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Neuroprotective effects of *Nigella sativa* extract on cell death in hippocampal neurons following experimental global cerebral ischemia-reperfusion injury in rats

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ABSTRACT

Background: Global cerebral ischemia followed by reperfusion, leads to extensive neuronal damage, particularly the neurons in the hippocampal CA region. Recent studies have demonstrated that pharmacological agents, such as *Nigella sativa* L. (Ranunculaceae) that is an annual herbaceous flowering plant, given at the time of reperfusion afforded protection against ischemia, which is referred to as pharmacological post conditioning.
Objectives: The aim of this study was to evaluate the neuroprotective effects of *Nigella sativa* in the hippocampus neurons of rats exposed to global ischemia/reperfusion.
Methods: In the present study 30 Wister rats (200–250 g) were divided into 5 groups namely sham (operated without treatment), control (operation with normal saline treatment), and 3 treatment groups with *Nigella sativa* 1 mg/kg, 10 mg/kg and 50 mg/kg. Firstly, the animals were anesthetized by ketamin and xylazine, and then the right carotid artery was operated upon dissection of the soft tissues around it and ligation by a clamp for 20 min. The *Nigella sativa* extraction was used during surgery through IP route and after 72 h the animals were euthanized and their brain removed, fixed and prepared for histopathological examinations.
Results: In treatment group (1 mg/kg) the interstitial neuron frequency which contains cytoplasmic edema, along with CA, was 28 cells, whereas the edematous astrocyte number along with CA in this group was 115 cells. In the treatment group (10 mg/kg) the interstitial neurons of cornua ammonis (CA) were 15 and the edematous astrocytes were 122 cells and in the treatment group (50 mg/kg) the number of edematous interstitial neurons was 7 cells in distance of 2900 μ of CA. In such group the number of edematous interstitial neurons was less as well. In this group the appearance of CA cells was more similar to control group, not only the edema decreased in interstitial and astrocyte cells, but it dramatically decreased in pyramidal cells. Our study revealed that the *Nigella sativa* extraction could prevent intracellular edema of interneurons in 50 mg/kg group significantly compared to sham group (91.6%) and the extraction (50 mg/kg) decreased edematous astrocytes 67.1% dramatically compared to sham group. Furthermore there was no significant difference between control and two treatment groups (1 and 10 mg/kg) ($P > 0.05$),
Conclusion: Our finding suggested that the *N. sativa* extraction could prevent the cerebral edema which the best result was obtained in 50 mg/kg group; consequently such extraction is able to prevent ischemia/reperfusion in the hippocampus tissue of the brain.

1. Introduction

Transient, severe global ischemia arising in humans as a consequence of cardiac arrest or cardiac surgery or induced experimentally in animals leads to selective and delayed neuronal death, particularly of pyramidal neurons in the hippocampal [1].

The hippocampus is the central structure of the memory system. Because of its unique vulnerability to ischemic insult and its simple anatomy, this structure is the model used most frequently to study ischemia-related phenomena. However, particular hippocampal subdivisions show different susceptibilities to ischemia/hypoxia. Sector CA1

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is known to be the most sensitive area, and exposure to even short ischemic insult triggers mechanisms of delayed neuronal death which results in marked neuronal loss in this structure [2]. Sectors CA2–3 were reported to be relatively resistant to ischemia. Prolonged ischemia results in reversal of ischemic, changes in sector CA2–3 neurons, but apparent neuronal loss in these sectors has seldom been demonstrated [3].

Global ischemia-induced neuronal death cannot be detected until 2– 4 d after induction of global ischemia in rats and gerbils. The relative contributions of apoptotic and necrotic death to ischemia-induced neuronal loss remain controversial [4–6]. Although the mechanisms underlying ischemia-induced death are as yet unclear, the substantial delay between insult and onset of death provides the opportunity to examine molecular events that destine these neurons to die. Several studies have reported that plant extracts have protective effects against ischemic damage in several organs such as the brain, heart and kidneys [7,8].

Nigella sativa (NS), family Ranunculaceae has been shown to contain >30% of fixed oil and 0.4–0.45% wt/wt of volatile oil. The volatile oil contains 18.4–24% thymoquinone (2-isopropyl-5-methyl-1, 4-benzoquinone) and 46% monoterpenes such as p-cymene [9] and grows spontaneously and widely in several southern Mediterranean and Middle Eastern countries [10]. Recently, clinical and animal studies have shown that the extracts of the black seeds have many therapeutic effects such as anti-inflammatory [11] and immuno-modulatory [12] antioxidative [13] and neuroprotective [14] activities. In addition, it prevents hippocampal neurodegeneration after chronic toluene exposure in rats [15]. Recently, there is an overwhelming attention to plant products and natural agents that can limit free radical-mediated injuries, for better therapeutic management of ischemia-reperfusion injury. The aim of this study was the histopathological study of the neuroprotective effects of *N. sativa* extract on hippocampal neurons after global ischemia/reperfusion.

2. Materials and methods

2.1. Animals

All experimental protocols were approved by the local animal care committee in accordance with the Faculty of Urmia Veterinary Medicine office regulations. In the current study, 30 Wister-albino rats of both sexes, weighing 200–250 g with averagely 3 days old were utilized. The animals were kept in individual propylene cages under standard laboratory conditions by the dimensions of $30 \times 50 \times 25$ cm^3 two by two. Rats were maintained on a 12 h light/dark cycle at 22 ± 1 °C and $50 \pm 10\%$ humidity. The animals were kept in standard room conditions and fed with standard rat diet and water ad libitum. Author version proof: 2047361423103295, Diagnostic Pathology.

2.2. Experimental protocol

The rats were divided into five groups and randomly allotted into one of five experimental groups, each group contains six animals: Group 1 was the sham group in which only surgery was done without induction of ischemia ($n = 6$), group 2 served as the ischemic control, to which saline solution plus 0.8% Tween 80 was injected intra peritoneally (10 ml/kg, IP) ($n = 6$), In groups 3–5, ischemic animals treated with 1, 10 and 50 mg/kg of NS (Ischemia + 1 mg/kg of NS, $n = 6$; Ischemia + 10 mg/kg of NS, $n = 6$; Ischemia + 50 mg/kg of NS $n = 6$), nigella sativa (NS) was administrated intra peritoneally 20 min after induction of ischemia/reperfusion. All drugs were administrated immediately at the onset of reperfusion. Nigella sativa and saline were also given every 24 h for 2 consecutive days (before the day of decapitation). After 72 h, animals were decapitated and the hippocampus was removed for the histopathology examination.

2.3. Chemicals and preparation of extracts

N. sativa seeds were purchased locally, and the identity was confirmed by Pharmacognosy Department, School of Veterinary Medicine, Urmia, Iran (Fig. 9). The seeds were washed, dried, and crushed to a powder. Twenty grams of the powdered seeds was added to 400 ml of distilled water and the extraction was carried out by steam distillation. The yield was equal to 0.4% with reference to dried seeds. The extracted materials were kept in screw-capped tubes in the dark at 20 °C until use. This dose corresponds to normal therapeutic dose administered to adult humans as calculated based on relative surface areas of human and rat [16]. All other chemicals were of the highest analytical grades commercially available.

2.4. Surgical procedure: occlusion of the right common carotid artery (global ischemia model)

The rat focal hippocampus ischemic reperfusion model was performed as previously described [17–19]. Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), which administered IP. Neurological investigation was performed to verify ischemic severity. Briefly, both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea [20] (Fig. 1). Ischemia was achieved by clamping the right carotid artery for 20 min using non-traumatic artery clamps (Microvascular Clamps, Micro Bulldog Clamps, Harvard Apparatus Ltd., Kent, UK). Recirculation of blood flow was established by releasing the clips and restoration of blood flow in the right carotid artery was confirmed by careful observation, and reperfusion was allowed for 72 h. At the end of this time, the rats were killed by decapitation after aortic exsanguination. The right carotid artery was exposed through a midline cervical incision under a dissection microscope and occluded for 20 min [19] (Fig. 1). The sham operated animals were subjected to the same surgical procedures, except that the right common carotid artery was not occluded. At 72 h after ischemia and reperfusion, all rats were sacrificed, and brain tissues were collected. The tissues were kept in a refrigerator at −20 °C for experimental use and histological evaluation, using optical and light microscopes.

2.5. Histological analysis of hippocampal CA1, CA2, CA3, and CA4 regions

3 days after ischemia, and after craniotomy, the whole brain of each animal was removed and immediately immersed in 10% formalin in phosphate-buffered saline (0.1 M; pH 7.4). and the brains were removed from the skull and fixed in the same fixative for 24 h. Multiple serial sagittal sections of each brain (hippocampus) were obtained (Leitz microtome) and routinely processed by embedding in paraffin blocks and 5 μm-thick sections were stained, using the histopathological hematoxylin/eosin method [21,22]. The segments of the hippocampal were CA1, CA2, CA3, and CA4 regions per 2900 μm lengths. The hippocampal damage was determined by counting the number of intact neurons in the stratum pyramidal within the CA1, CA1, CA2, CA3 and CA4 subfield at a magnification of 400× (Nikkon E 600, digital camera DXM1200F, Nikon Corporation, Tokyo, Japan). Only neurons with normal visible nuclei were counted. The mean number of CA1, CA2, CA3 and CA4 neurons per millimeter linear length for both hemispheres in sections of the hippocampus was calculated for each group of animals. An observer who was unaware of the drug treatment for each rat made all the assessments of the histological sections. In conclusion, the results of the present study indicate that NS has inhibitory effects against neuronal edema and supportive neuronal tissue process during cerebral global cerebral ischemia-reperfusion in rat hippocampus.

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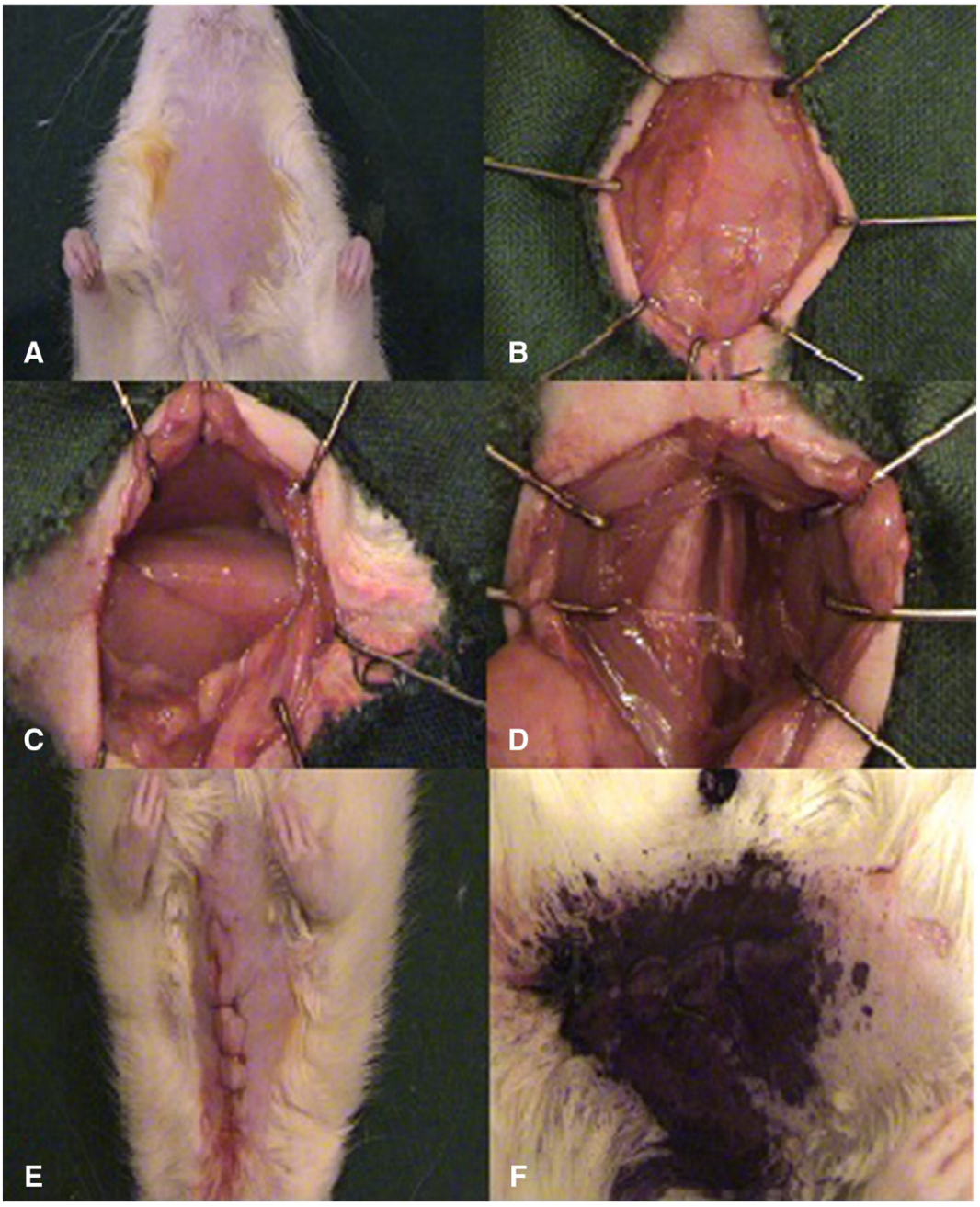

Fig. 1. (A–F): Surgical procedure: Both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea and ischemia was achieved by clamping the right carotid artery.

2.6. Data analysis

The data were expressed as the mean ± standard deviation (SD), and analyzed by repeated measures of variance. For histopathological evaluation, the difference between proportions was assessed by *t*-test and *p* value less than 0.05 was considered statistically significant.

3. Results

In assessment of global ischemia/reperfusion effects on hippocampus cornu ammonis cells the following results were obtained. In the case of sham group, it was revealed that the pyramidal neurons (Astrocytes) had peripheral edema and interstitial neurons has intracellular edema. In addition, the supportive neuronal tissue such as glial cells had cytoplasmic edema and vacuolization (Table 1, Fig. 2).

In the sham group the CA cells were counted by graded lens from CA1 to CA4, somehow the interstitial edematous neurons in such distance (nearly 2000 μ) were 84 cells, including mild to severe edematous cytoplasmic cells which the latter stance showed dark-bright appearance (Table 1, Fig. 3). The glial cells especially astrocytes, are bigger than microglia, had no any nucleolus developed edema and vacuolization. Furthermore, in the sham group the astrocyte numbers, showing the edema were 173. In addition, such cells, internal or external of cornu ammonis cells, counted in a 2000 micron distance (Table 1, Fig. 4).

In the control group, the cornu ammonis cells including pyramidal and interstitial have not shown any edema in cytoplasm and pericellular. Although a few cells of neuronal supportive tissue (24 cells) were observed in CA cells series which had a slight edematous and empty space in their cytoplasm.

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Table 1
The mean frequency of astrocyte and edematous neurons and their decrease percentage compared to the sham group.

Groups	The mean frequency of astrocytes in 2900 distance of CA micron ± SD	Decrease percentage compared to the show group.	The mean frequency of edema neurons in 2900 distance micron ± SD	The decrease percentage compared to the sham group.
1(Control)	24 ± 8.16	–	–	–
2(Sham)	173 ± 10.29	–	84 ± 12.1	–
3(1 mg/kg)	115 ± 25.23	33.5%*	28 ± 8.4	66.6%*
4(10 mg/kg)	122 ± 18.8	29.5%*	15 ± 7.02	82.1%*
5(50 mg/kg)	57 ± 5.8	67.1%*	7 ± 2.9	91.6%*

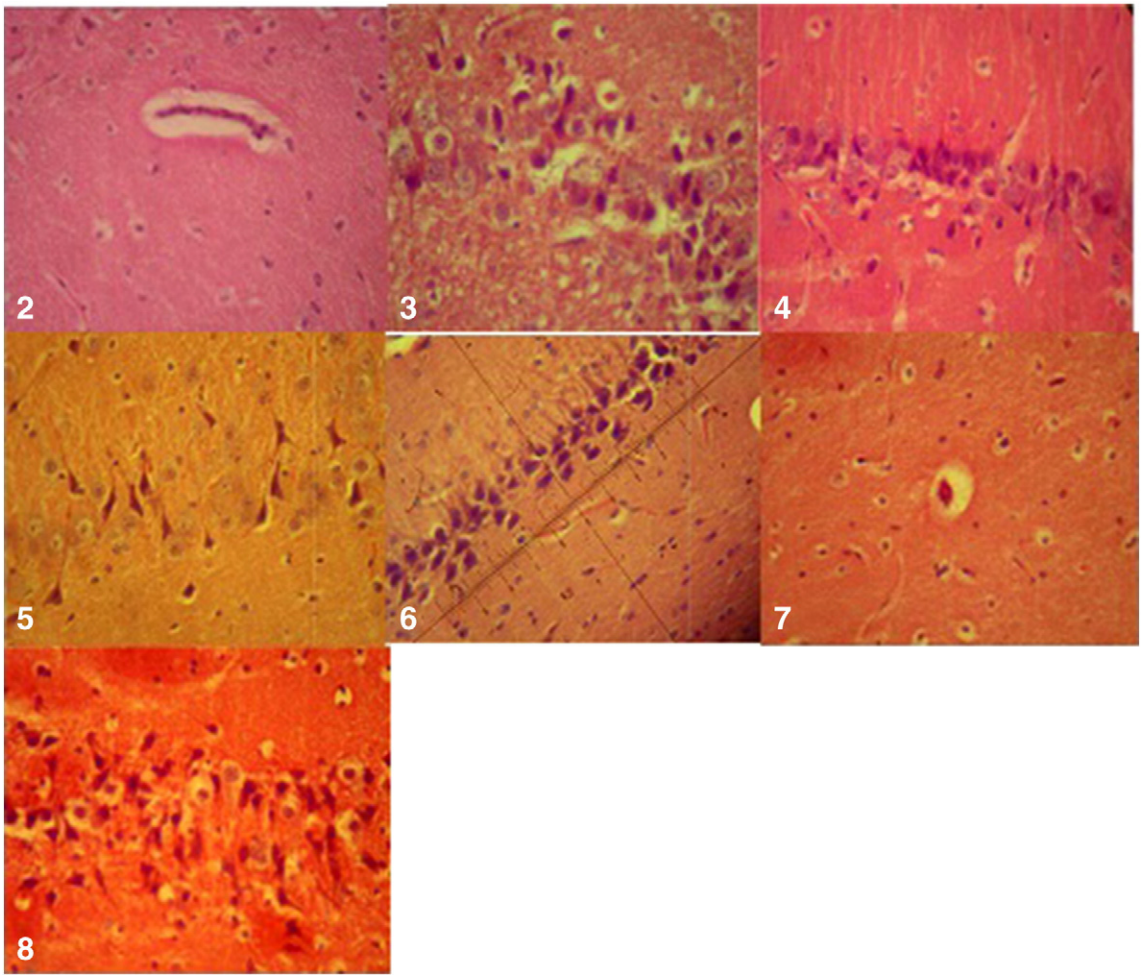
*$p < 0.05$

In the treatment group (1 mg/kg) the interstitial neuron frequency which contains cytoplasmic edema, along with CA, was 28 cells. It is noticeable that the majority of pyramidal neurons in this group had intercellular edematous so the counting was excluded (Table 1, Fig. 5), whereas the edematous astrocyte number along with CA in this group was 115 cells.

In the treatment group (10 mg/kg) the interstitial neurons of cornua ammonis were 15 and the edematous astrocytes were 122 cells (Table 1, Fig. 6).

In the treatment (50 mg/kg) group the number of edematous interstitial neurons was 7 cells in a distance of 2900 μ of cornu ammonis. In such group the number of edematous interstitial neurons was less as well, and the fluid volume and the white space around nucleus were less compared to other groups. On the other hand, a slight edema was observable (Table 1, Fig. 7). All of 57 edematous astrocytes, internal and external of pyramidal, and interstitial cells of CA were distinguished and contained slight edema similar to interstitial neurons. Actually, in this group the appearance of CA cells was more similar to control group, not only the edema decreased in interstitial and astrocyte cells, but dramatically decreased in pyramidal cells (Table 1, Fig. 8).

Table 1 shows that the difference between 1 and 10 mg/kg is not significant ($P > 0.05$), whereas both have remarkable difference with the sham group ($P < 0.05$). The results demonstrated that the extraction was able to prevent edema (10 mg/kg) although regarding astrocyte edema showed no dramatic difference between such two concentrations. Furthermore, Table 1 showed that the *N. sativa* extraction could



Figs. 2–8. Photomicrographs illustrate neurons within the CA region of the hippocampus stained with hematoxylin and eosin at a magnification of 400× after global cerebral ischemia–reperfusion. 2: Indicates that the rat brain glial cells, without nucleolus, have surrounding edema in the sham group (400×). 3: Represents the rat brain intracellular edema of interneurons and surrounding pyramidal cell edema in the treatment group (1 mg/kg) (400×). 4: Indicates that the rat brain edema decreases compared to the previous group in the treatment group (10 mg/kg) (400×). 5: Represents the rat brain having minor intracellular edema of interneurons and surrounding pyramidal cell edema (scale bar 50 μm, 50 mg/kg) (400×). 6: Indicates no edema in interneurons and pyramidal cells in the control group (400×). 7: The reddish proteinoid materials around vessels are observable (100×). 8: Represents the interneuron cytoplasmic edema of rat brain (scale bar 50 μm, 400×).

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Fig. 9. The seeds of the annual **flowering** plant, **Nigella Sativa**.

prevent intracellular edema of interneurons in 50 mg/kg group significantly compared to the sham group (91.6%). Table 1 and figures showed no significant difference between control and two treatment groups ($P > 0.05$), and the extraction (50 mg/kg) decreased edematous astrocytes 67.1% dramatically compared to the sham group.

4. Discussion

In accordance with Brierley and Excel (1996) the global ischemic in human and animal brain results in vigorous external anatomical zone damage such as cortex, cerebellum purkinje, striate bodies and hippocampus. Because hippocampus, a given mass located symmetrically at both sides of the brain, develops damage during ischemia, hence in the present study the ischemia and reperfusion effects of cornu ammonis cells were evaluated. In addition to cellular edema due to endothelial damage, the vasogenic edema will be created as well. This research evaluated the ischemic effects, produced as cytotoxic edema in glial cell, interstitial neurons and around pyramidal neurons. In addition, some reddish proteinoid material inclusion to peripheral space of vessels was visible; somehow the presence of such proteinoid materials indicated the endothelium damage and protein leakage to vessels anent. The most important pathological change in this study was cytotoxic edema in astrocyte cells and around pyramidal hippocampus neurons, pyramidal cells of CA, and other zones of the brain. Keltzo in 1967 claimed that the cytotoxic edema was one of the first brain responses to the lesion which appeared through astrocyte edema and caused extracellular space decrease as well [23]. It is remarkable that in addition to intra glial cell edema and around pyramidal neurons, it was observable in interneurons of CA cells layer unexpectedly.

Norenberg in 1994 named such granular neurons that are similar to dentate gyrus cells as interneurons which play interneuronal relationship role amongst pyramidal neurons [24]. Obviously, the cytoplasm surrounding nucleus contains a large amount of bright fluid, causing cellular enlargement and explicit, whereas the pyramidal cell edema occurs in the surrounding cells where the astrocyte pedicles are present (Fig. 7).Table 1 indicates that in the sham group 84 interneurons with cytoplasmic edema were recorded in 2900 micron distance from the cornu ammonis. In addition, no edematous interneurons were observed in the control group. Thus, such edema exactly originated from ischemic effects on the neurons. The results showed that the surrounding edema of pyramidal neurons was more severe than interneurons edema, inducing peripheral pressure to neurons which prohibited precise counting of edematous and non-edematous neurons. Therefore, the interneurons were evaluated due to limited frequency and obvious features. The edematous interneurons in treatment group (1 mg/kg) decreased to 28 that showed a significant difference ($P < 0.05$) with the sham group. On the other hand, the *N. sativa* extraction could prevent the edema that resulted from ischemic/reperfusion in interneurons (66.6%). In case of astrocytes the edematous neurons of the sham group were decreased averagely from 173 to 115 neurons, and actually prevented the astrocyte edema (33.5%). It is noticeable that the pyramidal neurons surrounding edema are observable with astrocyte pedicles which are around pyramidal neurons. Thus, the *N. sativa* extraction (1 mg/kg) could prevent the edema of pyramidal neurons (33.5%), and in the treatment group (10 mg/kg) the edematous interneurons declined to 17. On the other hand, the extraction could prohibit interneurons edema (82.1%) and astrocyte edema as well (29.5%). Table 1 shows that the difference between 1 and 10 mg/kg is not significant ($P > 0.05$), whereas both have remarkable difference with the sham group ($P < 0.05$). The results demonstrated that the extraction was able to prevent edema (10 mg/kg) although regarding astrocyte edema there was no dramatic difference between such two concentrations. However, entered water volume to astrocyte cells, was so trivial, compared to astrocyte edema volume of previous groups which showed significant difference. Some studies demonstrated that a disruption was produced in neuronal energy due to hypoxia and free radicle release in reperfusion stance, and resulted in Na^+-K^+ ATPase disorder and finally accumulation of Na^+ and water in supportive neuronal cells induces cell swelling [25–27].

Our study suggested that the extraction indicated significant difference regarding interneurons edema prevention and astrocyte swelling prevention with all groups. However, some prevented edema significantly compared to the sham group. Additionally, the *N. sativa* concentration was more efficient than two previous dilutions. In conclusion,

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the *N. sativa* extraction could prohibit edema in neurons and supportive neuronal tissue of hippocampus. Furthermore, our findings might raise a possibility of potential therapeutic application of *N. sativa* extract for preventing and treating cerebral ischemic and neurodegenerative diseases and NS treatment might be beneficial in hippocampus tissue damage, and therefore shows potential for clinical implications.

Conflict of interest

There are no competing interests.

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