Neurovascular protective effects of nutritional interventions in ischemic brain injury

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ABSTRACT

Background: Cerebral ischemia/reperfusion (I/R) injury is marked by oxidative stress, inflammation, and neuronal loss. Natural antioxidants such as fish oil and *Nigella sativa* oil have shown potential neuroprotective properties. This study evaluated the individual and combined effects of these oils in a mouse model of cerebral ischemia.

Methods: Forty mice were randomly assigned to five groups: sham, I/R, *Nigella sativa* oil, fish oil, and combined treatment. Cerebral ischemia was induced by 17 min bilateral common carotid artery occlusion followed by reperfusion. Treatments were administered orally. Motor coordination and cognitive function were assessed using behavioral tests, while neuronal damage was evaluated histologically. Oxidative stress markers (MDA, GSH, SOD) and the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) were measured in brain tissue.

Results: All treated groups showed significant improvements in motor and cognitive performance compared with the I/R group. Treatments reduced MDA and TNF-α levels and increased GSH and SOD activity. Histopathological analysis revealed attenuated hippocampal neuronal damage, with the combination therapy showing the greatest protection.

Conclusions: Combined fish oil and *Nigella sativa* oil therapy provides enhanced neuroprotection against cerebral I/R injury and may represent a supportive strategy for reducing ischemic brain damage.

Key words: ischemic stroke, fish oil, *Nigella sativa*, antioxidant.

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Introduction

Stroke is a leading cause of morbidity and mortality worldwide.[1] The World Stroke Organization reports that more than 12.2 million new stroke cases occur annually.[2] The risk of experiencing a stroke rises progressively with advancing age, with the likelihood approximately doubling beyond the age of 55 years.[3] Ischemic stroke (IS) constitutes most stroke incidents, accounting for about 80% of cases.[4] The pathophysiology of ischemic stroke involves several complex processes, including leukocyte invasion, complement and platelet activation, oxidative stress caused by free radicals, calcium overload, and disruption of the redox balance.[5] Altogether, these pathological processes contribute to damage of the blood-brain barrier and neuronal apoptosis.[6] Currently, the US Food and Drug Administration has approved only recombinant tissue plasminogen activator (rtPA) for managing acute stroke, which works by breaking down clots to reestablish blood flow to the brain.[7] However, rtPA has significant limitations, such as a narrow therapeutic window – only effective within 3 hours – risk of intracranial hemorrhage, absence of neuroprotective effects, and the potential for stroke recurrence.[8] Therefore, there is an urgent need to develop neuroprotective therapies that target oxidative stress and inflammation to prevent neuronal damage in stroke. To better understand the pathophysiological mechanisms of stroke, various local and global experimental models have been developed.[9] Among these, the bilateral common carotid artery occlusion

(BCCAO) model in mice is widely used to induce transient global cerebral ischemia, mimicking human conditions such as systemic hypoperfusion or cardiac-arrest related brain injury.[10]

Fish oil, a potent source of omega-3 fatty acids (ω-3PUFA) like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is crucial in modulating inflammation and oxidative stress, both of which are significant factors contributing to neuronal injury after an ischemic event.[11,12] ω-3 PUFAs play a crucial role in reducing the production of pro-inflammatory immune factors, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), while also enhancing the synthesis of specialized pro-resolving mediators like resolvins and protectins that contribute to the resolution of inflammation.[13] Furthermore, their antioxidant capabilities are essential for scavenging reactive oxygen species and strengthening the cell's natural defense systems. On the other hand, *Nigella sativa* volatile oil, abundant in thymoquinone and p-cymene, exhibits significant neuroprotective properties in models of cerebral ischemia.[14] These compounds help reduce oxidative stress and inflammation, preserving neuronal integrity and limiting brain damage following ischemic injury.[15]

Considering the established neuroprotective benefits of ω-3PUFAs present in fish oil, the present study aimed to investigate whether combining fish oil with *Nigella sativa* volatile oil enhances neuroprotection against cerebral ischemia through antioxidant and anti-inflammatory mechanisms.

Materials and Methods

Drugs and chemicals

Fish oil (16% EPA, 11% DHA) and cold-pressed *Nigella sativa* seed oil (3% thymoquinone and 55% linoleic acid) were purchased from Herbo Nutra Extract Private Limited, Greater Noida, India (batch no. EA/HN1611/23/12 and HN/BSO/23/11, respectively). All other reagents used in the study were of analytical grade and procured from Central Drug House (CDH) Fine Chemicals India.

Experimental animals

Male Swiss albino mice, aged 2-3 months and weighing 20-25 g, were obtained from the Animal House facility at Raj Kumar Goel Institute of Technology (Pharmacy), Ghaziabad, India. All animals were acclimatized under standardized environmental conditions, including a maintained temperature of 24±2°C, relative humidity of 60.4±5.1%, and a 12-hour alternating light and dark cycle. The mice were housed in polypropylene cages with adequate bedding and had free access to a standard rodent diet and tap water.

Experimental design

The mice were randomly assigned to five experimental groups (n=8 per group): i) Sham, ii) Ischemia/Reperfusion (I/R), iii) I/R treated with *Nigella* oil (Nig), iv) I/R treated with fish oil (FO), and v) I/R treated with a *Nigella* oil and fish oil (Nig/FO) emulsion. The sham group received oral saline, whereas the I/R group underwent BCCAO to induce transient global cerebral ischemia, followed by vehicle treatment. The Nig, FO, and Nig/FO groups were subjected to BCCAO and treated orally with *Nigella sativa* oil, fish oil, or their combination emulsion (200 mg/kg/day),[16] starting from reperfusion and continued once daily for seven days. Following the completion of neurobehavioral evaluations on the 8th day after treatment, the mice were euthanized, and their brains were carefully isolated. The collected brain tissues were either stored at −20°C for subsequent biochemical analyses or fixed in 4% paraformaldehyde for histopathological examination (Figure 1).

Induction of ischemia/reperfusion injury in mice

Global cerebral ischemia was induced in mice using the BCCAO model. Anesthesia was administered via intraperitoneal injection of ketamine (80 mg/kg) combined with xylazine (5 mg/kg). A midline incision was made in the neck region to expose the trachea. Subsequently, the right and left common carotid arteries were gently isolated from the surrounding vagus nerve. Bilateral occlusion of the common carotid arteries using 3-0 surgical sutures was performed to induce cerebral ischemia. After 17 minutes of induced cerebral ischemia, the sutures were carefully removed to restore blood flow through the carotid arteries, initiating reperfusion. Sham-operated mice were subjected to the identical surgical procedure, excluding the occlusion of the arteries. Throughout the operation, body temperature was kept within the range of 37.0-37.5°C using a heating pad.[17]

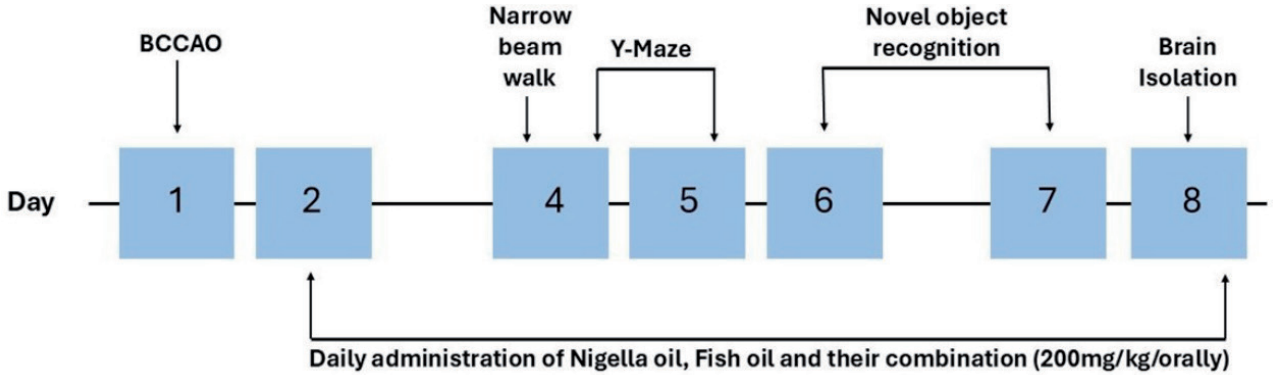

Figure 1. Experimental design of the study.

Preparation of different sets of oil emulsions for mice dosing

Three separate oil-in-water emulsions were formulated, all sharing an identical quantitative composition but varying only in the type of oil phase used. The oil phases included: whole crude oil of *Nigella sativa*; pure fish oil; and a blend of pure fish oil with *Nigella sativa* volatile oil fraction added at 2.6% of the fish oil's weight. This percentage was selected to reflect the typical natural concentration of volatile oil present in *Nigella sativa* crude oil. The three emulsions were prepared using a composition of 6% total oil phase, 1.2% Tween 80 as a surfactant, and 92.8% distilled water. Initially, each oil phase was individually mixed with the surfactant through mechanical stirring for 20 minutes to ensure proper dispersion. Then, distilled water was added drop by drop to each oil-surfactant mixture to create a coarse, milky white emulsion on its own. The three distinct oil emulsions were further homogenized using a high-speed rotor-stator homogenizer (Remi Electrotechnik LTD., Mumbai, India) set to 3000 rpm for three minutes to reduce the particle size of the dispersed oil droplets. The final emulsions were kept in the refrigerator at 4°C and administered to mice on the next day of formulation. Throughout the feeding study, all oil emulsions were stored for no longer than 10 days, even if no visible signs of phase separation were observed. To maintain consistency, fresh batches of each emulsion were prepared every 10 days until the conclusion of the experiment. Additionally, a control vehicle consisting of 1.2% surfactant by weight and 98.8% distilled water was formulated for comparison alongside the three oil emulsions.[16]

Behavioral parameters

After the 24-h reperfusion period, all behavioral tests were conducted during the light phase of the cycle by the investigators blinded to the experimental groups. The apparatuses were cleaned with 70% ethanol between trials to eliminate olfactory cues. Additionally, less demanding tasks were conducted first to reduce stress- related carryover effects, with short rest periods provided between tests.

Y-maze

The Y-maze test was employed to assess spatial working memory by measuring spontaneous alternation behavior. The apparatus comprises three arms, designated as A, B, and C, each 40 cm long, 9.5 cm high, and 4 cm wide, extending from a central junction at 120° angles. Each mouse was placed at the end of the designated start arm and allowed to freely explore the maze for eight minutes, without any form of reinforcement such as food, water, or electric shock. The total number of arm entries and their sequence were meticulously recorded. An entry was counted when the mouse had all four paws inside an arm. A successful alternation was defined as consecutive entries into three distinct arms.[18] The percentage of alternation was calculated using the following formula:

$$\text{Spontaneous alternation (\%)} = \frac{\text{No. spontaneous alternations}}{\text{total no. of arm entries} - 2} \times 100$$

Narrow beam walk test

The narrow beam walk test is designed to assess motor coordination and balance in mice with induced ischemia. Mice were pre-trained to cross the beam for at least one minute before the actual evaluation. The setup features a narrow, elongated horizontal beam measuring 1 cm in width and 100 cm in length, positioned above a soft, cushioned surface. Mice were directed from the starting point to the endpoint, using nesting material as a motivational cue. The time taken to traverse the beam was measured with a stopwatch, and the number of foot slips during the crossing was also recorded.[19]

Novel object recognition test

The novel object recognition (NOR) test was used to evaluate recognition memory. The procedure consisted of three sequential stages: habituation, training, and testing. During the habituation phase, the day before surgery, each mouse was introduced into a 50×80 cm arena with 40 cm high walls and allowed to explore freely for 5 minutes in the absence of objects. During the training phase, at the end of the reperfusion period, two identical objects were placed in the arena, and each mouse was allowed to explore them for 5 minutes.

In the testing phase, 3 hrs after the training phase (retention interval), one of the familiar objects was replaced with a novel object, and the mouse was allowed to explore the arena for 5 minutes. Exploration was defined as directing the nose toward the object at a distance of ≤2 cm and touching or sniffing the object; sitting or climbing on the object was not considered exploration. The position and type of the new object were switched between animals to avoid bias. Total exploration time was recorded, and recognition memory was quantified using the discrimination index (DI).[20]

$$\text{Discrimination index (DI)} = \frac{\text{Time spent exploring the novel object} - \text{time spent exploring the familiar object}}{\text{Total time spent exploring both objects}}$$

Biochemical parameters

Determination of brain malondialdehyde content

The level of brain malondialdehyde (MDA) in brain homogenate was ascertained by the reaction with thiobarbituric acid, as reported by Ohkawa *et al*.[21] The values were expressed as nanomoles of MDA per milligram of protein.

Determination of brain glutathione content

The brain glutathione (GSH) assay was conducted following the method described by Moron *et al*.[22] The amount of reduced GSH was expressed as micrograms of GSH per milligram of protein (µg/mg protein).

Determination of brain superoxide dismutase content

The superoxide dismutase (SOD) activity was evaluated using the pyrogallol auto-oxidation method by Marklund and Marklund.[23] The values were expressed as Units of SOD per milligram of protein (Units/mg protein).

Determination of brain tumor necrosis factor content

The measurement of the inflammatory marker tumor necrosis factor (TNF-α) was carried out using an ELISA kit. It is a solid-phase ELISA designed for use with a multimode microplate reader to estimate inflammatory markers.[24] The values were expressed as picograms per milligram of protein (picograms/mg protein).

Histopathological assessment

Hematoxylin and eosin staining

The brain tissues fixed in formalin solution underwent solvent treatment for histopathological analysis. Subsequently, paraffin-embedded tissue blocks were cut into sections of 5µm thickness and stained with hematoxylin and eosin and observed under a light microscope (400×). The counts of healthy and damaged neurons were then analyzed using ImageJ software.[25]

Statistical analysis

All data were expressed as mean ± standard error mean, and the statistical analysis of the results was performed by one-way analysis of variance, using GraphPad Prism 10, followed by Tukey's *post-hoc* test for multiple comparisons. P≤0.05 were considered significant.

Results

Effect of *Nigella* oil, fish oil, and *Nigella* oil + fish oil emulsion on behavioral parameters

In the Y-maze test, BCCAO significantly reduced spontaneous alternation (Figure 2A) and total arm entries (Figure 2B) compared with the Sham group ($p<0.0001$). Treatment with Nig ($p<0.05$), FO ($p<0.01$), and Nig/FO emulsion ($p<0.0001$) significantly improved both parameters compared to the ischemic control group.

The discrimination index in the NOR (Figure 2C) paradigm was significantly decreased in the ischemic control group compared to Sham ($p<0.05$). Nig ($p<0.05$), FO ($p<0.01$), and Nig/FO emulsion ($p<0.0001$) significantly increased the discrimination index relative to the ischemic control group.

Ischemic control animals showed significantly increased beam-crossing time (Figure 2D) and foot slips (Figure 2E) compared with Sham ($p<0.0001$). Treatment with Nig and FO ($p<0.01$) and Nig/FO emulsion ($p<0.0001$) significantly reduced these motor deficits compared to the ischemic control group.

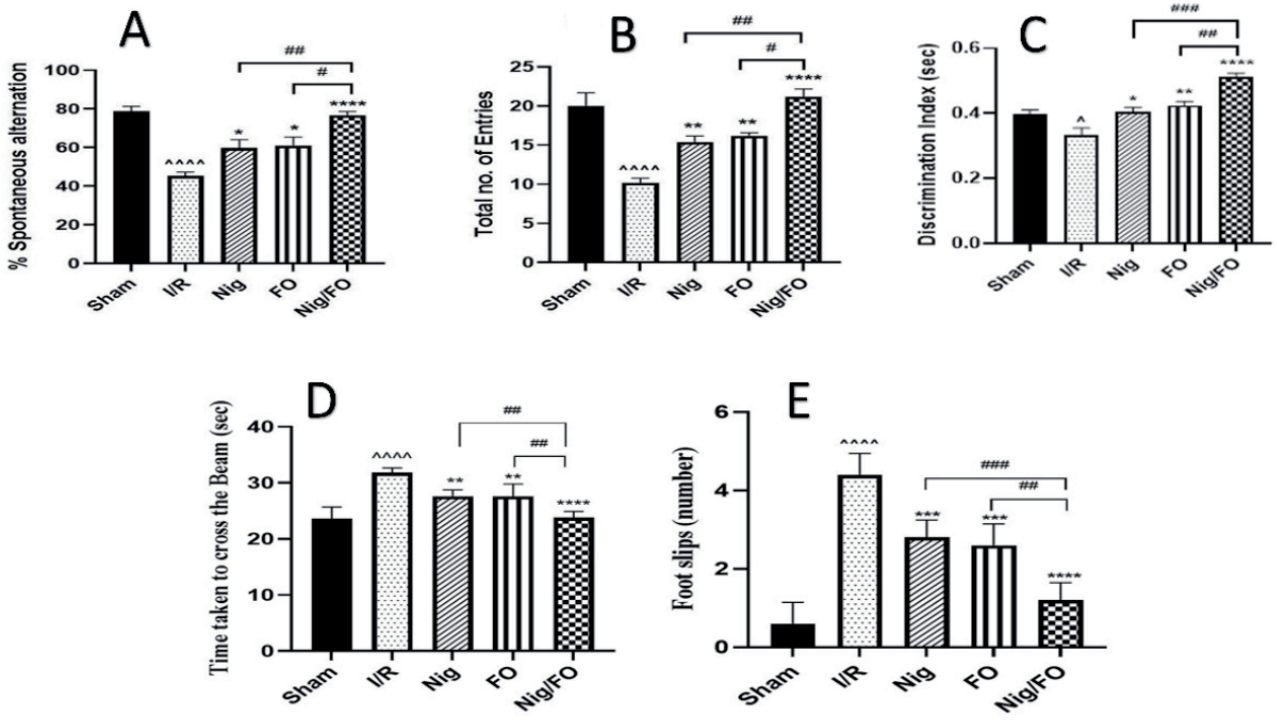

Figure 2. Effect of *Nigella* oil (Nig), fish oil (FO), *and Nig/FO* administration on cognitive and behavioral deficits in ischemic (I/R) mice: A) % spontaneous alternation and B) total no. of arm entries in Y-maze test. C) discrimination index in Novel Object Recognition test; D) time taken to cross the beam and E) foot slips in the narrow beam walk test. Data are expressed as mean ± standard error mean. ^^^^p<0.0001, ^p<0.05 *vs* Sham; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 *vs* I/R; #p<0.05, ##p<0.01, ###p<0.001 *vs Nigella* oil/fish oil group.

Effect of *Nigella* oil, fish oil, and *Nigella* oil + fish oil emulsion on oxidative stress and inflammatory parameters

The oxidative stress parameters (MDA, GSH, and SOD) were significantly altered following I/R injury. MDA levels were markedly increased ($p<0.01$), while GSH and SOD levels were significantly decreased ($p<0.0001$) compared to the Sham group, indicating elevated oxidative stress. Treatment with Nig ($p<0.05$-0.01), FO ($p<0.001$-0.05), and Nig/FO ($p<0.0001$) significantly restored these markers relative to the I/R group, with the Nig/FO combination exhibiting a more pronounced effect than Nig and FO alone. Similarly, the inflammatory marker TNF-α was found to be significantly elevated in the I/R group ($p<0.0001$). All treatments resulted in a considerable reduction in TNF-α, with Nig ($p<0.01$), FO ($p<0.01$), and Nig/FO ($p<0.0001$) showing efficacy. The Nig/FO demonstrated pronounced anti-inflammatory activity compared to Nig ($p<0.0001$) and FO ($p<0.01$) individually (Figure 3).

Effect of *Nigella* oil, fish oil, and *Nigella* oil + fish oil emulsion on the histopathological assessment of brain tissues

Histological examination of the hippocampus showed clear differences between the groups. The Sham group had a normal structure, with well-organized layers and healthy pyramidal neurons, except for a few apoptotic cells. In the Ischemic (I/R) group, the overall layering remained intact, but noticeable neuronal damage was present − especially in CA2 and CA3 − marked by scattered pyknotic neurons, though no necrosis or inflammation appeared.

Both *Nigella* oil and fish oil treated groups showed mostly preserved hippocampal architecture. *Nigella* oil caused only mild apoptotic changes in CA2 and CA3, while fish oil showed a slightly thicker pyramidal layer and more apoptotic cells in these regions, but still no signs of necrosis or inflammation. The combined *Nigella* and fish oil treatment provided the strongest protection. Neurons in CA1-CA3 and the dentate gyrus appeared

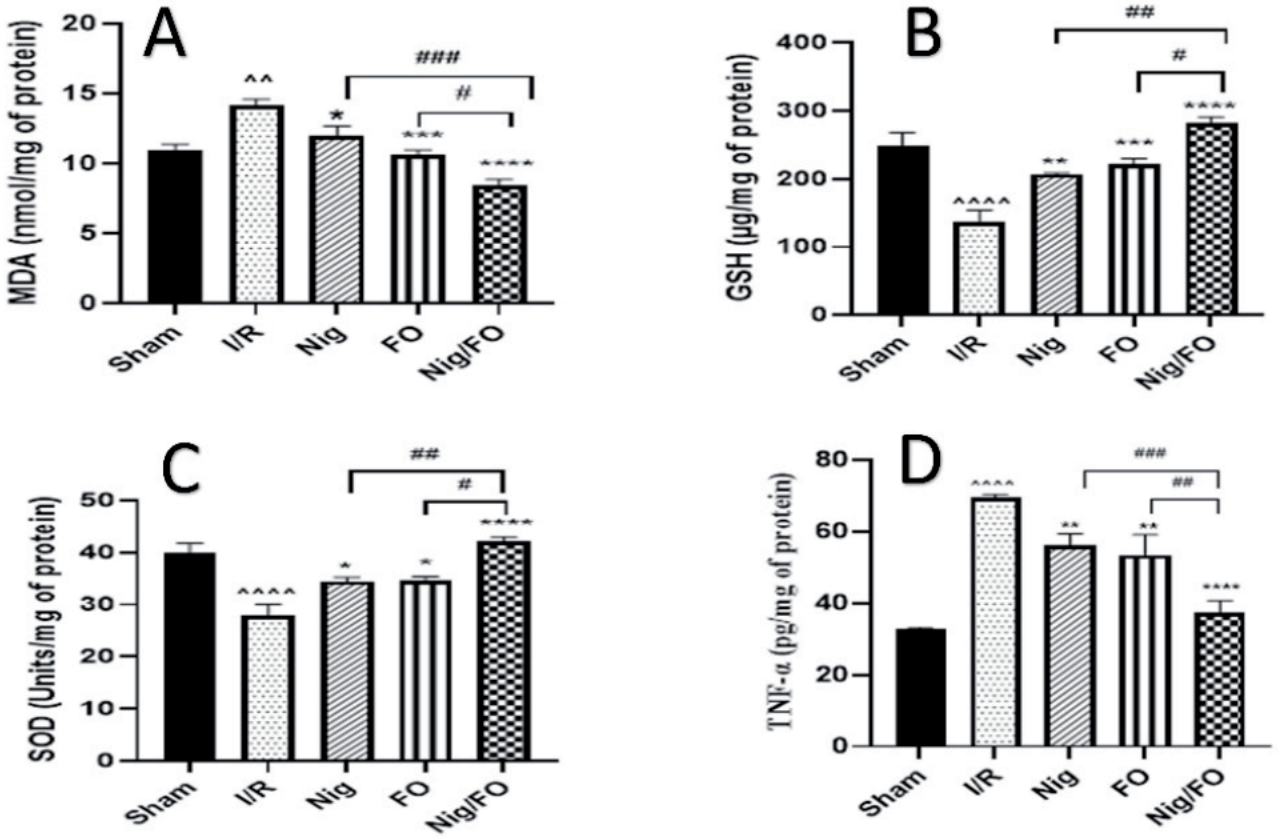

Figure 3. Effect of *Nigella* oil (Nig), fish oil (FO), and Nig/FO emulsion on oxidative stress and inflammatory markers in mice brain: A) MDA, B) GSH, C) SOD, and D) TNF-α (n=5). Ischemia/reperfusion (I/R). Results are presented as mean± standard error mean. ^^$p<0.01$, ^^^^$p<0.0001$ *vs* sham; *$p<0.05$, **$p<0.01$ (Nig), *$p<0.05$, **$p<0.01$, ***$p<0.001$ (FO), ****$p<0.0001$ (Nig/FO) *vs* I/R group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.05$ (Nig) and $^{\#}p<0.05$, $^{\#\#}p<0.01$ (FO) *vs* Nig/FO group.

healthy, with uniform morphology and no evidence of pyknosis or inflammation. Quantitative neuron counts using ImageJ supported these observations (Figure 4).

Discussion

Our findings indicated that fish oil, *Nigella sativa* oil, and their combination possess neuroprotective potential in a mouse BCCAO-induced model of transient cerebral ischemia. Using behavioral, biochemical, and histological parameters, we investigated the extent of neuronal damage and the efficacy of each treatment approach. The findings demonstrate that while both fish oil and *Nigella sativa* oil confer protective effects against ischemic injury, the combination therapy exhibits enhanced neuroprotective effects compared to the single treatment alone. The BCCAO model primarily reflects systemic hypoperfusion rather than focal ischemic stroke; the findings should be interpreted within the context of ischemia/reperfusion injury.

The Y-maze, narrow beam walk test, and novel object recognition tests were used to evaluate cognitive and motor functions.[26] Spatial working memory, as well as motor coordination and balance, was significantly impaired in mice from the I/R group, highlighting the harmful effects of cerebral ischemia.[27] Studies showed that Nig treatment was more effective than the untreated group in % spontaneous alternation, total entries in the Y-maze, reducing foot slips, shortening beam-crossing time, and increasing the discrimination index. Although the behavioral effects of fish oil were less pronounced than those observed with Nig, improvement was still evident. Combining fish oil and Nig resulted in the greatest improvement in mouse behavior, suggesting complementary neuroprotective actions.[28] Importantly, cognitive performance was evaluated using normalized scores (% spontaneous alternation and discrimination index) to minimize the impact of locomotor differences.

Oxidative stress contributes significantly to ischemic brain injury, as reflected by increased MDA and reduced SOD and GSH levels.[29] We observed elevated MDA and decreased concentrations of SOD and GSH in the I/R group, indicating oxidative damage following ischemia. Nig extract reduced MDA levels and restored SOD and GSH, demonstrating strong antioxidant activity. The antioxidant effect of fish oil was comparatively moderate. Combined treatment produced the most substantial reduction in oxidative markers and enhancement of antioxidant defenses, supporting an additive protective effect.[30]

TNF-α, a key mediator of post-ischemic inflammation and neuronal injury, was significantly elevated in the I/R group.[31] *Nigella sativa* oil reduced TNF-α levels, reflecting its anti-inflammatory properties. fish oil provided moderate anti-inflammatory benefit, whereas combined therapy achieved the greatest suppression of TNF-α, supporting the concept that simultaneous targeting of oxidative stress and inflammation enhances neuroprotection.[32] Emerging evidence suggests that omega-3 PUFAs exert neuroprotection partly through activation of the Nrf2 antioxidant pathway, enhancing transcription of endogenous antioxidant enzymes such as SOD and glutathione-related enzymes. Additionally, ω-3 PUFAs modulate inflammatory cascades by suppressing NF-κB activation and reducing downstream cytokines, including TNF-α, IL-1β, and IL-6, while promoting anti-inflammatory mediators such as IL-10.[33] *Nigella* sativa oil, particularly its active component thymoquinone, has also been reported to inhibit NF-κB signaling

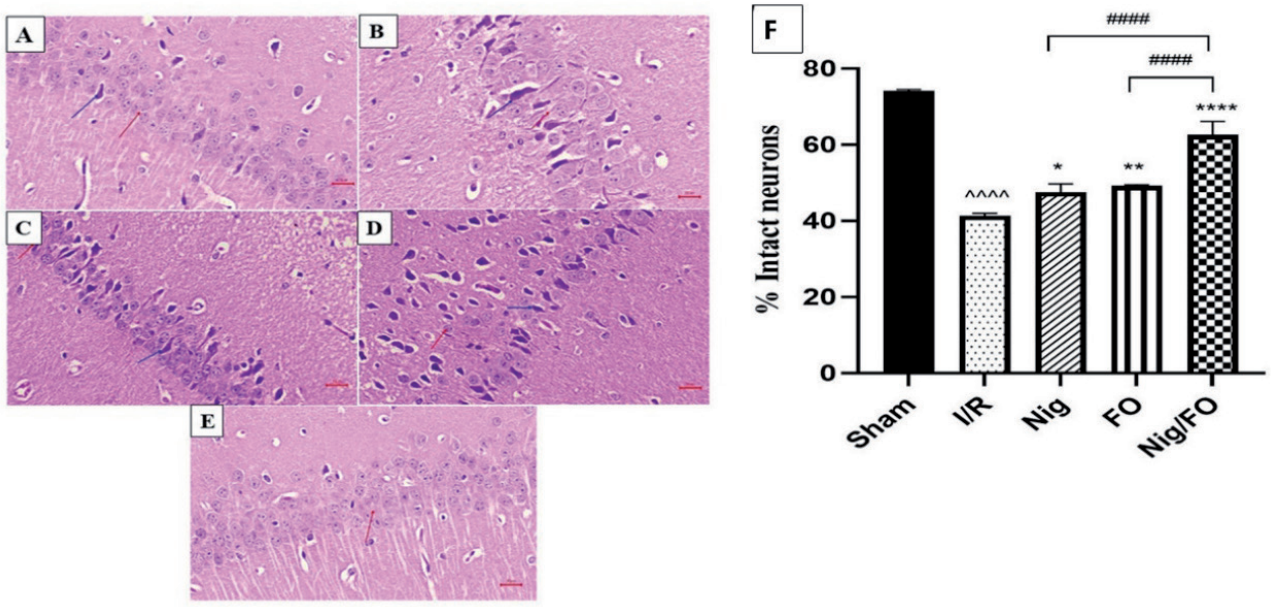

Figure 4. Histopathological analysis of brain tissue by Hematoxylin and Eosin staining. A) Sham, B) Ischemia/reperfusion (I/R) group, C) *Nigella* oil group, D) fish oil group, E) Nig/FO group. F) Quantitative estimation of % intact neurons in the hippocampal CA1 region. All data are expressed as mean ± standard error mean.
For % intact neurons, ^^^^p<0.0001 *vs* sham; *p<0.05, **p<0.01, and ****p<0.0001 *vs* I/R; ####p<0.0001 and ####p<0.0001 *vs* Nig/FO group.

and attenuate oxidative stress via modulation of redox-sensitive pathways. Furthermore, omega-3-derived specialized pro-resolving mediators (SPMs), including resolvins and protectins, may contribute to the resolution of post-ischemic inflammation. The combined administration of fish oil and *Nigella sativa* oil may therefore target complementary molecular pathways involving NF-κB inhibition and Nrf2 activation, contributing to the enhanced neuroprotective effects observed in the present study. However, direct molecular confirmation of these pathways was beyond the scope of this investigation.

The hippocampal architecture remained largely preserved in sham-operated animals, with minimal apoptosis observed.[34] In contrast, the ischemic group demonstrated numerous pyknotic nuclei, particularly in CA2 and CA3 regions. *Nigella sativa* oil improved neuronal survival and reduced structural damage. fish oil provided partial protection, whereas combined treatment showed the greatest preservation of pyramidal neurons with minimal evidence of pyknosis or inflammation, thereby substantiating the hypothesis that the combined treatment produced a more pronounced neuroprotective effect than either intervention alone.[35]

The study has several limitations. Outcomes were assessed only over a short-term period, and long-term neurobehavioral recovery was not evaluated. The study did not evaluate key molecular signaling pathways and inflammatory cytokines, including IL-1β and IL-6. Only male mice were included, limiting generalizability across sexes. Future studies should include long-term evaluation and comprehensive molecular analyses in both sexes to better clarify the underlying mechanisms.

Conclusions

This study demonstrates that oral administration of *Nigella sativa* oil and fish oil, particularly when used in combination, exerts enhanced neuroprotective effects against cerebral ischemia through antioxidant and anti-inflammatory mechanisms. These findings highlight the potential of *Nigella sativa* oil and fish oil as promising natural interventions for the prevention and management of ischemia-induced neurological impairments.

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