradual depressive behavior (Fig. 1) [12]. Animals were also subjected to the training session for step down latency test (SDL) on 8th day.

Spontaneous alternation behavior test (*SAB*) for cognitive dysfunction and retention test for SDL was performed on alternate days (i.e., on 9th, 11th, 13th and 15th, day) [13]. Retention test for SDL was performed on Cook's pole climbing apparatus that consists of a grid floor composed of stainless-steel rods which delivered electric shock (20 V, 50 Hz, 1 mA). Mice were gently placed on the wooden platform in the center of the grid floor that served as a shock-free zone. As the mouse stepped down and placed all its paws on the grid floor, electric shock at 20 V, 50 Hz, 1 mA was delivered for 15 s and the SDL was recorded. SDL is defined as the time taken by the mouse to step down from wooden platform to grid floor. After performing the above tests, animals were euthanized on 15th day and brains were removed. Sections of brain tissues (hippocampus and frontal cortex) were stored in 10% formalin for histopathology and the remaining parts were preserved at -20° for biochemical estimations as shown in Fig. 1.

Table 1
Grouping and treatment schedule.

S. No.	Group (n = 6)	Dose, route, and duration
1.	Vehicle control	0.1 ml Tween 80 p.o. for 14 days
2.	CP 200 (Toxic)	CP 200 mg/kg, i.p. once on 7th day
3.	NER per se	NER 400 mg/kg, p.o. for 14 days
4.	NER 200 + CP 200	NER 200 mg/kg, p.o. for 14 days + CP 200 mg/kg, i.p. once on 7th day
5.	NER 400 + CP 200	NER 400 mg/kg, p.o. for 14 days + CP 200 mg/kg, i.p. once on 7th day

CP: cyclophosphamide, **NER**: Nerolidol.

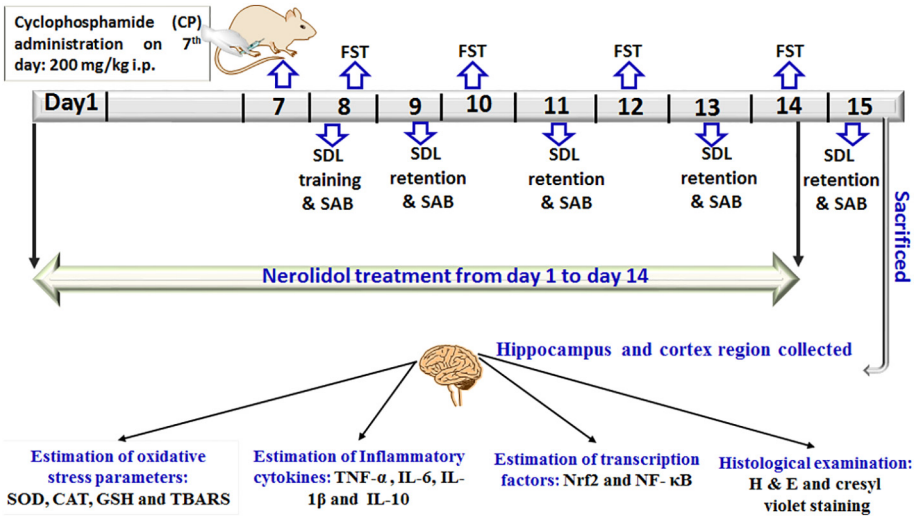

Fig. 1. Treatment plan for dose administration, paradigms for the neurobehavioral study and biochemical estimations at different time points. FST: Forced swim test, SDL: Step dawn latency, SAB: Spontaneous alternation behavior, SOD: Superoxide dismutase, CAT: Catalase, TBARS: Thiobarbituric acid reactive substances and GSH: Glutathione.

2.5. Preparation of brain tissue homogenate

Hippocampus and frontal cortex sections were obtained, rinsed with the ice-cold saline and homogenized in phosphate buffer (0.1 M; pH 7.4 at 4 °C) (10 times the volume of tissue). Homogenates were then centrifuged at 800 ×*g* at 4 °C for 5 min. and used for biochemical estimations.

2.6. Preparation of 0.5% tween 80 NER emulsion

Nerolidol was prepared according to the method of Reinaldo et al., 2016 with slight modification [15]. Weighed amount of nerolidol was mixed with the pre-heated (45 °C) Tween 80 drop by drop. This mixture was stirred on a magnetic stirrer at 45 °C at 650 RPM for 20 min. Pre-heated (45 °C) double distilled water was then added to the mixture of Tween 80 and NER precisely at the rate of 26 drops per minute. Immediately after the addition of 3–4 drops of water, the solution turned into milky color, the intensity of which reduced gradually with the subsequent addition of water. This emulsion was further stirred at 800 RPM for 15 min at 45 °C and exposed to bath sonication for 7 min to further increase the solubility.

2.7. Oxidative stress and inflammatory marker estimation

Thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and nitrate levels were estimated by the methods published Earlier [13]. Estimation of TNF-α, IL-6, IL-1β and IL-10 was done using ELISA kits according to the manufacturer's instruction.

2.8. Histopathological and immunohistochemical analysis

For histopathological examination, brain tissue was fixed in 10% formalin then sliced and embedded in paraffin wax. 5 µm thick sections were cut transversally and stained with hematoxylin and eosin dyes to detect histological alterations. For immunohistochemical analysis 5 µm thick sections were cut and deparaffinized in xylene followed by rehydration with a graded series of ethanol, washed under running double distilled water and further proceeded according to the method published earlier [29]. Photomicrographs were taken with a computer-enabled Motic microscope. Fiji (Image J) software was used for semi-quantification of the protein expression by reciprocal intensity method.

2.9. Quantification for the neuronal damage

Quantitative assessment for the neuronal damage was done in the hippocampus and frontal cortex region by H & E staining, Cresyl violet staining and by immunohistochemical staining, respectively. For these assessments, 6 random regions in the hippocampus and frontal cortex were selected under high resolution (40×). After sacrificing, mouse brain was removed, rinsed with the ice-cold saline and coronal section was cut to get hippocampus and frontal cortex (anteroposterior extension from bregma at coordinate around −2.12 mm). Before proceeding for quantification of degenerated neurons, brain regions were further confirmed under the microscope by comparing the sections to image on the Allen Mouse Brain atlas (Allen Institute for Brain Science, 2004). In H & E and Cresyl violet stained slides, pyknotic and necrotic neurons in the hippocampus and frontal cortex were counted and represented as percentage degenerated neurons. While, in immunohistochemically stained slides of Nrf2 and NF-κB, the percentage positive neurons in the hippocampus and frontal cortex regions were counted and the data were analyzed statistically.

2.10. Statistical analysis

Data were expressed as mean ± SEM. One-way ANOVA followed by post-hoc Tukey-Multiple Comparison test was applied for determining the significance of data. In all the tests, values were considered statistically significant when $P < 0.05$. The statistical analysis was performed using Graph Pad Prism 4.0 software (Graph Pad Software San Diego, California, USA).

3. Results

3.1. Docking simulation study against Nrf2 receptor

The docking result of nerolidol showed significant interaction and binding pattern to the catalytic amino acid residues present at the active binding site of Nrf2 protein. The molecular docking results revealed that nerolidol displayed docking score of - 4.043 and displayed two hydrogen bonds with the side chain of amino acid residues ASN 414 and ASN382. (Table 2 and Fig. 2). Nerolidol expressed high Van der Waals interactions and represented hydrophobic interactions with the other amino acid residues ARG 380, GLY 364, SER 363, GLY 603, ARG 415, PHE 577, ALA 556, TYR 572, TYR 334 and SER 602 surrounding the active binding site of Nrf2. The results of docking and molecular interaction studies revealed that nerolidol has the potential to activate Nrf2 protein by binding to its active catalytic domain (Table 2 and

Table 2
Molecular docking results of nerolidol at the active binding site of Nrf2 (PDB ID: 5CGJ) and residues involved in forming hydrogen bonds and hydrophobic interactions.

Ligand	Docking score	Glide energy (cal/mol)	Glide emodel	Glide evdw	Glide ecoul	Residues involved in hydrogen bonding interactions
Nerolidol	−4.043	−36.994	−44.841	−49.464	−9.750	ASN 414, ASN382

Fig. 2).

3.2. Effect of nerolidol on the hippocampus and frontal cortex Nrf2 expression and oxidative stress parameters (SOD, CAT, TBARS & GSH)

We observed intense dark brown positive staining (showed with blue arrow in Fig. 3), that indicated the Nrf2 positive neurons in the hippocampus and frontal cortex when exposed to CP 200. Administration of CP 200 slightly increased the percentage of Nrf2 positive neurons ($P < 0.001$) (transient adaptive response of Nrf2 towards oxidative stress). CP 200 also caused significant reduction ($P < 0.001$) in SOD, CAT, GSH and elevation in the level of TBARS in hippocampus and in the frontal cortex regions, respectively when compared with the control (Fig. 3 and Table 3). Treatment with NER 400 significantly ($P < 0.001$) increased the percentage of Nrf2 positive neurons and also increased SOD, CAT, GSH and reduced the level of TBARS in the hippocampus and in the frontal cortex regions, respectively. Treatment with NER 200 showed varied response in terms of reversal of the above parameters to normal. It was found ineffective in reversing SOD, GSH and TBARS in hippocampus, whereas reversed CAT and all other

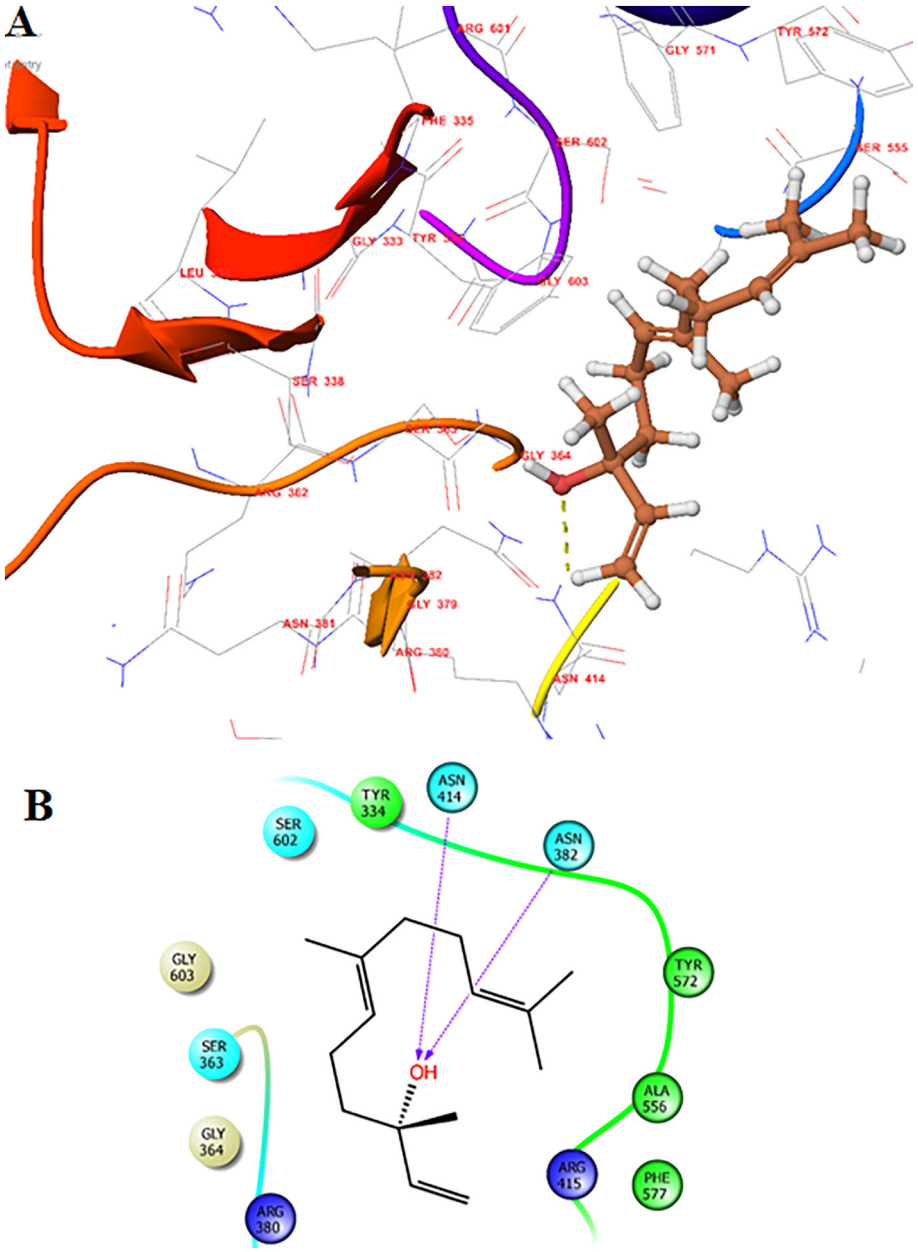

Fig. 2. (A) 3D Binding mode and ligand interaction of nerolidol in the catalytic pocket of Nrf2. (B) 2D Binding mode and ligand interaction of nerolidol in the catalytic pocket of Nrf2 (PDB ID: 5CGJ).

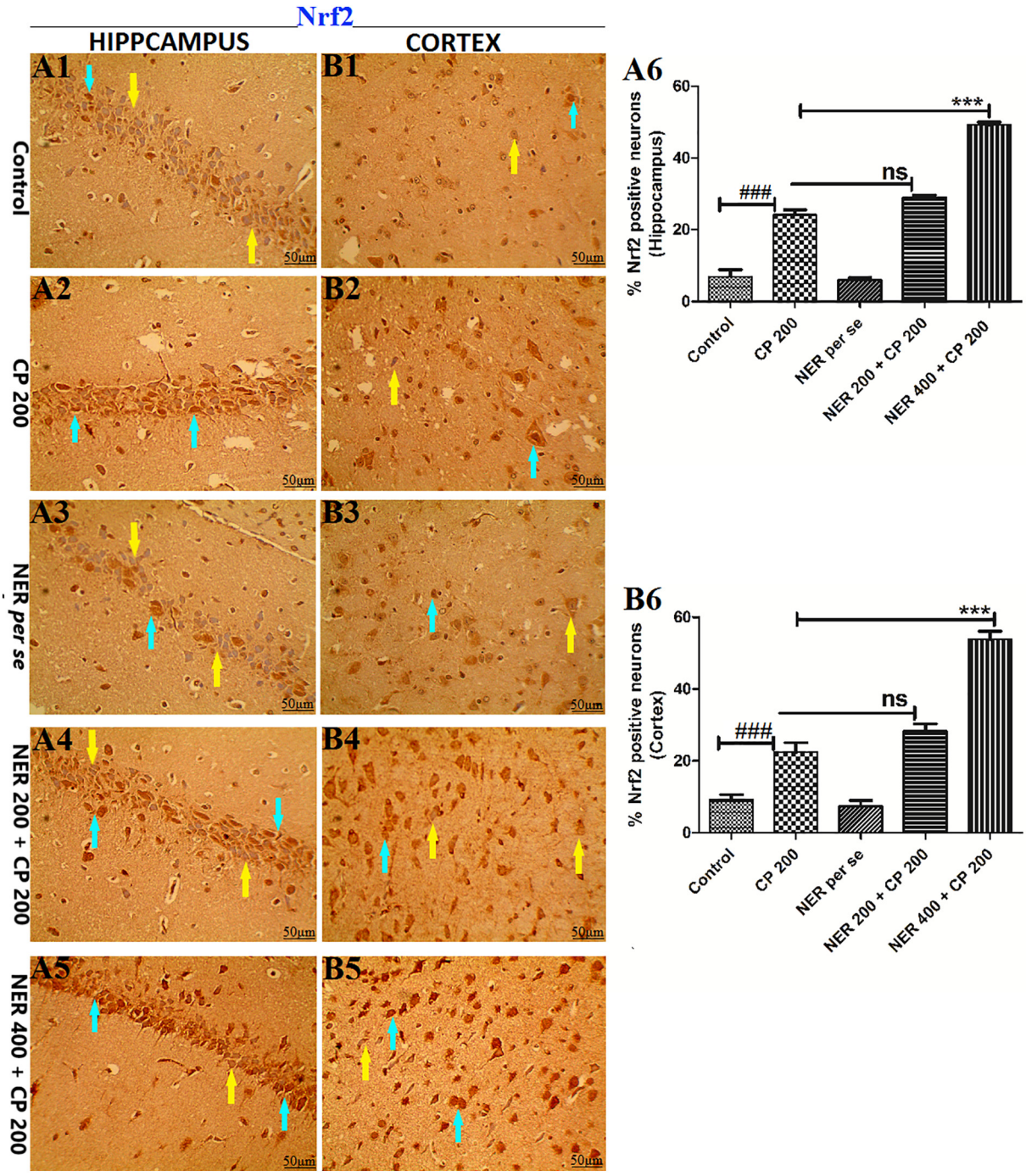

Fig. 3. Showing the immunohistochemistry of Nrf2 expression in the hippocampus and frontal cortex of different groups (A1–A5 and B1 to B5, respectively). Blue arrow showing positively stained Nrf2 whereas yellow arrow showing negatively stained Nrf2 neurons. Figure 3A6 and B6 showing the quantitative analysis of positively stained Nrf2 neurons in the hippocampus and frontal cortex regions. Values are expressed as mean ± SEM ($n = 6$). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data. $^{###}p < 0.001$ significant, versus control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant, versus CP and ns is non-significant versus CP. [scale bar- 50 µm]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

parameters to normal in the frontal cortex as shown in Table 3 and Fig. 3.

3.3. Effect of nerolidol on NF-κB expression and inflammatory cytokines in the hippocampus and frontal cortex

We observed intense dark brown positive staining (showed with yellow arrow in Fig. 4), that indicated the activated p65 NF-κB in the hippocampus and frontal cortex when exposed to CP 200. Administration of CP 200 increased the percentage of p65 NF-κB positive neurons ($P < 0.001$). CP 200 also significantly increased the proinflammatory (TNF-α, IL-6, and IL-1β) and decreased anti-inflammatory (IL-10) cytokines level ($P < 0.001$) in the hippocampus and frontal cortex (Figs. 4 and 5). Treatment with NER 400 reduced the percentage of p65 NF-κB positive neurons ($P < 0.001$) and also reduced the level of proinflammatory (TNF-α, IL-6, and IL-1β) and increased anti-inflammatory (IL-10) cytokines ($P < 0.001$ for TNF-α and IL-6) and ($P < 0.01$ for IL-1β and IL-10) in hippocampus and frontal

Table 3
Effect of NER on SOD, catalase, TBARS and GSH content in hippocampus and frontal cortex.

Group	SOD U/mg protein	Catalase nmol of H_2O_2/min/mg protein	TBARS nmol of MDA/mg of protein	GSH μmol/mg of protein
Hippocampal oxidative stress				
Vehicle control	20.1 ± 1.01	16.06 ± 0.91	10.75 ± 0.54	32.19 ± 1.77
CP 200 (Toxic)	6.83$^{###}$ ± 0.67	8.20$^{###}$ ± 1.08	28.13$^{###}$ ± 1.2	9.41$^{###}$ ± 0.5
NER per se	18.88 ± 1.04	17.2 ± 0.76	11.98 ± 0.49	33.17 ± 1.34
NER 200 + CP 200	10.53^{ns} ± 0.68	12.78* ± 1.12	25.16^{ns} ± 0.66	13.67^{ns} ± 0.16
NER 400 + CP 200	16.58*** ± 1.2	16.21*** ± 1.09	19.83*** ± 0.69	28.74*** ± 0.86
Frontal cortex oxidative stress				
Vehicle control	20.74 ± 1.04	18.42 ± 0.86	9.93 ± 0.44	32.19 ± 1.77
CP 200 (Toxic)	6.84$^{###}$ ± 0.35	7.33$^{##}$ ± 1.04	27.52$^{###}$ ± 1.87	9.41$^{###}$ ± 0.5
NER per se	19.23 ± 1.04	17.02 ± 0.97	10.82 ± 0.40	32.22 ± 1.08
NER 200 + CP 200	10.96* ± 0.36	12.52** ± 0.47	22.23** ± 0.66	16.01* ± 0.71
NER 400 + CP 200	14.22*** ± 1.15	15.27*** ± 1.39	19.83*** ± 0.69	27.94*** ± 73

Values are expressed as mean ± SEM (n = 6). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data.
$^{###}$ $p < 0.001$ significant, versus control.
* $p < 0.05$ significant, versus CP.
** $p < 0.01$ significant, versus CP.
*** $p < 0.001$ significant, versus CP.
^{ns} Non-significant versus CP.

cortex, respectively (Figs. 4 and 5). However, treatment with NER 200 was found to be ineffective in reducing this percentage of p65 NF-κB positive neurons ($P > 0.05$) and against increased proinflammatory (IL-6, and IL-1β) and decreased anti-inflammatory (IL-10) cytokines in the hippocampus and frontal cortex ($P > 0.05$). However, NER 200 was found to be effective against increased TNF-α ($P < 0.05$) in the hippocampus and frontal cortex as shown in Figs. 4 and 5.

3.4. Effect of nerolidol on spontaneous alternation behavior (SAB) test

SAB is one of the well-validated neurobehavioral methods for cognitive assessment where percentage alternation is positively correlated with cognition. SAB was calculated as number of quintuples/possible entries × 100. Treatment with CP 200 significantly reduced the % alternation ($P < 0.001$) when assessed after 24 h of CP administration as shown in Fig. 6A. There was a gradual reduction in % alternation from day 8 to day 15 as compared with the control group ($P < 0.01$ on day 8; $P < 0.001$ from day 9 onwards to day 15). Treatment with NER 400 significantly increased the % alternation from day 8 to day 15 ($P < 0.01$ on day 8 and $P < 0.001$ from day 9 onwards to day 15) as shown in Fig. 6A. However, NER 200 was found to be ineffective on day 8, day 9 and day 11 but showed increased % alternation on day 13 onward ($P > 0.05$ on day 8, day 9 and day 11; $P < 0.05$ on day 13 onward to day 15) as shown in Fig. 6A.

3.5. Effect of nerolidol on the step-down latency (SDL)/passive avoidance test

Retention transfer latency was measured after 24 h of acquisition period. Treatment with CP 200 significantly reduced the retention transfer latency ($P < 0.001$) on alternate days from day 9 to day 15. Treatment with NER 400 significantly elevated the retention transfer latency period ($P < 0.01$ on day 9; $P < 0.001$ from day 11 onwards to day 15). However, treatment with NER 200 was found to be ineffective in reducing the retention latency from day 9 to day 13 ($P > 0.05$) but elevated the retention transfer latency period ($P < 0.05$) on day 15 as shown in Fig. 6B.

3.6. Effect of nerolidol on forced swim test (FST)

Forced swim test is a well-validated and well-established model to access the depressive behavior. In FST, the duration of immobility represents the extent of depression. Treatment with CP 200 significantly increased the duration of immobility ($P < 0.001$) on alternate days from day 8 to day 14. Treatment with NER 400 significantly reduced the duration of immobility from day 8 onwards to day 14 ($P < 0.01$ on day 8; $P < 0.001$ from day 10 onwards to day 14). However, treatment with NER 200 was found to be ineffective in reducing the duration of immobility from day 8 to day 12 ($P > 0.05$) but reduced the duration of immobility ($P < 0.05$) on day 14 as shown in Fig. 6C.

3.7. Histopathological analysis by H & E staining and Cresyl violet staining/Nissl staining in the hippocampus and frontal cortex

To get a better picture of histological damage induced by the CP administration and to assess the protective role of nerolidol against this toxicity, we performed H & E staining followed by Cresyl violet staining (CV staining)/Nissl staining in the hippocampus (CA1, CA2 and CA3 regions) and frontal cortex, respectively (Figs. 7 and 8). Administration of CP 200 showed marked chromatolysis, pyknosis, vacuolization and neuron damage which is cumulatively represented as % degenerated neurons. CP-treated mice showed increased % degenerated neuron in the hippocampus (CA1, CA2 and CA3) and frontal cortex ($P < 0.001$) as shown in Figs. 7 and 8. Administration of NER 400 significantly improved the histological damage and reduced the % degenerated neurons in the hippocampus and frontal cortex region, respectively ($P < 0.001$). However, administration of NER 200 was ineffective in improving these histological alterations but only found effective against CA3 region of the hippocampus ($P < 0.05$) as shown in Fig. 7.

4. Discussion

CP has been documented to cause oxidative stress and cognitive dysfunction in the brain [5]. In the current study we have tried to see the neuroprotective effect of NER against CP-induced neurotoxicity in the brain, specifically in the hippocampus and frontal cortex region in terms of antioxidant status, inflammation, histological damage and behavioral changes. Previously, NER has been reported to exert protective action against rotenone, PTZ and trypanosoma evansi-induced neurotoxicity by modulation of biochemical and inflammatory mediators like SOD, CAT, TNF-α, Il-6 and IL-β [15,20–23]. However, till date NER has not been studied for its neuroprotective role in CP-induced neurotoxic model. Concerning CP-induced neurotoxicity, to the best of our knowledge, all the studies has constrained to the report of oxidative stress and cognitive dysfunction and evidence for CP-induced neuroinflammation and histological alteration is lacking [8,9,24–26]. Meanwhile, we encountered only one study by Yang et al., 2010 that had shown CP-induced reduction in hippocampal neurogenesis in the

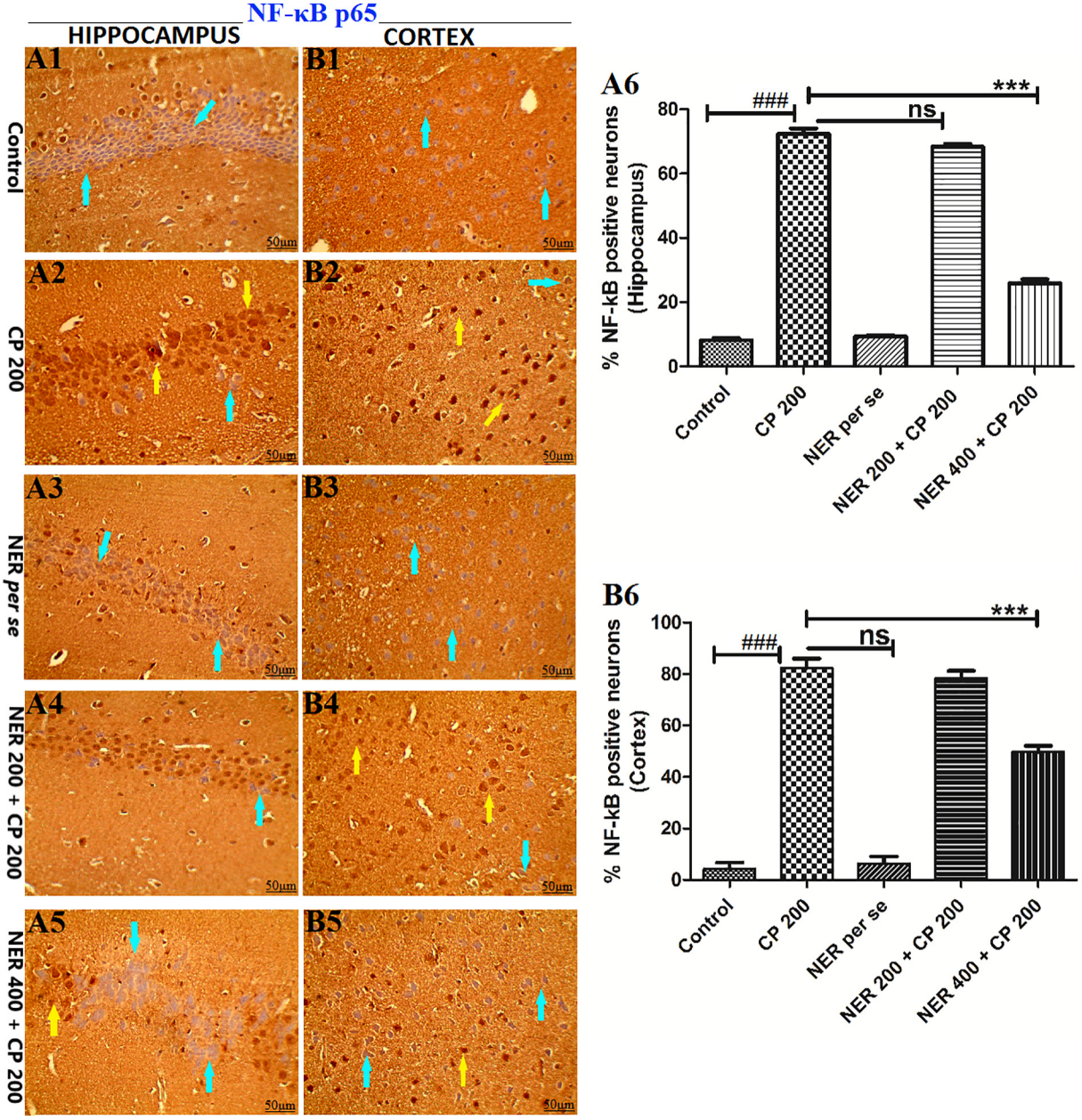

Fig. 4. Showing the immunohistochemistry of NF-κB expression in the hippocampus and frontal cortex of different groups (A1-A5 and B1 to B5, respectively). Yellow arrow showing positively stained NF-κB whereas blue arrow showing negatively stained NF-κB neurons. Figure 4A6 and B6 showing the quantitate analysis of positive NF-κB neurons in the hippocampus and frontal cortex. Values are expressed as mean ± SEM (n = 6). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data. $^{###}p < 0.001$ significant, versus control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant, versus CP and ns is non-significant versus CP. [scale bar- 50 µm]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dental gyrus region of the hippocampus [27]. CP-induced inhibition of neurogenesis was found to be responsible for altered learning and memory impairment. Therefore, this is a first study to explore the protective role of NER in CP-induced neuroinflammation, histological aberrations and cognitive dysfunctions by explicating the role of NF-kB, Nrf2 and associated downstream in the hippocampus and cortex regions, respectively.

Hippocampus is one of the essential parts of the limbic system and is involved in cognitive function, storage and retrieval of episodic memory (ability to recall the spatial, personal and temporal context of a past event) [12]. It is also reported to be actively involved in motivation, learning, and appetite in primates [27,30]. It is important to highlight that the hippocampus functions as a temporary relay structure that transmit the newly encoded hippocampus-dependent memory to the permanent storage structures in the cortex [31,32]. Cortex is connected to the thalamus and basal ganglia and involved in the information processing via efferent and afferent connections, voluntary movement and the movement according to the self-generated thoughts [32]. Therefore, hippocampus and cortex together maintain the appropriate and effective neuronal homeostasis in the brain and any damage to these organs reflect neurobehavioral abnormalities [12,32]. CP is already known to cause behavioral abnormalities in mice. As hippocampus and cortex together maintain the appropriate and effective neuronal homeostasis in the brain, damage to these organs reflects in neurobehavioral abnormalities. Keeping this in mind we focused on the hippocampus and cortex region of the brain and performed various

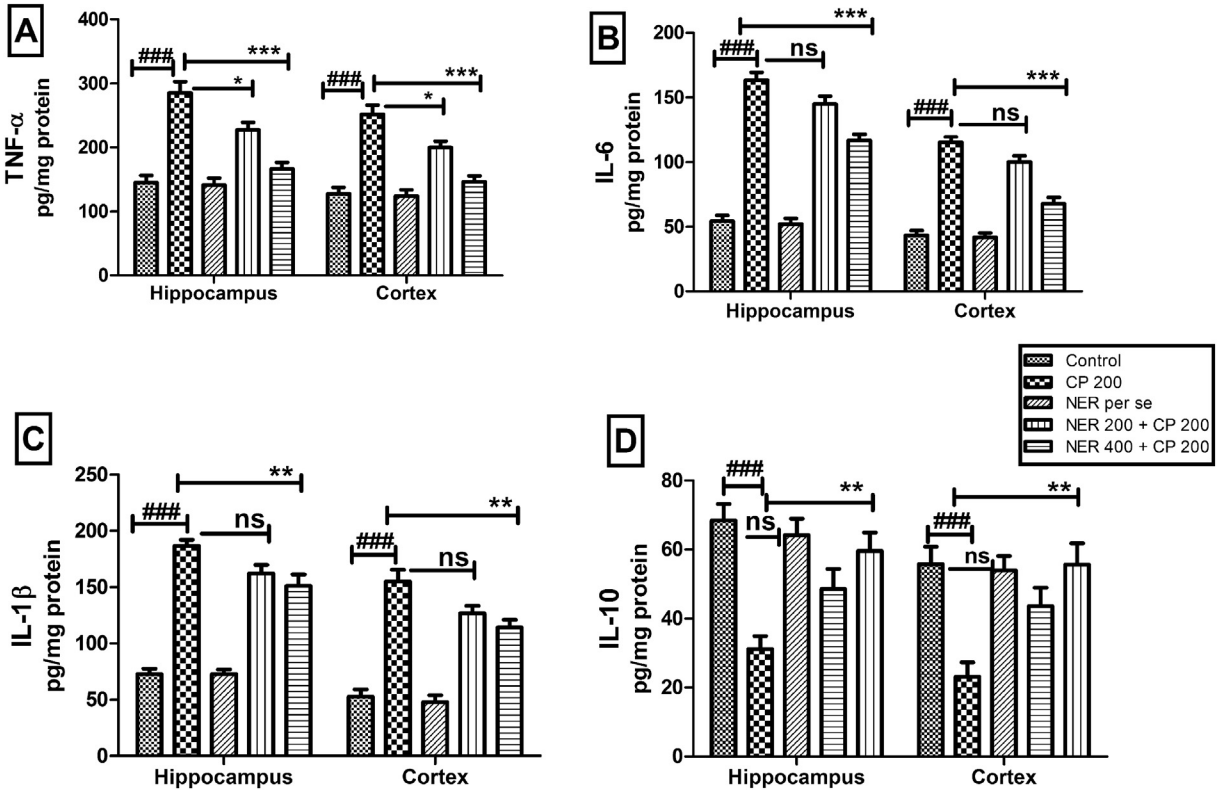

Fig. 5. Showing the effect of CP 200, NER 200 and NER 400 on cytokine levels (A) TNF α, (B) IL- 6 (C) IL-10 (D) IL-1β. Values are expressed as mean ± SEM ($n = 6$). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data. $^{###}p < 0.001$ significant versus, control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant versus, CP and ns is non-significant versus, CP.

biochemical, histopathological and immunohistochemical studies to correlate the hippocampal and cortical damage with the behavioral change in mice when administered CP200 and the neuroprotection offered by nerolidol.

The brain is considered vulnerable towards oxidative stress because of the limited supply of antioxidants, as neurons can't synthesize

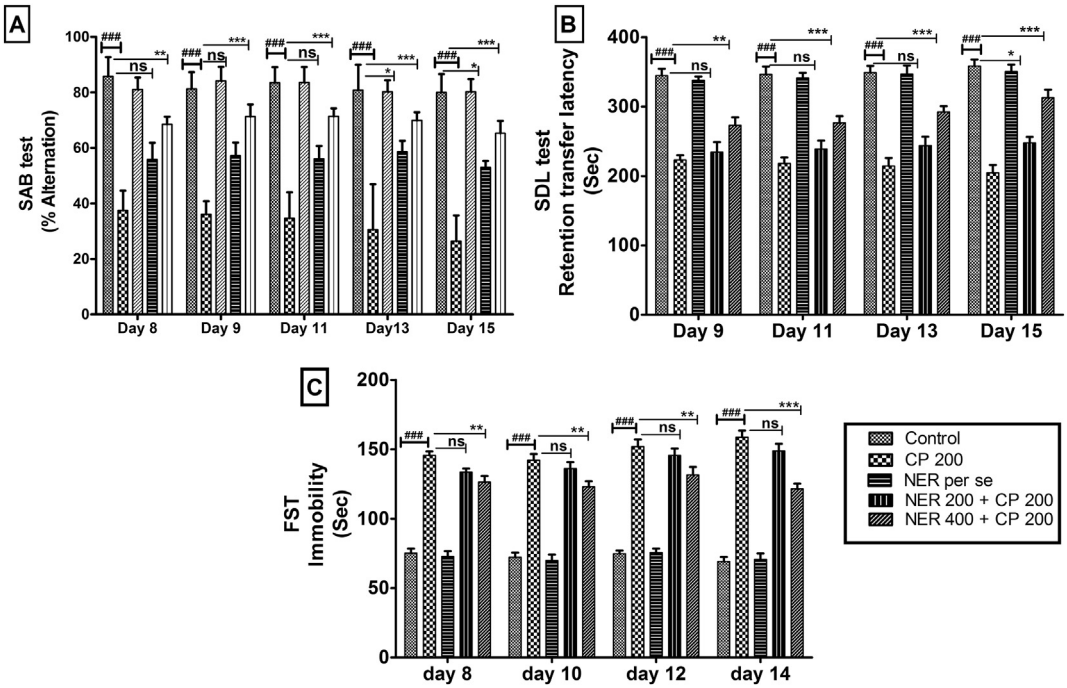

Fig. 6. Showing the effect of CP 200, NER 200 and NER 400 on neurobehavioral changes. (A) Spontaneous alternation behavior (B) Retention transfer latency/ Passive avoidance test and (C) Forced swim test. Values are expressed as mean ± SEM (n = 6). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data. $^{###}p < 0.001$ significant, versus control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant, versus CP and ns is non-significant versus, CP.

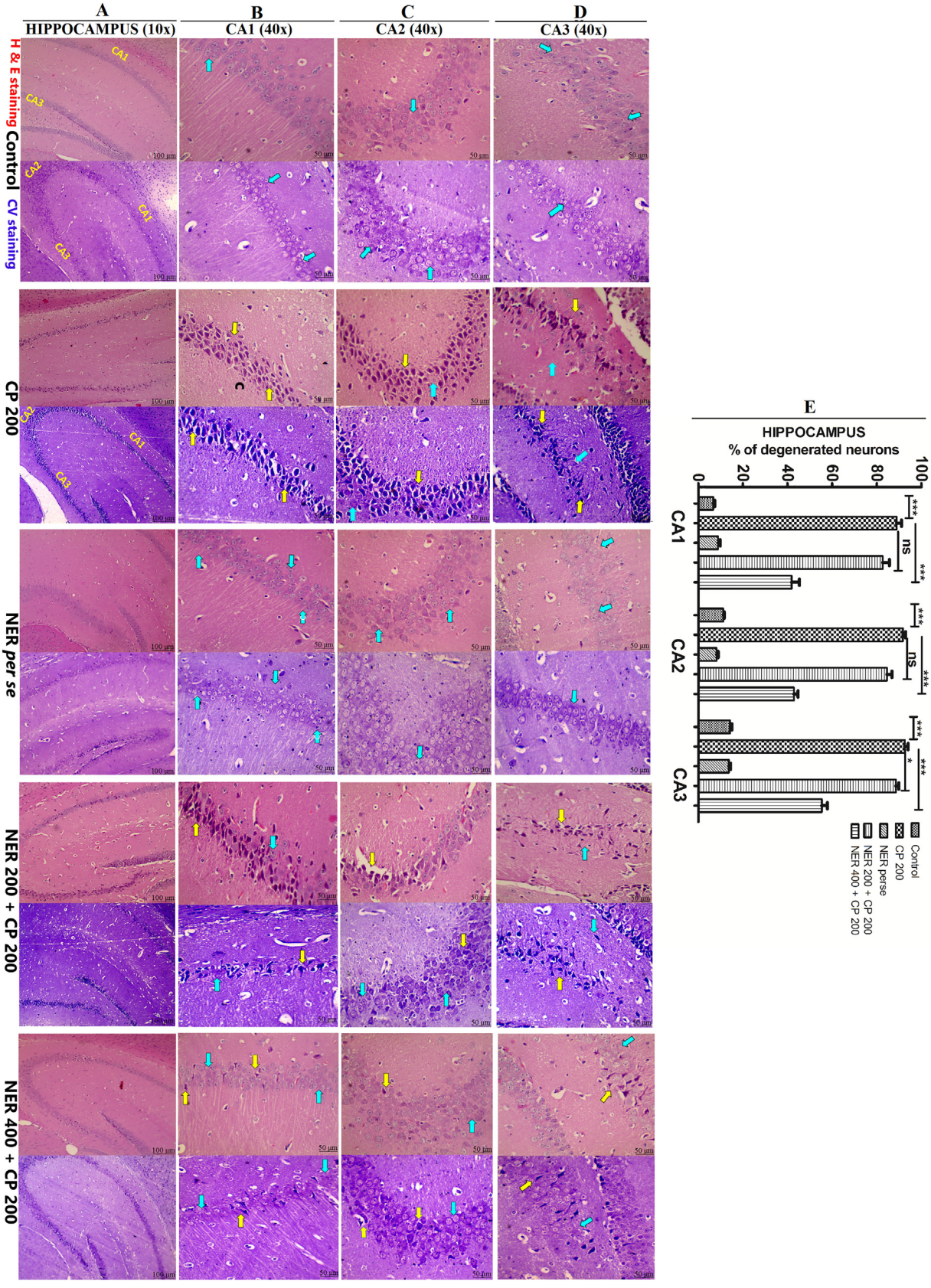

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Fig. 7. Showing H & E and Cresyl violet stained (CV stained) histological changes in the hippocampus and in its CA1, CA2 and CA3 sub-regions. Upper panel of each group represents H & E stained changes and the lower panel represents CV stained changes. Panel A shows changes in the hippocampus [scale bar- 50 μm]. Panel B, C and D shows changes in CA1, CA2 and CA3 regions of the hippocampus, respectively. Yellow arrow is showing pyknotic nucleus and degenerated neurons whereas blue arrow is showing healthier neurons. Fig. 7E showing the quantitative analysis of % degenerated neurons in CA1, CA2 and CA3 regions of the hippocampus. Values are expressed as mean ± SEM (n = 6). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data. $^{###}p < 0.001$ significant, versus control; $^{*}p < 0.05$, $^{**}p < 0.01$., $^{***}p < 0.001$ significant, versus CP and ns is non-significant versus CP [scale bar- 50 μm]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

important antioxidant like glutathione in sufficient amount and it contains more amount of polyunsaturated fatty acids (PUFA) [12,33]. MDA produced from the oxidation of PUFA, ROS produced as a by-product of metabolic process and during phagocytosis by macrophages, a severe oxidative stress in the brain is created [12,33,34]. Endogenous antioxidants regularly detoxify these ROS and maintain the redox homeostasis. Thus, an imbalance in the redox homeostasis or limited availability of antioxidants render the neurons towards free radicals attack leading to oxidative stress, neuroinflammation and multitudinous pathogenicity including neurodegeneration, cognitive dysfunction and depression as shown in Fig. 9 [33,35]. Additionally, oxidative stress is positively correlated with Nrf2 expression and its nuclear translocation. Nrf2, belong to the Cap 'n' Collar (CNC) subfamily and found widely distributed in CNS. Increased expression of Nrf2 result in neuroprotection by activating antioxidant responsive element (ARE), causes transcription of Phase II detoxifying enzymes and antioxidant proteins like GSH, SOD, HO-1, etc. However, reduced nuclear Nrf2 is reported to be associated with neuroinflammatory, neurodegenerative disorders and associated comorbid conditions [36,37]. Thus, looking into the prominent role of Nrf2 in neurological disorders, in the present study, we performed molecular docking study of NER with Nrf2. Findings showed effective binding of NER within the pocket domain of Nrf2 and thus NER can act as Nrf2 activator and can increase the expression of Nrf2 (Fig. 2 and Table 2). CP administration caused mild increase in the level of Nrf2 as compared to control group in the hippocampus and frontal cortex (Fig. 3 and Table 3). Additionally, CP administration also increased the level of TBARS and diminished SOD, CAT, and GSH in the hippocampus and frontal cortex region (Fig. 3 and Table 3). Treatment with NER 400 significantly increased the expression of Nrf2 and also elevated the level of the antioxidant defense system as compared to CP 200 in the hippocampus and frontal cortex which goes fine with the previous findings [8,9,16,20–22,24–26,38].

It is well established that neuroinflammation has always remained a significant contributor to neurobehavioral abnormalities in neurological disorders like Alzheimer's disease, parkinsonism, seizure, encephalopathy, ataxia and multiple sclerosis [39]. NF-κB and Nrf2 are considered as the central player of neuroinflammation and neurodegeneration [7,40]. Under normal condition, inactive NF-κB is located in the cytoplasm in association with the natural biological inhibitor, IκB [7,41]. Stimuli like ROS, reduced Nrf2 expression or CP causes phosphorylation of IκB, resulting into activation/translocation of NF-κB into the nucleus [42]. Translocated NF-κB binds with a domain of DNA via p65 subunit and regulates the encoding of inflammatory cytokines and chemokines like TNF-α, IL-6, IL-1β and IL-10 [7,29]. Thus, Pharmacological blockage of NF-κB p65 can reduce the level of IL-1β, TNF-α and IL-6 which in turn can reduce the severity of neuroinflammation and cognitive dysfunction. In the current study, CP 200 significantly increased the expression of NF-κB p65 in the hippocampus and frontal cortex region along with its downstream proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and reduced the level of anti-inflammatory cytokines (IL10) as shown in Figs. 4 and 5. Treatment with NER 400 caused reduced expression of NF-κB p65 as well as proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and elevated the level of anti-inflammatory cytokines (IL-10) in the hippocampus and frontal cortex which was in accordance with the earlier findings [15,22]. Treatment with NER 200 significantly reduced the level of TNF-α in the hippocampus and frontal cortex but found to be ineffective against elevated NF-κB level and other inflammatory chemokines as shown in Figs. 4 and 5.

Interestingly, there has been report of a negative correlation between NF-κB p65 activation and Nrf2 expression. NF-κB p65 has been shown to inhibit the binding of Nrf2 with ARE and also promotes ubiquitination and proteasomal degradation of Nrf2 leading to a reduced level of phase II detoxifying enzymes like SOD, CAT and GSH [43]. Findings by Yu et al., 2008 showed that p65 subunit of NF-κB is associated with Keap1 which prevent the translocation of Nrf2 into the nucleus [44]. At the same time, activated Nrf2 has been reported to inhibit IkB phosphorylation via autophagocytosis and thus inhibit the activation/translocation of NF-κB, leading to a reduced level of proinflammatory cytokines. Thus, decline in the Nrf2 expression and persistent increase in the expression of NF-κB p65 results in neuroinflammation whereas reduced NF-κB expression and persistent increase in Nrf2 expression results in neuroprotection [45]. Interestingly our findings are in line with this report.

One of the major consequences of oxidative stress and neuroinflammation is cognitive dysfunction and depression which is well documented. Additionally, there are ample published reports which show prominent cognitive dysfunction and depression upon CP administration [8,10,24–26,38]. In the present study, we also found depressive and cognitive abnormalities in mice when treated with CP 200 as shown in Fig. 6. CP 200 administration resulted into a reduced percentage of alternation and retention time in SAB and SDL tests. In SAB test, mice were not able to enter into the open arm and in SDL test, mice could not remain on shock free zone for significant time duration even when they were trained for the same, 24 h before the experimental procedure. Similarly, CP administration resulted into significant depression, where CP 200 treated mice exhibited a higher duration of immobility as compared to vehicle-treated mice. NER 400 was found to be effective against altered behavioral abnormalities, right from the day 8 to day 15. However, NER 200 showed a positive result on neurobehavioral aberrations only after 3 doses of CP administration, i.e., from day 12 onwards. We hereby assume that this delayed response of NER 200 could be because of not achieving effective therapeutic concentration in the brain before day 12. However, biodistribution studies or estimation of drug concentration in the brain tissue is needed to strengthen this assumption.

Histological findings of the hippocampus and frontal cortex upon CP administration showed significant damage to neuronal architecture, pyknosis in certain areas, vacuolization, and nuclear degeneration, as shown in Figs. 7 and 8. As of now, the histological finding of any of these parts of the brain has not been reported in CP-induced neurotoxic model. Additionally, we observed some typical changes in mice after CP administration, like the sluggish movement, piloerection, stretching, twisting and bending of the neck and hind limb seizure (supplementary file attached). Therefore, we conclude that these behavioral and motor alteration in mice might be because of damage to vital parts of the brain (hippocampus and frontal cortex) resulting into impaired neurotransmitters release, abnormality in nerve conduction, alteration in motor-sensory reflex and altered neuronal plasticity. Treatment with NER 200 and 400, reversed the histological aberration in a dose dependent manner and thus exerted neuroprotective effect.

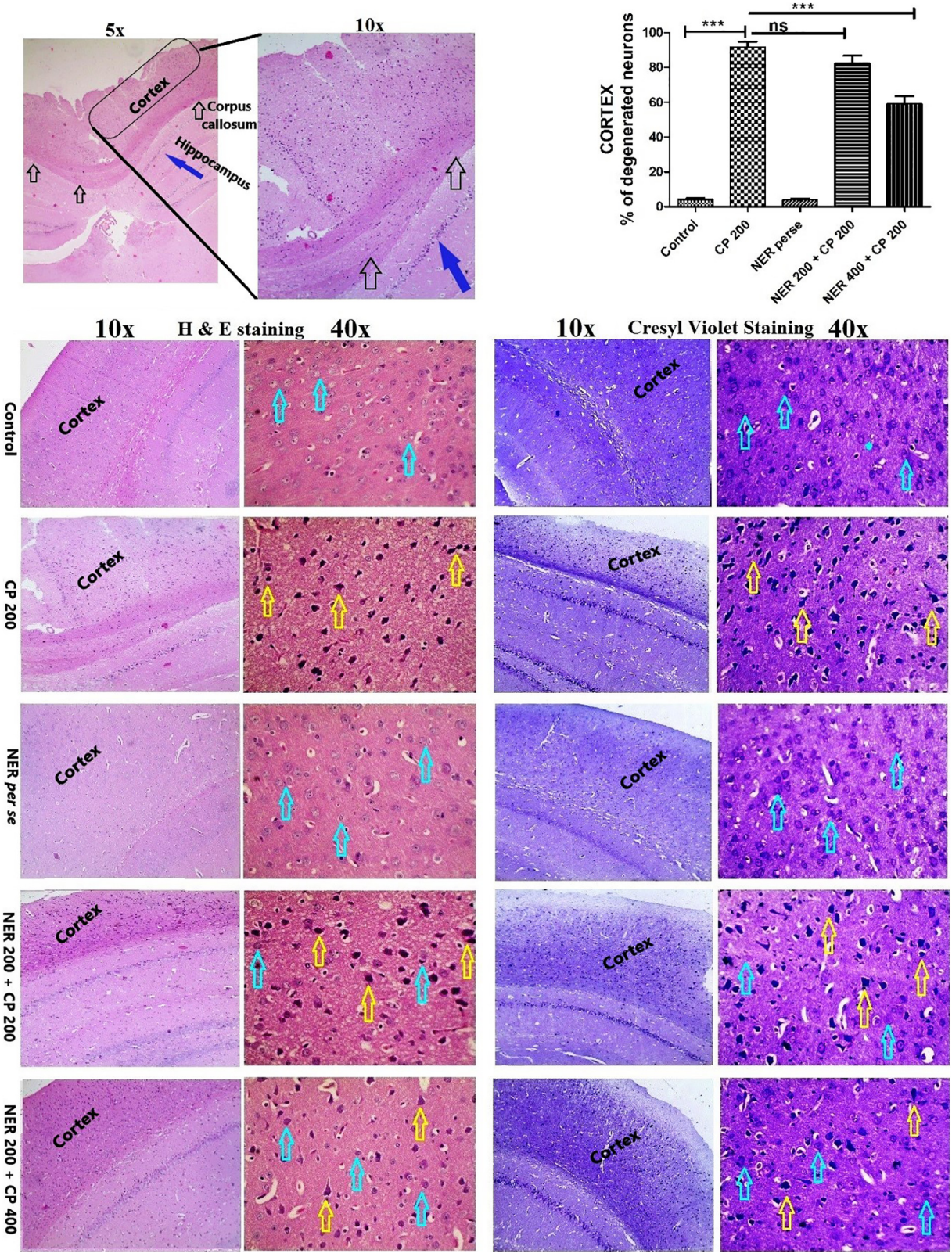

Fig. 8. Upper panel (left side) showing the hemi section area of brain where the area shown within the black enclosure represents cortex region which was used in histopathological analysis and for quantification of degenerated neurons (5×). Magnified area of hemi section is shown at 10×. Upper panel (right side) showing the quantification of degenerated neurons from the cortical region. Lower panel showing H & E and Cresyl violet stained histological alterations induced by CP 200, and the effect of NER 200 and NER 400 in the cortical region of the brain at 10× and 40×, respectively. Yellow arrow is showing pyknotic nucleus and degenerated neurons whereas blue arrow showing healthier neurons. Values are expressed as mean ± SEM ($n = 6$). One-way ANOVA followed by Tukey's multiple comparison test was applied for determining the significance of data. $^{###}p < 0.001$ significant, versus control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant, versus CP and ns is non-significant versus CP. [scale bar- 50 μm]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

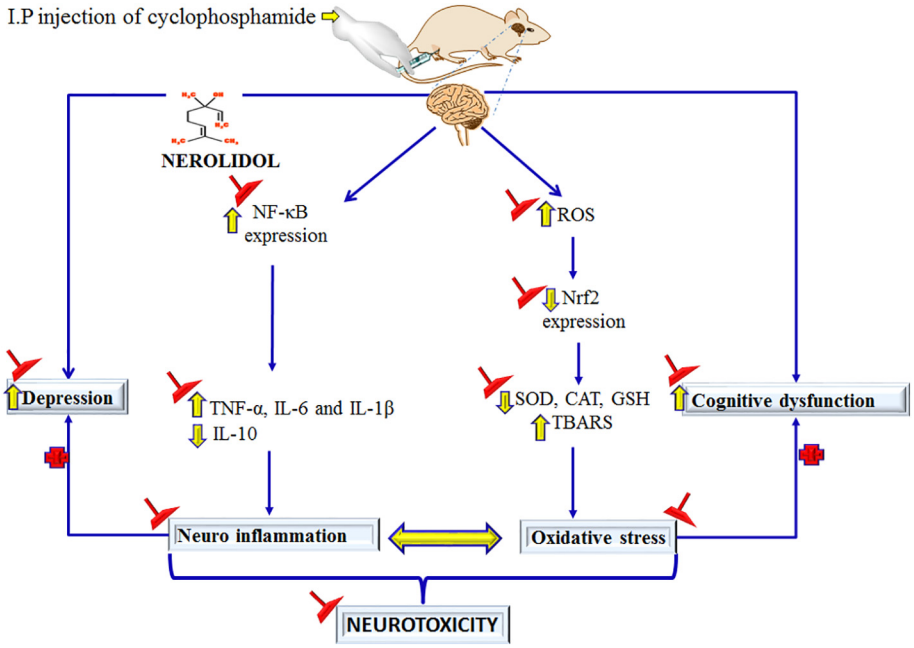

Fig. 9. Showing the proposed therapeutic implication of nerolidol against CP-induced neurotoxicity. CP administration induces oxidative stress in the hippocampus and frontal cortex that results into reduced Nrf2 expression. Reduced Nrf2 expression further diminished the level of antioxidant enzymes and proteins. CP administration also causes increased NF-κB expression that resulted into increased level of inflammatory cytokines in the hippocampus and frontal cortex. Thus, reduced Nrf2 level and increased NF-κB level resulted into neuronal oxidative stress and inflammation that collectively caused depression and cognitive dysfunction. Nerolidol by virtue of its antioxidant and anti-inflammatory effect, ameliorated these neurotoxic aberrations and reduced the extent of depression and cognitive dysfunction.

5. Conclusion

Based on the Nrf2, NF-κB, antioxidant and histological examinations, we came to the conclusion that cyclophosphamide caused neurotoxicity in terms of oxidative stress, inflammation and histological damage and nerolidol 400 mg/kg significantly reversed the deranged parameters to normal as shown in Fig. 9. These biochemical and histopathological findings had been very well supported with the behavioral studies that we performed in terms of passive avoidance test, forced swim test and spontaneous alternation behavior test. It was also observed that the NER alone administration did not cause any adverse effect, which was evident from the results of per se group. In conclusion, our findings added a novel target to the mechanism of CP-induced neurotoxicity and explored the neuroprotective role of nerolidol through modulation of Nrf2 and NF-κB. However, more detailed study like estimation of neurotransmitters, radiological imaging of brain, gamma scintigraphy study and ultrastructural study of different parts of the brain are needed to strengthen the findings so that nerolidol can be used as an adjuvant with CP in cancer therapy.

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Declaration of competing interest

The authors declare no conflict of interests.

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