Prevention of Brain Hypoperfusion-Induced Neurodegeneration in Rat's Hippocampus by Black Cumin Fixed Oil Treatment

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ABSTRACT

Introduction: The oil extract of black cumin seeds *Nigella sativa* (NSO) demonstrated considerable preservation of spatial cognitive functions in rats subjected to chronic brain hypoperfusion (CBH). The hippocampal CA1 region pyramidal cells are the earliest neurons suffering neurodegeneration following CBH. **Objective:** The current study was devoted to assess the protective effects of *Nigella sativa* (NSO) treatment on CA1 hippocampal pyramidal cells of rats subjected to chronic brain hypoperfusion (CBH) that was achieved through permanent two vessel occlusion (2VO) procedure. **Methods:** Twenty four rats were equally divided into three groups; sham control, untreated 2VO and NSO treated group (2VO with daily oral NSO treatment. After the 10th postoperative week coronal sections of the hippocampus were collected for histopathological and electron microscopical examinations. **Results:** The number of viable pyramidal cells within CA1 hippocampal region in sham control and NSO treated groups was significantly higher than that of untreated 2VO group, while the difference was not significant when comparing the viable pyramidal cells number of sham control with NSO treated groups. Furthermore, 2VO group showed marked intracellular ultrastructural distortions that were less pronounced in NSO treated group. **Conclusion:** NSO displayed a robust potential to protect hippocampal pyramidal cells from CBH induced neurodegeneration putting forward its prospective neuroprotective activity against age related cognitive decline of Alzheimer's disease and vascular dementia.

KEYWORDS: Cerebral hypoperfusion, neurodegeneration, 2VO, *Nigella sativa*, hippocampus, neuroprotection

INTRODUCTION

Neurodegenerative disorders including Alzheimer's disease (AD) and particularly dementia are group of diseases arising from a chronic breakdown and weakening in the neurons of the central nervous system (CNS) and associated with aging and reduced cerebral blood flow (CBF). AD patients are affected by cognitive and memory loss, impaired thinking, progressive deterioration of thinking abilities severe enough to interfere with occupational, intellectual and social functions. It also causes personality changes, inability of self-care and problems with communication and reasoning as well as irreversible cerebral degeneration, with selective neuronal death.[1, 2] These diseases have significant morbidity, mortality and cause medical, social and financial burden to society.[3] AD is the most common progressive, degenerative brain disease, manifests in elderly people and it is associated with destruction of hippocampal and cortical neurons. [4] Severity of neurodegeneration in the later stages of AD is widespread, with massive synapse loss and an overall decline in grey matter resulting from neuronal loss in cortical and hippocampal regions.[5] A similar condition of chronic cerebral hypoperfusion due to reduced CBF is experimentally produced by permanent bilateral occlusion of the common carotid

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arteries (2-vessel occlusion, 2VO) in rats results in a significant reduction of CBF and cause severe histopathological damage in the CA1 region of the hippocampus and related behavioral deficits.[6,7] The chronic neurodegenerative changes taking place in the pyramidal cells of the hippocampus in rats following 2VO intervention were described as closely resembling human age related neurodegenerative changes of sporadic AD.[6]

Herbal medicines and their constituents have been used traditionally for a long time as an alternative method to treat cerebral vascular diseases and dementia-like symptoms.[8,9] The black seed or “Habbatul-barakah” in Arabic (*Nigella sativa*) is a spice, and herbaceous, flowering plant. The black seed and its oil extract (NSO) were traditionally used for centuries in the Mediterranean, South Asia and the Far East as a food flavor and to treat a broad array of diseases against different disease processes.[10] Evidence based studies have asserted that NSO has antioxidant,[11] anti-cancer,[12] and anti-inflammatory with immunomodulatory properties,[13, 14] that were the basis for further *in vivo* neuroprotective testing against inhaled toluene hippocampal intoxication,[11] and in rats suffering from chronic cerebrovascular hypoperfusion.[15]

The ultrastructural changes in 2VO rat brains were mainly directed towards detection of capillary basement membrane (BM) thickening, glial cell activation, astrocytosis as well as the neurodegenerative changes affecting the perikaria and the nuclei of pyramidal cells.[16, 17] These cellular disruptions jointly may result in cognitive decline both in AD patients (Coleman et al., 2004) and 2VO operated rats.[17] Up to the authors' knowledge, there has been no published data concerning the effects of NSO on the morphology of the CA1 area of hippocampus. Thus the aim of the current study was to assess the neuroprotective effects of NSO treatment on rats subjected to mild but chronic cerebral ischemia (oligemia) through 2VO using the conventional light microscopy and TEM. The assessment was confined to CA1 hippocampal neurons.

MATERIALS AND METHODS

Medicinal plant extraction

Ten kg of raw black cumin (*Nigella sativa*) seeds purchased from a standard local herbal supplier in Kuala Lumpur were recognized and authenticated by a Taxonomist in the Faculty of Pharmacy, IIUM. After washing and drying the seeds, they were ground to powder, then dissolved in methanol and the mixture was left overnight under magnetic stirrers and filtered on the next day. The oil extract of *Nigella sativa* (NSO) was separated from the solvent using rotary evaporation apparatus (BUSHI RotaVapor). The pure yield of NSO (114 ml/kg of raw seeds) was stored at -20°C until later used for oral treatment.

Animals

Twenty four male Sprague Dawley rats, weighing 250-300 gm were housed in cages (2 rats per cage) at temperature of 22 ± 1° C and 12 h light/dark cycle. All animals had free access to tap water and food pellets *ad libitum*. The research work was conducted in accordance with the international principles of good laboratory practice as well as the guidelines and recommendations of IIUM ethics committee and Malaysian National Animal Welfare Foundation.

Experimental design

After a week of acclimatization rats were randomly assigned into 3 study groups of equal number (n=8). Sham operated control group; 2VO group and NSO treated 2VO group. The NSO treated 2VO group received NSO orally by gavage every morning, commenced 10 days prior to 2VO intervention and was continued as a daily oral dose of 1ml/kg bodyweight until the end of the 10th postoperative week.

2VO surgery

2VO intervention was performed under complete aseptic conditions. The rats were anaesthetized intraperitoneally with ketamine 90mg/kg and xylazine 20 mg/kg. The common carotid arteries were exposed via a ventral cervical incision, and separated from the carotid sheaths and vagal nerve; both arteries were doubly legated with silk sutures. The rats were left under a heating lamp to prevent hypothermia until full recovery from the general anesthesia.

Euthanasia

Rats were sacrificed at 10th postoperative week. Rats’ brains were quickly dissected on ice cold metal tray. The right hemisphere was fixed in 10% formalin for 72 hours and processed for light microscopy. Sections of 5 mm thickness were stained by Cresyl Violet. The left hemisphere was further dissected to excise the hippocampus that was preserved in McDowell’s and Trump fixative for 24 hours, post-fixed in 1% osmium tetroxide for 2 hours.

They were then dehydrated in acetone and embedded in Spurr’s resin. Semi-thin sections (1 µm) were stained with tulidine blue and studied by light microscopy for localization of the hippocampal CA1 area; followed by ultrathin sectioning (60-70nm), collected on copper mesh grids, stained with 5 % uranyl acetate and lead citrate and examined under Technai G2 12 Biotwin TEM.

Neuronal counts

Neuronal counts were performed on Cresyl violet-stained sections; the number of viable neurons (cells of stratum pyramidal) in CA-1 region of the hippocampus was examined and counted;[15] for calibration purpose, neurons were counted within 1 mm horizontal distance of CA1 hippocampus of all slides.

Statistical and observational analysis

For histopathological examination one way ANOVA with post hoc Tukey's test were used to compare mean values viable hippocampal cells of different study groups. Results were expressed as mean $\pm$ SEM. *P* values <0.05 were considered statistically significant. For TEM examination all sections were thoroughly studied at different magnifications. Images that showed considerable ultrastructural differences from sham control group were captured and analyzed by two experienced histologists who were blind to study groups.

RESULTS

Histopathological observations

Morphologically, normal pyramidal cells with well-defined cell membrane, lightly colored cytoplasm and a well delineated nucleus as in sham control sections (Figure 1A) were regarded and counted as viable. Distorted, shrunken pyramidal cells with irregular boundaries and a dark cytoplasm with indistinct pyknotic nucleus were considered neurodegenerated (non-viable) as in 2VO group (Figure 1B). Normal pyramidal cells dominated NSO treated rat hippocampal sections with clear and well demarcated nucleus (Figure 1C). The mean viable CA1 pyramidal cells number of sham control (233 $\pm$ 4.8) and NSO treated group (214.5 $\pm$ 12.8) proved significantly higher than mean viable CA1 pyramidal cells number of 2VO group (106.5 $\pm$ 17.2) [F(2,21) = 28.78, P<0.001]. However, when comparing the mean values of sham control with that of NSO treated group the difference was insignificant (P=0.56) (Figure 1D).

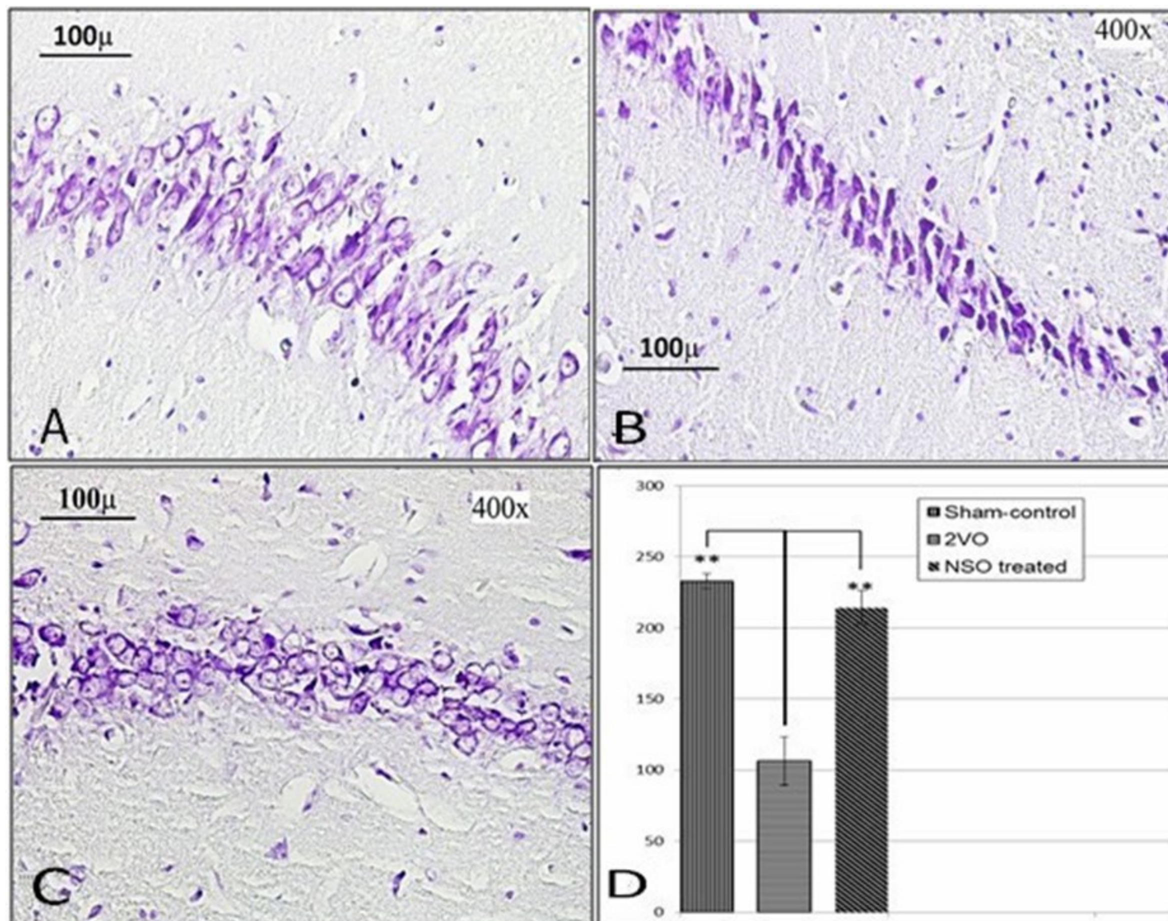

Figure 1. Hippocampal pyramidal cells sections stained with cresyl violet from **(A)** sham control **(B)** untreated 2VO **(C)** NSO treated group **(D)** Bar chart comparison of the mean viable neurons among groups. ***P* < 0.01 vs. untreated 2VO group.

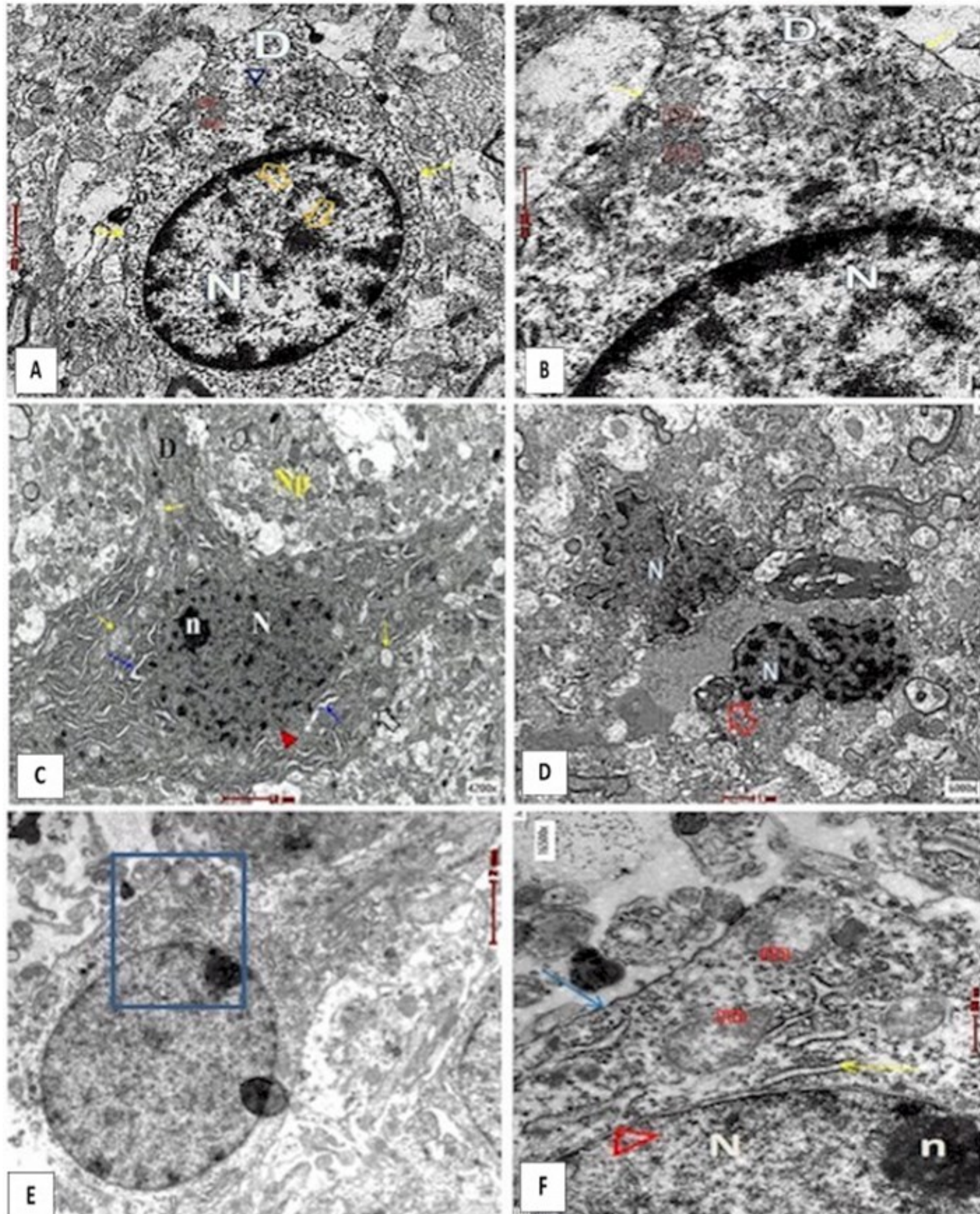

Figure 2. Electron photomicrograph of **(A)** sham control rat showing a dorsal hippocampal CA1 pyramidal neuron (9,900x), **(B)** Abundant intracellular organelles easily recognized on higher magnification (20,500x). N=Nucleus, D= Apical dendrite, m= mitochondria, s= Golgi apparatus, open arrows= chromatin patches, yellow arrows = neuronal cell membrane. **(C)** Large necrotic pyramidal neuron of2VO rat with its apical dendrite "D". (4,200x). Yellow arrows = swollen mitochondria with distorted cristae, blue arrows= ER, and the neuropil (Np) area can be observed in the vicinity. The highly electron dense cytoplasm is distinguishable from nuclear membrane (arrow head). Disintegrated plasma membrane (open black arrow). **(D)** Degenerated neurons with formation of apoptotic body (open red arrow) (6,000x). **(E)** CA1 hippocampal neuron from NSO treated group (4,200x), **(F)** Magnified part from E photomicrograph (16,500x).

Electron microscope observations

Hippocampal neurons in the sham control group showed normal nuclei and intracellular organelles, with smooth outer cell membrane and intact cytoplasm. There were no signs of edema or injury (Figure 2 A and B). Various stages of apoptotic and necrotic neurodegenerative changes were observed in 2VO group. Necrotic alterations existed in the form of increased electron density of the cytoplasm evident from condensation and darkening of the cytoplasm, intracellular dissolution with decreased density of organelles, altered cell volume, dissolution of plasmalemma, swelling of mitochondria with disrupted cristae and fragmentation of Golgi apparatus (Figure 2 C). In other neurons of 2VO group apoptotic degenerative changes were evident. These were manifested by excessive chromatin aggregation and formation of apoptotic bodies. The nucleus assumed a lobular shape and contained condensed chromatin which accumulated at the nuclear envelope and in the central nuclear region. Invaginations and shrinkage of the nuclear membrane signified nuclear lysis (Figure 2 D). On higher magnification (16,500x), the intracellular organelles were not readily observed, most probably indicating intracellular dissolution. Hippocampal neurons of NSO treated group revealed ultrastructural features that were closer to those of sham control than 2VO group, such as the accumulation of polyribosome and mitochondria adjacent to the nuclei, normal cisterns of rough and smooth endoplasmic reticula (ER) and normal un-fragmented Golgi complexes. The chromatin was evenly dispersed throughout the nucleus and along the nuclear envelope which contained a prominent nucleolus usually peripherally located (Figure 2 E and F).

Astrocytes cell bodies of healthy sham control group generally contained round nuclei and relatively clear cytoplasm which contained glycogen particles, normal mitochondria, RER and bundles of intermediate filaments; the heterochromatin distribute uniformly at the nuclear envelope and in the central area (Figure 3 A). The astrocyte processes were normal with no signs of swelling or deformity and contained abundant bundles of fibrils (Figure 3 B). Some 2VO astrocytes demonstrated prominent ultrastructural abnormalities. Astrocyte cell bodies were shrunken and the detection of intact mitochondria was not possible. It was also obvious that the cytoplasm was disrupted and vacuolated. Lobulation and shrinkage of the nuclei with clumped chromatin material were noticed too (Figure 3 C). In other fields swollen astrocytic processes were filled with amorphous material and sparse glycogen granules (Figure 3 D). On the other hand, CA1 hippocampal astrocytes of NSO treated animal showed minimal vacuolization of the cytoplasm. The mitochondria and the astrocytic processes appeared normal. Minimal degenerative changes were observed at the synapses (Figure 3 E and F).

Axons of sham control group appeared normal, contained microtubules and neurofilaments; the myelin sheath had a lamellated structure and was surrounded by oligodendrocytic processes (Figure 4 A and B). Conversely, axons of untreated 2VO group showed moderate disorganization of neurofibrils. Splitting of the lamellae in the myelin sheaths was noticed (Figure 4 C and D). Myelinated axons of NSO treated rats looked morphologically closer to those of sham control group with minimal signs of axonal degeneration. Normal axonal structure and intact fibers with preserved axoplasm were predominantly observed (Figure 4 E and F).

DISCUSSION

The strong prophetic medicinal recommendations for the use of black cumin seeds and the concept of its protective role in almost every disease process have prompted researchers to a more thorough exploration of its therapeutic values. Black cumin seeds have been reported to have potent anticancer and superoxide anion scavenging abilities in animal models and cell culture systems.[18] Robust neuroprotective capacity of NSO and its isolated active ingredient has been asserted on rats subjected to chemical brain insult,[11] as well as in ischemia-reperfusion injury to the brain *in vivo*.[19] It was reported that NSO significantly preserved spatial memory and learning in 2VO operated rats.[15] These evidences, together with the fact that CA1 dorsal hippocampal subfield is the most sensitive area to global cerebral hypoperfusion,[20] prompted us to focus on histopathological and ultrastructural changes, specifically within this region, and consider them as parameters to assess the neuroprotective action that can result from NSO treatment. The 10 days NSO pretreatment strategy ensured adequate enrichment of hippocampal antioxidant stores and protective anti -inflammatory activity prior to cerebral hypoperfusion that was produced by 2VO intervention.

In accordance with the finding of Kim et al,[21] who subjected SD rats to 2VO surgery, the significantly lower number of viable pyramidal cell number observed in our study 10 weeks postoperatively in untreated 2VO group as compared to sham control group indicated significant hippocampal neurodegeneration secondary to critically attained threshold of cerebral hypoperfusion (CATCH). This 2VO induced neurodegeneration was also noticed with variable severities on different time intervals after surgery10 weeks,[7] 40 days,[22] 2 months,[23] and 4 months.[24]

The significantly higher viable pyramidal cell count of NSO treated rats as compared to untreated 2VO group implied a preservative/ neuroprotective effect of NSO treatment precluding CATCH induced neurodegeneration. This notion was further consolidated by the close similarity in numbers of

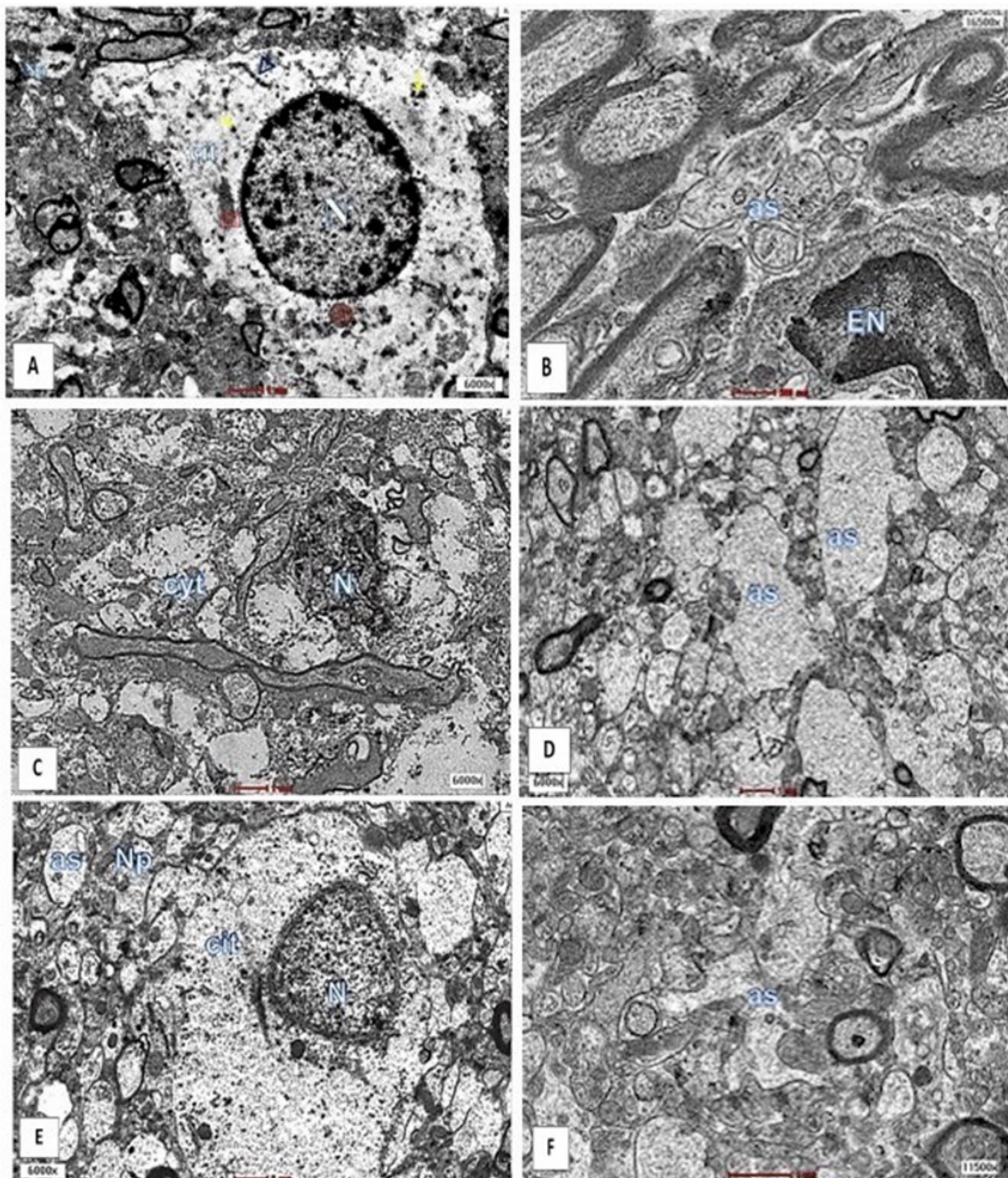

Figure 3. Electron photomicrographs of sham control group **(A)** Astrocyte. Cyt=cytoplasm, N= nucleus, m= mitochondria, glycogen granule (yellow arrows), ER (arrow head) (6,000x, scale bar=1μm), **(B)** Astrocytic processes (as) around endothelial cell (16,500x, scale bar=500nm). **(C)** Degenerating astrocyte of 2VO group; nucleus (N), cytoplasm (cyt) (6,000x), **(D)** hypertrophied astrocyte processes (as) of 2VO group; (11,500x). Scale bar=1μm. **(E)** Astrocytes of NSO treated rat (6,000x), **(F)** Astrocytic processes of NSO treated rat (11, 500x). N= nucleus, cyt= cytoplasm, as= astrocyte processes. Scale bar=1μm.

viable pyramidal neurons of NSO treated group with that of sham control group. These findings are in agreement with previous studies that reported thymoquinone, an active ingredient of NSO, significantly reduced the number of dead pyramidal cells after 7 days of transient global cerebral ischemia.[25] The increase in viable hippocampal neurons has been attributed to the antioxidant defensive properties of NSO which significantly lowered the malondialdehyde, an oxidative stress parameter, in the thymoquinone treated than untreated animal group of transient cerebral ischemia.[25]

The presence of both necrotic and apoptotic changes within the pyramidal cell layer of the hippocampus in untreated 2VO group on ultrastructural examination reflects the complex and combined forms of neurodegenerative mechanisms which eventually lead to extensive CA1 hippocampal pyramidal cell loss. This conjoint picture of neuronal cell death type may also contradict the theory which hypothesized that the type of neurodegeneration is phase dependent, i.e. neurodegeneration within the acute phase following 2VO is necrotic while it is apoptotic in nature within the late phase, based on the principle of intracellular ATP presence/ absence related

neurodegeneration.[26] Furthermore, these findings were in contrast to other studies which deduced that the neurodegeneration in permanent focal cerebral oligemia is exclusively apoptotic.[16] Even if it was a transient model of cerebral ischemia, Zeng and Xu's findings were similar to ours regarding the mixed necrotic/apoptotic mechanisms that lead to 2VO induced pyramidal CA1 hippocampal neurodegeneration.[27] The ultrastructural morphological similarities of NSO treated group to that of sham control group in addition to the relatively lower proportion of pyramidal nuclear, cytoplasmic and plasmalemmal disfigurements than 2VO group suggest that NSO treatment was capable of reducing the harmful oligemic impact of 2VO intervention probably through enrichment of intracellular antioxidant and anti-inflammatory stores that would prevent the initiation and/or progression of the necrotic/ apoptotic neurodegenerative events as a result of CATCH. This NSO dependent protection of the hippocampal neurons was in accordance with earlier reports which affirmed NSO neuroprotection against chemically induced neurodegeneration.[11, 28]

Astrocytes activity in the CNS functioning offers a wide range of new possibilities to unravel physiological and pathological mechanisms.[29] Astrocytes have been implicated as actual mediators of inflammation, as observed in sites of injury, ultimately limiting neuronal repair and remyelination.[30] The degenerative changes found in CA1 hippocampal astrocytes of 2VO group of this study is in agreement with previous observations which supposed that the astrocytic degeneration would play a deleterious role in the progression of other neuronal degeneration through amplifying glutamate excitotoxicity as a result of loss of cell membrane glutamate receptors on degenerated astrocytes.[29, 31] Another harmful effect of astrocytic degeneration in 2VO group can result from diminished interaction among astrocytes, nerve cells and the hippocampal microcirculation which is critical to maintain neural energy metabolism and synaptic plasticity.[32] The relatively lower occurrence of astrocytes degeneration in NSO treated group as compared to untreated 2VO infers another neuroprotective activity of NSO opposing oligemia initiated neurodegeneration. Reactive astrocytosis, demonstrated by astrocyte proliferation, is a late event after 2VO which may not be discerned within the 10th postoperative week.[6]

The absence of significant axonal demyelination among all study groups may indicate that the hippocampal axons, contrast to the optic tract fibers and corpus callosum,[33, 34] are resistant to CATCH induced ultrastructural damage. However, the obvious reduction in microtubular density in axonal cross sections of 2VO group as compared to sham control group may reflect disturbed axoplasmic function. Previous studies have demonstrated a significant reduction in synapse density of the hippocampal CA1 and parietal cortex in the 2VO model,[17, 35] the ultrastructural similarity in neuropil area of CA1 stratum radiatum of sham control and NSO treated groups in our study may be indicative of the conserved integrity of the synaptic density in pre and post-synaptic neuronal junctions within stratum radiatum.

Electron microscopic study of the cingulate cortex from post mortem samples revealed that the capillary basement membrane was a favored site of ultrastructural degradation in AD and Parkinson's disease with dementia.[6] In the present study, thickening of endothelial BM in 2VO group was noticed at higher rate than in sham control group. This is in agreement with other studies which stated that CA1 hippocampal subfield was the most sensitive to CATCH among other hippocampal subfields.[36] The local thickening and distortion of the basement membrane, and extracellular deposits in the vessel walls (that often contained collagen) appeared to be a prominent degenerative feature.[6] The infrequent BM thickening in NSO treated rats may point out an additional stabilizing factor through which neurons maintained their nutritional support of glucose and oxygen without being impeded by the BM pathology that should result from CATCH as in untreated 2VO group.

The expected mechanism of neuroprotection offered by NSO involves free radicals scavenging and antitoxic activities. Moreover, dual anti-inflammatory mechanism of black seed is believed to play a central neuroprotective role through inhibition of both cyclooxygenase (COX) and 5-lipooxygenase (LOX) enzymes which represent key factors for synthesis of inflammatory mediators,[13] whereas some studies emphasized the role of thymoquinone (TQ) as the most active ingredient in NSO,[11, 25] TQ has been reported to suppress NF-κB activation induced by various carcinogens and inflammatory stimuli.[37]

The inhibition of NF-κB activation thereby led to down-regulation of gene products involved in inflammation (cyclooxygenase COX-2), thus abrogating the apoptosis induced by tumor necrosis factors (TNF) and chemotherapeutic agents as well as suppressing TNF-induced cellular attack.[37] Flavonoids which have anti-oxidative activity and free-radical scavenging capacity are present in NSO.[38] They modulate critical neuronal signaling pathways involved in processes of memory and, therefore, are expected to affect synaptic plasticity and long-term potentiation mechanisms.[39] The flavonoid-induced improvements in behavior have been associated with specific changes in protein expression in the hippocampus.[40]

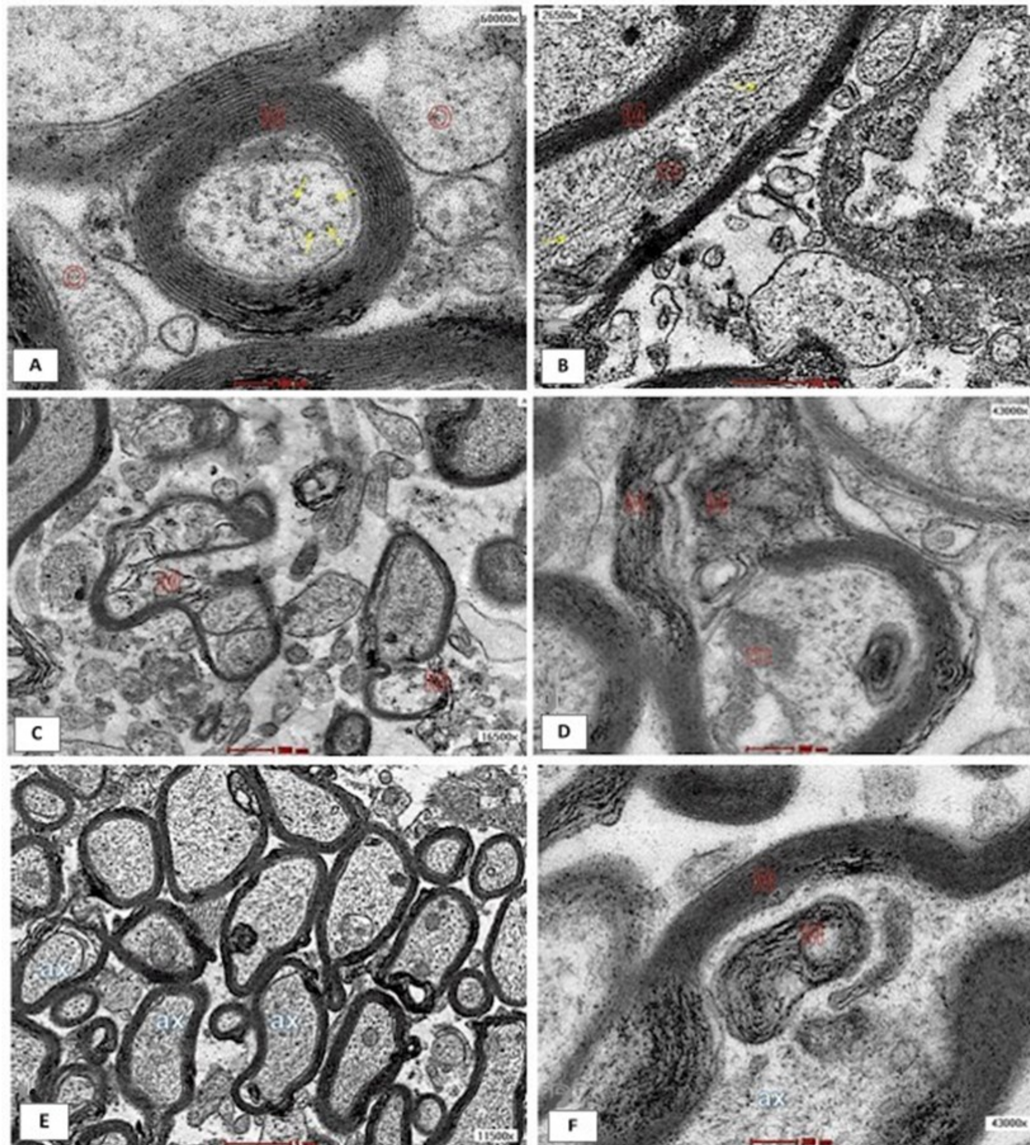

Figure 4. Myelinated axons of sham control group in **(A)** cross sectional view showing intact microtubules (arrows) and the myelin sheath (M) surrounded by oligodendrocytic processes (O); scale bar=100nm, **(B)** longitudinal section depicting microtubules (arrows), intact myelin sheath and mitochondrion (m); scale bar= 500nm. **(C)** Disrupted axonal myelin sheath of 2VO group (16, 500x), **(D)** myelin sheath of 2VO group showing axoplasmic distortions (43,000x). M=Myelin sheath, m= mitochondria. **(E)** Myelinated axons of NSO treated rats showing normal contents. M=myelin sheath, m=mitochondria, ax=axoplasm (11,500x, scale bar=1μm) **(F)** normal axoplasmic organelles of NSO treated rats (43,000x, scale bar=200nm). Notice the similarity of axonal appearance to that of normal control group.

CONCLUSION

It can be concluded that NSO through its fractional ingredients (hexane, ethyl acetate and aqueous) exhibited neuroprotective effects to CA1 hippocampal neurons that are involved in spatial memory and learning faculties of the brain, the fact that makes it highly recommended to investigate these prospective NSO neuro-prophylactic activities on high risk individuals or even in clinical trials on premorbid conditions of AD especially elderly with mild cognitive impairments.

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Conflict of interest statement

The authors declare no potential conflicts of interests.

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