Protective effect of treatment with black cumin oil on spatial cognitive functions of rats that suffered global cerebrovascular hypoperfusion

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The fixed oil of black cumin seeds, *Nigella sativa* L. (NSO), has shown considerable antioxidant and anti-inflammatory activities. Chronic cerebral hypoperfusion has been linked to neurodegenerative disorders including Alzheimer's disease (AD) and its subsequent cognitive impairment in which oxidative stress and neuroinflammation are the principal culprits. Cerebrovascular hypoperfusion was experimentally achieved by bilateral common carotid arteries occlusion (2VO) in rats. Morris water maze (MWM) test was employed to assess the effects of NSO on spatial cognitive function before and after 2VO intervention. Rats were divided into long-term memory (LTM) and short-term memory (STM) groups, each was further subdivided into 3 subgroups: sham control, untreated 2VO and NSO treated 2VO group. All subgroups were tested with MWM at the tenth postoperative week. Working memory test results for both sham control and NSO treated groups showed significantly lower escape latency time and total distance travelled than untreated 2VO group. Similarly, LTM and STM MWM tests for sham control and NSO treated groups revealed significantly better maze test performance as compared to untreated 2VO group. Sham control and NSO treated 2VO groups demonstrated superior probe memory test performance as compared to untreated 2VO group. The fixed oil of *Nigella sativa* seeds has demonstrated noticeable spatial cognitive preservation in rats challenged with chronic cerebral hypoperfusion which indicates a promising prospective neuroprotective effect.

Key words: *Nigella sativa*, spatial learning, spatial memory, working memory, Morris water maze, 2VO, neurodegeneration, Alzheimer's disease, rat

INTRODUCTION

The triggering factors of neurodegenerative changes in Alzheimer's disease (AD) are not yet fully understood. Despite dozens of studies within the last two decades relating AD to beta amyloid hypothesis, treatments that can halt neurodegenerative process or revert the deterioration in cognitive performance back to premorbid state, are still distant (de la Torre 2010c). It is now widely accepted that amyloid plaques are not pathognomonic, though characteristic of AD since they were found in brain autopsies of Non-AD patients (Reisberg et al. 2002). Furthermore, the density of amyloid plaques did not correlate with the degree of memory and learning impairment (Giannakopoulos et al. 2003, Aizenstein et al. 2008) and removing the plaques failed to improve the disease symptoms or increased its survival rate (Holmes et al. 2008). These evidences encouraged many scientists to hunt for other explanations as for the onset of age related neurodegenerative changes in AD other than the standard beta amyloid hypothesis which has undergone relative regression (Obrenovich et al. 2006).

The vascular hypothesis for AD, as it tries to interpret the neurodegenerative processes, is starting to gain more acceptances since it relates the strongest non-modifiable risk factor for neurodegenerative diseases, which is aging, with the onset of the disease process and neurodegenerative brain damage (Milionis et al. 2008, de la Torre 2010b). This fact has led some

of the scientists to call for reclassification of AD as primarily a brain vascular disease (Obrenovich et al. 2009) in which chronic reduction in cerebral blood flow results in decreased metabolic supplies of glucose and oxygen to cerebral neurons which in turn would undergo a series of intracellular dysfunctional events ending with neurodegeneration and the eventual cognitive decline (Farkas et al. 2007).

Two vessel occlusion (2VO) rat model of cerebral hypoperfusion has been increasingly used as a paradigm for neurodegenerative disorders with permanent bilateral ligation of common carotid arteries (Farkas et al. 2007) creating a state of oligemia (cerebral hypoperfusion) that on the long run leads to neurodegeneration predominantly to pyramidal hippocampal neurons in charge of spatial (place) learning and memory (Nakazawa et al. 2004). The resultant spatial reference and working memory deficits can best be evaluated through Morris water maze (MWM) test (Morris 1984, Vorhees and Williams 2006). Spatial working memory (WM) denotes instantaneous memory that a person or animal operates during the process of learning new information about the surrounding space (Conrad 2010). Spatial reference memory on the other hand, whether short or long-term, usually refers to the brain activity required by the subject to recall more consolidated positions and places (Cechetti et al. 2010, Conrad 2010). Amongst the earliest signs of AD is the progressive deterioration in spatial memories (Baddeley et al. 1991). Likewise, reports indicated that 2VO operated rats have shown significantly deteriorated MWM performance in both learning and memory at varying test times after 2VO surgery as compared to healthy control rats (Liu et al. 2005).

Seeds of *Nigella sativa* L. (*N. sativa*) have been widely studied due to its strong traditional claims and beliefs of having therapeutic role in almost every disease process (Randhawa 2008). Several therapeutic roles have been asserted thereafter such as antioxidant (Erkan et al. 2008), anti-inflammatory, analgesic, antitumor, antihistaminic, oral hypoglycemic (Kanter et al. 2004), hepatoprotective (Yildiz et al. 2008), nephroprotective (Bayrak et al. 2008), and gastroprotective actions (Al Mofleh et al. 2008). The main active ingredients isolated from *N. sativa* seeds were thymoquinone, alkaloids (nigellidine, nigellimine, and nigellicine), vitamins like thiamine, riboflavin, pyridoxine, niacin, folic acid, minerals and proteins (Salem 2005).

An *in vitro* study stated that pretreatment with NSO has drastically improved neuronal cell viability as compared to untreated cerebellar neurons cell culture before beta-amyloid protein intoxication (Ismail et al. 2008). A different *in vitro* study revealed that the methanolic extract of *N. sativa* modulates the neuronal release of amino acid neurotransmitters including GABA, glycine, aspartate and glutamate on cultured cortical neurons (El-Naggar et al. 2010). Substantial histological improvement was found in the morphology of cerebral cortex and brain stem sections of rats chemically intoxicated with toluene upon using *N. sativa* treatment (Kanter 2008b). Additionally, NSO and thymoquinone have been recognized as neuroprotective agents for hippocampal cells of rats subjected to transient cerebral ischemia *via* four vessel occlusion procedure for 20 minutes, an ischemia-reperfusion model of brain insult (Hosseinzadeh et al. 2007). The present study aimed at assessment of NSO treatment on preservation of spatial reference long-term memory (LTM), short-term (STM) and spatial working memory (WM) of cerebrally hypoperfused rats induced by 2VO surgery. The assessment was undertaken by applying modified protocols of MWM test.

METHODS

Methanolic extraction of NSO

Raw seeds purchased from an established herbal supplier in Kuala Lumpur were recognized and authenticated by a botanist in the Faculty of Pharmacy, IIUM. A voucher specimen was deposited at the Faculty's herbarium. *N. sativa* seeds were ground to powder and later dissolved in methanol. The mixture was stirred overnight under magnetic stirrers and filtered after 24 hours. The solvent was separated from *N. sativa* extract using rotary evaporation apparatus (BUSHI RotaVapor). The extracted oil (NSO) was stored at −20°C until later used for forced oral treatment (Ismail et al. 2008). The yield of NSO was 114 ml/kg of raw *N. sativa* seeds.

Animals

Sixty male Sprague Dawley (SD) rats used in this study were purchased from the Faculty of Veterinary Medicine, University Putra Malaysia. Animals' body weights ranged from 250 to 350 grams at the date of

surgery. Rats had free access to tap water and food pellets *ad libitum*. For a week of acclimatization rats were randomly distributed in pairs per cage with 12/12 hours light/dark cycle. Lights were switched on at 07:00 AM. All procedures complied with the guidelines of the U.S. Public Health Service and NIH regarding the care and use of animals for experimentation as well as the guidelines and recommendations of IIUM ethical committee and Malaysian National Animal Welfare Foundation. Rats were divided into 2 main groups, the first was the long-term memory (LTM) group (*n*=30) which received MWM training prior to 2VO surgery (preoperative training) and was retested 10 weeks post 2VO surgery (Fig. 1A). The second group was the short-term memory (STM) and working memory (WM) tests group (*n*=30) in which rats were naïve to MWM at the time of 2VO surgery and tested *via* MWM only at the 10th postoperative week (Fig. 1B).

Each main group was further subdivided into 3 subgroups according to the type of treatment received: (1) (Sham-C) Sham operated control group (*n*=10) – rats were operated but neither double ligated nor NSO treated; (2) Untreated (2VO) group (*n*=10) – rats were operated with bilateral double ligation of both common carotid arteries but not NSO treated; (3) (NSO) treated group (*n*=10) – rats were operated, double ligated and NSO treated. The oral NSO treatment was started 10 days prior to 2VO surgery and continued with the daily oral dose of 1 ml/kg for further 70 days (10 weeks) post 2VO surgery (Fig. 1). The daily oral dose of NSO was given to the treated group *via* forced feeding using (gavage) cannula.

2VO procedure

Surgery was performed under complete aseptic conditions. Preanesthetic atropine sulfate (0.1 mg/kg) was given intramuscularly to prevent respiratory distress. Combined intraperitoneal general anesthesia was used consisting of ketamine and xylazine at the dose of 90 mg/kg and 10 mg/kg, respectively. A 2 cm ventral midline skin incision was made at the neck area just above the sternal bone and after gentle tweezing of neck muscles the carotid sheath was identified on both sides and the common carotid arteries were carefully separated from the vagus nerve before they were doubly ligated *via* silk suture just below the bifurcation into internal and external carotid arteries (Sivilia et al. 2009) and the arteries were cut between the two ligatures. Rats were left under a heat lamp to prevent hypo-
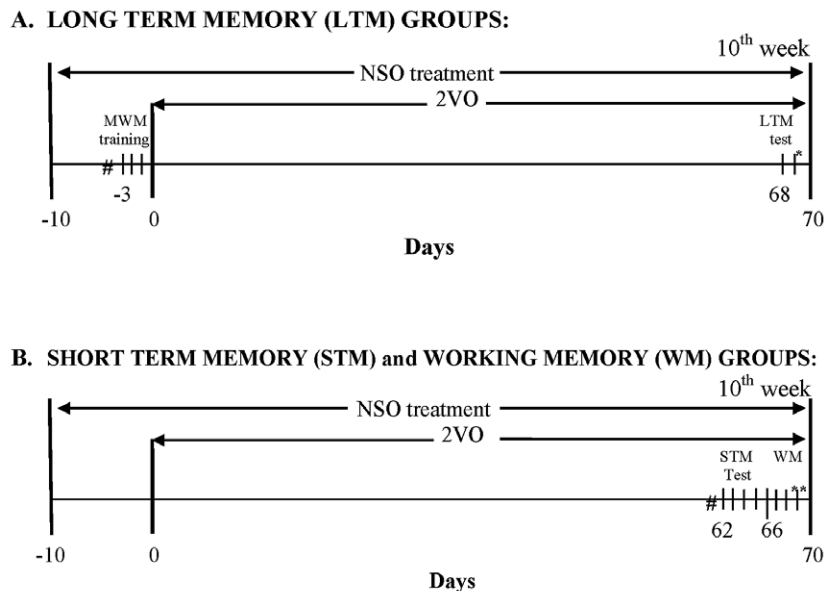

Fig.1. Experimental design of the three study groups. (A) Long-term memory protocol with preoperative acquisition trials started on day (-3) and postoperative test on day (68) followed by 1 day retention probe memory test. (B) Short-term and working memory groups with postoperative MWM test started on day (62) followed by retention probe memory test on day (66). Working memory test started on day (67) for 3 successive days. Day (-10) commencement of oral treatment, day (0) = 2VO operation date and sham operation for sham-C group. # Habituation training, * Probe memory test then cued version MWM test, ** WMT then cued version MWM test.

thermia until full recovery from the general anesthesia. For rats which did not commence water drinking following recovery from anesthesia a single intraperitoneal injection of buprenorphine (0.05 mg/kg) was given to reduce pain from neck muscles separation.

Apparatus

A modified model of MWM was employed in which a circular black fiberglass tank, 2 meter in diameter, was used. The height of the tank was 60 cm and water was filled up to 30 cm. A black 10 cm in diameter cylindrical escape platform (EP) was used as target rescue island for the rats during the test. The water temperature was maintained at $26 \pm 1°C$ throughout the training sessions and test trials, and the water was changed periodically. Colored posters were affixed to the walls surrounding the pool serving as extra-maze visual cues for the rats to orient themselves to the surrounding space and build up their spatial memory. Care was taken to avoid deposition of urine or fecal debris inside the pool to prevent development of olfactory cues. Rats swimming time, distance and speed were tracked, recorded and analyzed using ANY-maze video tracking software (Stoelting Co., USA). All MWM tests were performed

between 09:00 AM and 05:00 PM. The pool had 4 hypothetical poles (N, S, E and W) representing the 4 random starting point of the test for each rat and two hypothetical lines connecting between each two opposing poles dividing the pool into 4 equal imaginary quadrants (SW, SE, NW and NE) (Fig. 2). The order of starting points for the four daily test trials was not repeated on the subsequent test days.

Habituation training was performed on the 5th and 4th days prior to surgery and on the 61st and 62nd postoperative days for LTM and STM groups respectively. Each animal received within two successive days a four trial per day MWM training. Each trial lasted for a maximum of 120 seconds starting from a different starting point, with visible EP (2 cm above water surface) situated at the center of the pool. Animals which did not make their way to the visible EP were gently guided by the experimenter to the EP. Upon reaching the EP animals were left there for 30 seconds before they were helped out to adapt to the water tank and the
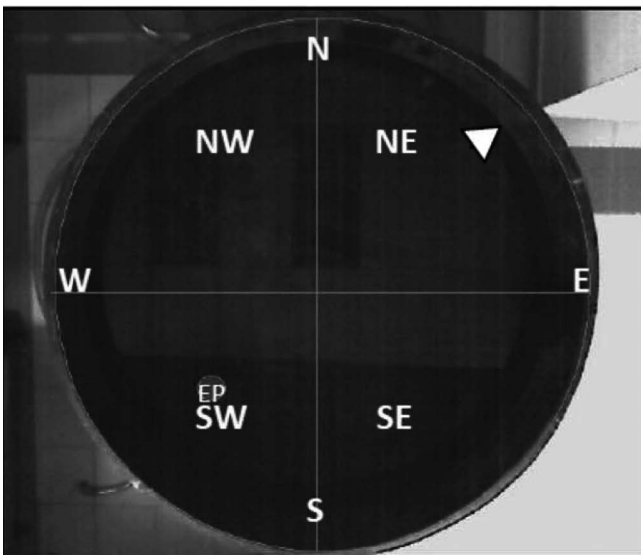

Fig. 2. MWM pool as it looks from above. This figure shows the four hypothetical random starting points for the test (N, S, E and W), the constant location of EP in the SW zone throughout all memory tests (LTM and STM). Probe memory trials started from NE quadrant (arrow head). (EP) escape platform; (NE) north east; (NW) north west; (SE) south east; (SW) south west.
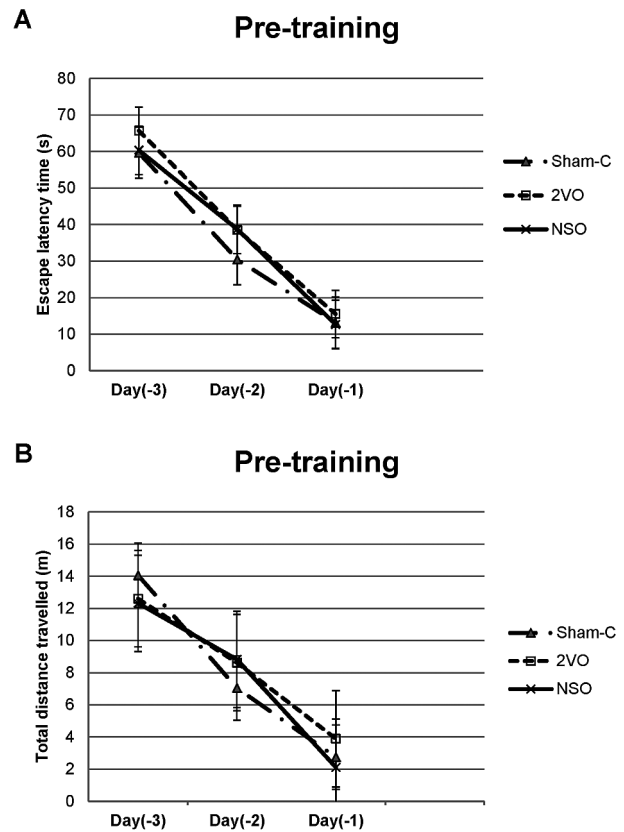

Fig. 3. Preoperative MWM acquisition test demonstrating negligible inter-group differences during the three successive pre-training days in: (A) Escape latency time, (B) Total distance travelled.

surrounding extra-maze visual cues before the actual acquisition test was done.

Acquisition test was done on the 3rd, 2nd and 1st pre-operative days for LTM groups (Fig. 1A) and on the 62nd to 65th postoperative days for STM groups (Fig. 1B). For this phase of the test the position of EP was permanently set in the SW zone of the pool, 33 cm away from the wall of the pool (Fig. 2) and water level was increased up to 2 cm above the EP surface so that it became invisible to the tested rat. Every rat was given 4 trials per day for 3–4 consecutive days; each trial had maximum test duration of 120 seconds with a 4 min inter-trial interval in a resting side cage. Parameters measured were the time required (in seconds) and the distance travelled (in meters) by each

animal until it reached the hidden EP. Maximum test duration of 120 seconds was given to those animals which failed to locate the EP within the test duration.

LTM test was done at the end of the 10th postoperative week namely on the 68th postoperative day for LTM group only (Fig. 1A). LTM test encompassed 4 trials for each animal during one test day only. For each trial the same test duration, parameters and rules of the acquisition test were applied to rats in LTM test.

Retention (probe) memory test was carried out on the 69th post-operative day for LTM groups and on the 66th post-operative day for STM groups. The EP was removed from the pool and each animal was given a 60 second single swimming trial starting from the NE pole (Fig. 2 arrow head). The time spent by the animal
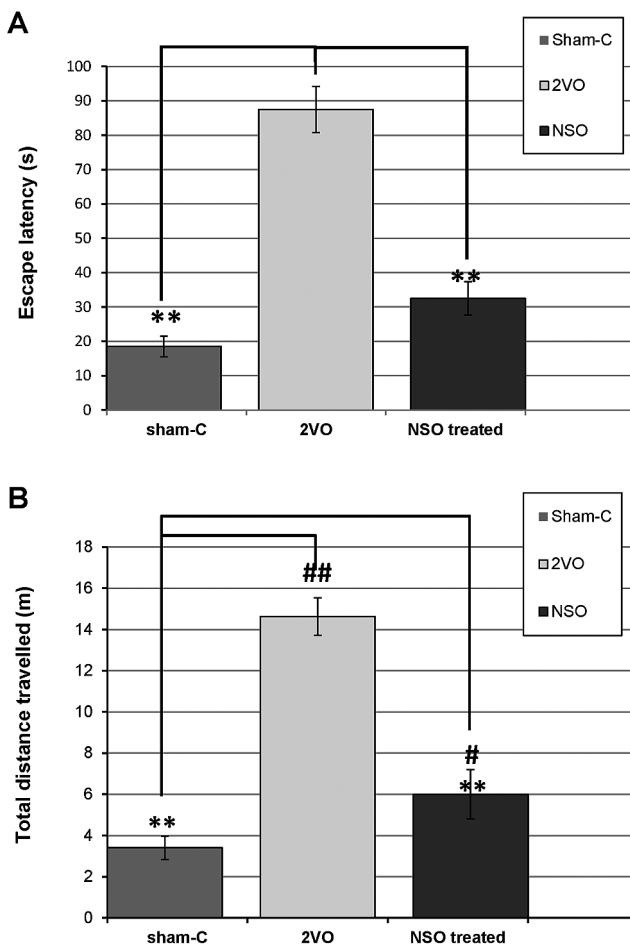

Fig. 4. LTM test on 68th day post-2VO showing differences in: (A) Escape latency time (B) Total distance travelled among sham-C, NSO treated and untreated 2VO group. **$P<0.01$ versus untreated 2VO, #$P<0.05$ versus sham-C, ## $P<0.01$ versus Sham-C group.
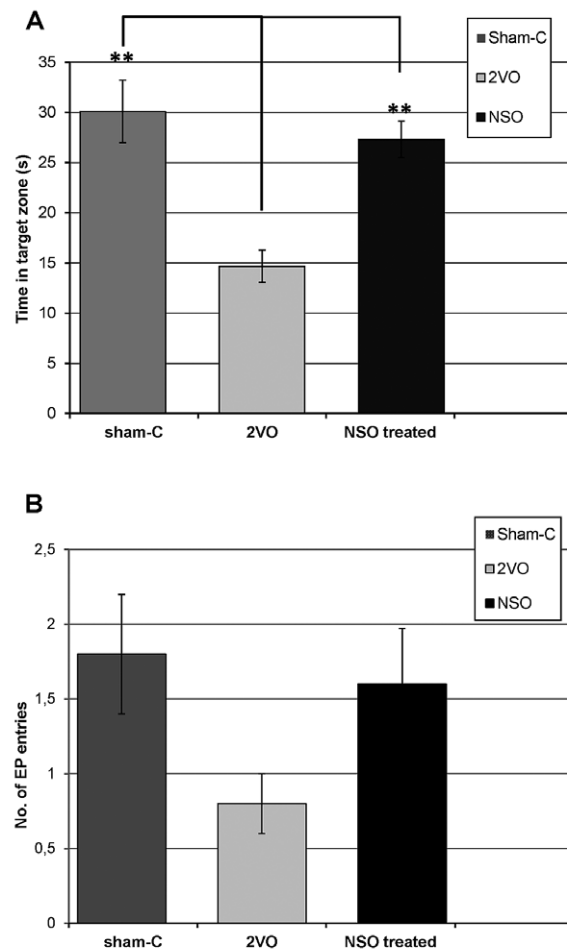
Fig. 5. Retention probe LTM test. This figure shows differences among study groups in: (A) Time spent in the target (SW) zone (B) Number of annulus crossings. **$P<0.01$ versus untreated 2VO group.

in the target zone and number of annulus crossings were recorded.

Working memory test (WMT) was performed on the 67th, 68th and 69th postoperative day for STM groups. In this test the position of EP was changed on daily basis among the NE, SE and NW zones respectively (Fig. 2). Each rat was given 4 trials for each day. Each trial lasted for 120 seconds with a one min inter-trial interval.

Cued version test was performed after the last test trial. A single trial was performed for all rats starting from the SE pole. Here, the platform was raised 1 cm above the water surface, and a square-shaped yellow flag 10-cm height with horizontal dark lines was placed on the platform. Each panel of the flag was 1 cm × 5 cm in dimensions.

Statistical analysis

Two way analysis of variance (ANOVA) with one repeated measure (both factors fixed) was used to ana-

lyze the statistical differences in latencies, total distance travelled (path-lengths) and swimming speeds. *Post-hoc* comparison (Duncan's test) was employed to assess the overall significance of treatment as compared to untreated 2VO and sham control groups. For retention probe memory test data one-way ANOVA was applied. Results were expressed as mean ± SEM. *P* value less than 0.05 was considered statistically significant.

RESULTS

Mortality and blindness rates

In untreated 2VO group, the postoperative mortality rate was 42.11% (22 survived out of 38 operated rats), 2 of the surviving rats suffered from blindness during the first postoperative week (blindness rate = 9%) and were excluded from the MWM test. In NSO treated group, the postoperative mortality rate was 16.7% (20
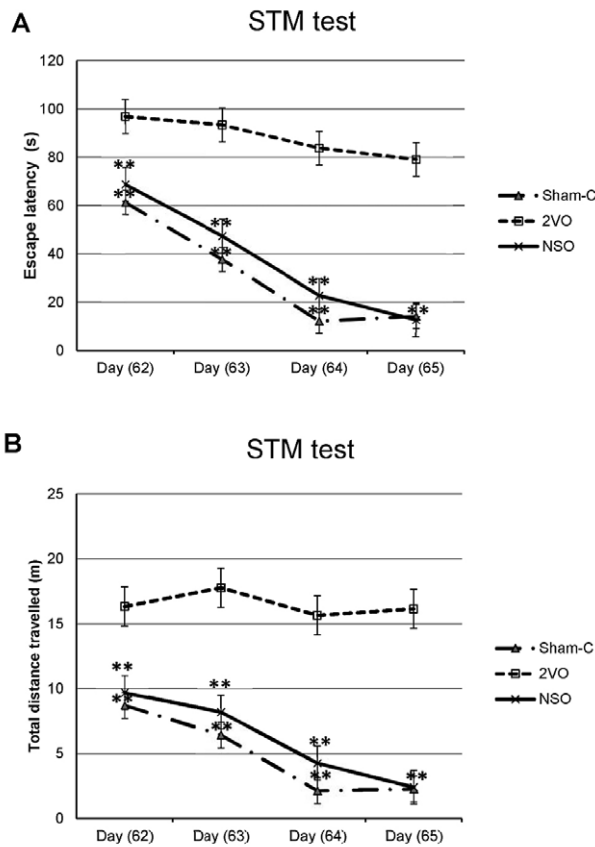

Fig. 6. STM test. This figure shows significant intergroup difference in: (A) Escape latency (B) Total distance travelled. ** *P*<0.01 *versus* untreated 2VO group.
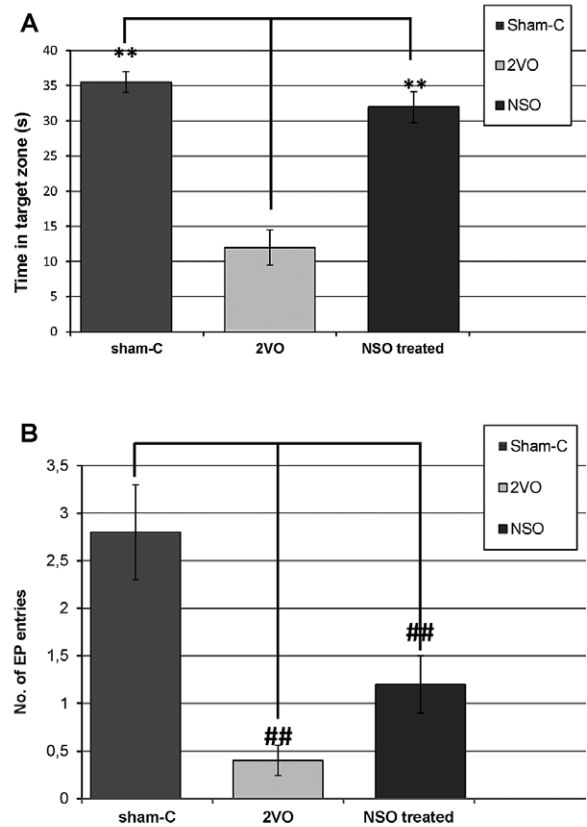

Fig. 7. Retention probe STM test. This figure shows differences among study groups in: (A) Time spent in the target (SW) zone (B) Number of annulus crossings. ***P*<0.01 *versus* untreated 2VO group, [##]*P*<0.01 *versus* sham-C group.

survived out of 24 operated rats) without any postoperative blindness. Sham-C group did not have any postoperative mortality or blind cases.

LTM test

All study groups showed relatively comparable results on each of the pre-training days in the escape latency time ($F_{3,156}=0.56$, $P=0.63$) and the total distance travelled ($F_{3,156}=1.79$, $P=0.15$) till reaching the EP zone (Fig. 3).

Postoperatively, the mean escape latency time for Sham-C group (18.52 ± 3.03 s) and NSO treated group (32.54 ± 4.89 s) were significantly shorter than that of untreated 2VO group (87.57 ± 6.77 s) ($F_{2,27}=47.81$,

A

WM test

B

WM test

Fig. 8 WM test. This figure shows intergroup differences in: (A) Escape latency time. (B) Total distance travelled with daily changing of EP location **$P<0.01$ *versus* untreated 2VO group, ##$P<0.01$ *versus* sham-C group.

$P<0.01$), while the difference between mean escape latencies for Sham-C as compared to NSO treated group was not statistically significant ($P=0.056$) (Fig. 4A). Significant difference in swimming distance was also found when comparing each of sham-C group (3.4101 ± 0.57 m) and NSO treated group (6.0006 ± 0.91 m) with untreated 2VO group (14.6319 ± 1.19 m), ($F_{2,27}=28.43$, $P<0.01$). However, the difference in swimming distance was also significant between Sham-C and NSO treated groups ($P=0.039$) (Fig. 4B).

The mean time spent in the target (SW) zone for the Sham-C and NSO treated groups was 30.09 ± 3.16 s and 27.32 ± 1.79 s, respectively. Both of which proved to be significantly longer than that of untreated 2VO rat group with mean of 14.67 ± 1.58 s ($F_{2,27}=8.89$, $P<0.01$). In contrast, the comparison between mean time spent in the target SW zone for the Sham-C and NSO treated groups did not show significant difference ($P=0.39$) (Fig. 5A). Although the average number of annulus (EP zone) crossings for sham-C and NSO groups was higher than untreated 2VO group, this differences were not statistically significant ($P=0.111$) during probe memory test (Fig. 5B).

STM test

Results from acquisition test of MWM on postoperative day 62–65 revealed a significant statistical difference ($F_{2,27}=35.41$, $P<0.01$) of untreated 2VO group as compared to Sham-C and NSO treated groups in mean escape latency time and total distance travelled (Fig. 6), while there was no significant difference between sham-C and NSO treated groups ($P=0.77$).

The mean time spent in the target zone by 2VO untreated rat group (11.99 ± 2.46 s) was significantly shorter than that of Sham-C (35.50 ± 1.42 s), and NSO treated group (31.97 ± 2.22 s) ($F_{2,27}=37.20$, $P<0.01$). However, the difference was not significant between sham-C and NSO treated groups ($P=0.24$) (Fig. 7A).

For 2VO untreated, Sham-C and NSO treated rat groups the average number of crossing the EP zone (annulus crossing) within 60 seconds of the test were 0.40 ± 0.16, 2.80 ± 0.49, and 1.20 ± 0.29, respectively. Substantial difference was found on comparing Sham-C average with both untreated 2VO group and NSO treated group ($F_{2,27}=12.75$, $P<0.01$). Furthermore, the difference between 2VO and NSO treated group was not statistically significant ($P=0.11$) (Fig. 7B).
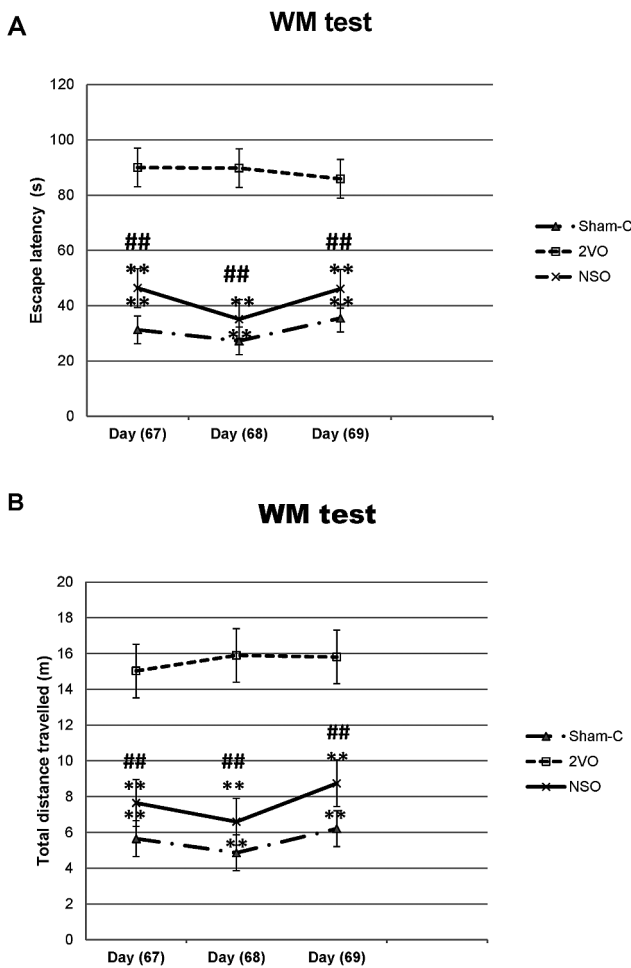
WMT results

Both Sham-C and NSO treated groups showed mean escape latency result for 3 successive test days with highly significant difference from that of untreated 2VO ($F_{2,27}$=44.62, $P<0.01$) (Fig. 8A). The differences in mean latency time was also significant between sham-C and NSO treated groups ($P=0.03$). The mean distance travelled by sham-C and NSO treated groups exhibited significant difference from that of untreated 2VO group ($F_{2,27}$=42.38, $P<0.01$). Additionally, the difference in swimming distance was significant between sham-C group and NSO treated group too ($P<0.05$) (Fig. 8B).

DISCUSSION

The murine model in this study was employed to produce progressive global oligemia to the cerebral neurons resulting in chronic cerebral hypoperfusion which in turn leads to hippocampal neurodegeneration and its subsequent cognitive deficits. The principal objective was to conduct an experimental behavioral test to answer the question of whether NSO can prove beneficial for cognitive learning and memory performance on live animals. Indeed, reports from previous *in vivo* studies indicated that NSO can protect the brain, *via* its antioxidant activities, from oxidative stress resulting from lipid peroxidation in transient global ischemia to the brain (Hosseinzadeh et al. 2007). Moreover, NSO and its active ingredient (thymoquinone) were stated to prevent hippocampal neurodegeneration after chronic toluene inhalation in rats (Kanter 2008a). Nevertheless, no experimental behavioral test, to the best of our knowledge, has yet been performed to cognitively assess the effect of NSO on 2VO operated rats. The oral dose of NSO was selected according to chronic toxicity test performed by Zaoui and colleagues (2002) who asserted that 2ml/kg/day of orally administered NSO caused significant leukopenia and thrombocytopenia after the 8th treatment week. Ten days of NSO treatment prior to 2VO ensured adequate enrichment of antioxidant stores and anti-inflammatory activity to cerebral neurons before oligemic brain insult took place. The concept of pretreatment is particularly crucial if we consider the consequent neurodegeneration is incurable though preventable (de la Torre 2010a).

Blindness as a result of acute ischemic injury to the optic tract within the first postoperative week is a common complication of 2VO (Stevens et al. 2002, Takizawa et al. 2003, Wakita et al. 2003). Blind rats from untreated 2VO group were excluded from MWM test which requires satisfactory visual acuity to adjust to the extra maze visual cues. Since cued version water maze test with visible platform was performed as a single trial test per animal, the development of alternative olfactory cues (fecal material or urine drops if any) would not have any influence.

Preanesthetic atropine was used to prevent respiratory distress during surgery. However, early postoperative mortalities were preceded by generalized tonic-clonic convulsions most probably indicating that lost rats could not escape acute vital centers ischemia from permanent 2VO. The mortality rate was drastically higher in untreated 2VO rat group (42.11%) than other studies with similar procedure (de Wilde et al. 2002, Kim et al. 2008, Choi et al. 2011). Knowing that all animals were lost within the first 48 hours after acute attacks of tonic-clonic convulsions might indicate that these rats could not escape the acute ischemic insult to the vital brain centers. Nonetheless, it has been mentioned by other researchers that even higher fatality rates (Cechetti et al. 2010) can result from strain and vender related differences leading to a wide range of postoperative survival rate even within the same lab or institute (Marosi et al. 2006). The significantly lower mortaity rate in NSO treated group (16.7%) than untreated 2VO group presents an important clue for the possible neuroprotective effect of NSO guarding cerebral neurons from intracellular oxidative stress and neuroinflammation associated with acute ischemia.

The first 2 day habituation training aimed to adapt the animals to the swimming task and to train them to search for the visible EP zone location; therefore no results were calculated for this phase (Nunez 2008), whereas the cued version MWM task on the last day of the test which revealed no significant differences among all groups (results not shown) aimed at ensuring intact sensory-motor function of the tested animals. Analysis of swimming speed measured throughout the test did not show any statistical difference among the three study groups (results not shown) indicating that the significant differences in escape latency time and swimming distance results were not confounded by rats swimming speed (Ahmadiasl et al. 2003).

Not unexpectedly, the preoperative difference among all study groups for both parameters – escape latency and swimming distance – was trivial since all animals

were cognitively healthy at this stage. However, 10 weeks after the operation the untreated 2VO rat group displayed considerable deterioration in LTM test performance in comparison with the sham control group. This worsening in LTM for the untreated 2VO group was confirmed by the retention memory test one day later indicating severe impairment of remote spatial memory. This was consistent with reports from previous studies on 2VO rats (de la Torre and Aliev 2005, Liu et al. 2005, Peng et al. 2007) . Similar findings were declared as early as the 4th postoperative week when the cerebral hypoperfusion is thought to attain a steady critical threshold (Liu et al. 2005, Peng et al. 2007). This decline in MWM performance was affirmed to continue for as late as 1 year after the time of 2VO surgery (Pappas et al. 1996, De Jong et al. 1999). The time period when cerebral hypoperfusion becomes chronic is thought to lie between the 8th and the 12th postoperative week. After that the cerebral blood flow returns to near normal levels; however the MWM performance keeps deteriorating suggesting an irreversible neurodegenerative process (Farkas et al. 2007).

NSO treated group revealed comparable performance to that of the sham control group denoting attenuation of 2VO induced LTM impairment with a clearly significant difference from untreated 2VO group. This improved LTM water maze performance by NSO treated rat group was confirmed by the probe memory test, with removed EP, which is essential to exclude spatial bias, adoption of non-spatial strategies and possibility that rats reached EP by chance (Maei et al. 2009). NSO treated group did not only show significant difference from untreated 2VO group but it also demonstrated highly comparable results to sham control group in target zone swimming time indicating a strong capacity of NSO to preserve retrieval of remote spatial reference memories (Nakazawa et al. 2004). Despite the fact that LTM test swimming distance of NSO treated group was significantly longer than Sham-C group (P=0.039) (Fig. 4B), it is likely that this distance was spent in the target (SW) zone where the EP was located as evident by the probe memory test which revealed comparable time spent in the target zone by both groups, thus minimizing the significance of difference in swimming distance of LTM test.

Substantially improved MWM performance in STM test for NSO treated group noticed from the 1st test day onward points out a similar conclusion to that obtained by LTM results with one major difference. Although the

mean escape latency and swimming distance travelled of NSO treated group were considerably lower than those of untreated 2VO group and despite the fact that the time spent in the target zone by NSO treated group was substantially different from that spent by untreated 2VO during probe STM test, this performance was still lower than that of sham-C group and we believe that the more accurate path efficiency indicator for NSO treated rat group was the average number of crossing the EP (annulus) zone which was trivially different from that of untreated 2VO. What's more, this average number of annulus crossing for NSO treated rat group was significantly lower than that of sham-C group raising the issue of residual deficit in STM with NSO treatment contrast to its action on LTM. One possible explanation to this finding is related to time of first training i.e. in LTM test the first trials were preoperative giving the opportunity to standard memory consolidation which is time dependent (Barondes and Cohen 1968, McGaugh 2000), while in STM MWM test the NSO treated group was naïve to training till the 10th postoperative week when the test was carried out. Another explanation could be due to the long-term effect of NSO on decreasing glutamate activity on NMDA receptors (El-Naggar et al. 2010) which plays a vital role in consolidating memories (Nakazawa et al. 2004).

The fact that daily working memory test results for untreated 2VO was significantly worse than the sham control group performance comes in agreement with what has been previously reported in other studies (de Wilde et al. 2002, Weinstock and Shoham 2004, Cechetti et al. 2010). Even though the MWM performance in WM test for NSO treated group was substantially superior to untreated 2VO group (P<0.01), this performance was significantly (P<0.05) worse than the sham control group, again signifying modestly enhanced learning with NSO treatment. This difference in the efficiency of NSO mediated cognitive enhancement can be due to the difference in mechanisms involved in constructing intact reference and working memories namely the role of adenylate cyclase (AC) enzyme. While AC 1 isoenzyme is thought to play a crucial role for mentaining intact hippocampal impulse transmission for reference memory (Wu et al. 1995), intracellular AC 8 isoenzyme is recently thought to play the most vital role in working memory,i.e.for the acquisition of newly learned spatial information presynapticly (Zhang et al. 2008). Hence it is possible that the neuroprotective effect of NSO was more pro-

nounced at the postsynaptic than the presynaptic neurons creating a significant improvement in the reference memory and only an intermediate enhancement of spatial working memory. However, the question of whether NSO may prove beneficial to treat established conitions of chronic cerebral hypoperfusion was not answered within this study. This objective would require commencing the treatment shortly after induction of chronic cerebral hypoperfusion.

CONCLUSIONS

Since all (hexane, ethyl acetate and aqueous) fractions of NSO were found to markedly augment the viability of cerebellar cell culture intoxicated with beta-amyloid protein (Ismail et al. 2008), it is likely that the vast majority of these fractional ingredients are collectively engaged in mnemonic effect of NSO, the fact that makes it quite reasonable to investigate NSO on humans in clinical trials especially those with mild cognitive impairments. The overall improvement in memory and learning after NSO treatment to 2VO operated animals can be attributed to either of two reasons or both jointly. The first is the ability of NSO to modulate neurotransmitters within the CNS, thereby enhancing the cognitive function (El-Naggar et al. 2010). The second is the neuroprotective action offered by NSO *via* its antioxidant, anti-inflammatory and immunomodulatory activity (Salem 2005). Further studies are prompted so as to elucidate the underlying mechanism(s) of this mnemonic activity of NSO.

ACKNOWLEDGEMENT

REFERENCES

Ahmadiasl N, Alaei H, Hanninen O (2003) Effect of exercise on learning, memory and levels of epinephrine in rats hippocampus. J Sport Sci Med 2: 106–109.

Aizenstein HJ, Nebes RD, Saxton JA, Price JC, Mathis CA, Tsopelas ND, Ziolko SK, James JA, Snitz BE, Houck PR (2008) Frequent amyloid deposition without significant cognitive impairment among the elderly. Arch Neurol 65: 1509–1517.

Al Mofleh IA, Alhaider AA, Mossa JS, Al-Sohaibani MO, Al-Yahya MA, Rafatullah S, Shaik SA (2008) Gastroprotective effect of an aqueous suspension of black cumin Nigella sativa on necrotizing agents-induced gastric injury in experimental animals. Saudi J Gastroenterol 14: 128–134.

Baddeley A, Bressi S, Della Sala S, Logie R, Spinnler H (1991) The decline of working memory in Alzheimer's disease. Brain 114: 2521–2542.

Barondes SH, Cohen HD (1968) Arousal and the conversion of" short-term" to" long-term" memory. Proceedings of the National Acad Sci U S A 61: 923–929.

Bayrak O, Bavbek N, Karatas OF, Bayrak R, Catal F, Cimentepe E, Akbas A, Yildirim E, Unal D, Akcay A (2008) Nigella sativa protects against ischaemia/reperfusion injury in rat kidneys. Nephrol Dial Transplant 23: 2206–2212.

Cechetti F, Worm P, Pereira L, Siqueira I (2010) The modified 2VO ischemia protocol causes cognitive impairment similar to that induced by the standard method, but with a better survival rate. Braz J Med Biol Res 43: 1178–1183.

Choi BR, Lee SR, Han JS, Woo SK, Kim KM, Choi DH, Kwon KJ, Han SH, Shin CY, Lee J, Chung CS, Kim HY (2011) Synergistic memory impairment through the interaction of chronic cerebral hypoperfusion and amlyloid toxicity in a rat model. Stroke 42: 2595–2604.

Conrad CD (2010) A critical review of chronic stress effects on spatial learning and memory. Prog Neuro-Psychopharmacol Biol Psychiatry 34: 742–755.

De Jong GI, Farkas E, Stienstra CM, Plass JR, Keijser JN, de la Torre JC, Luiten PG (1999) Cerebral hypoperfusion yields capillary damage in the hippocampal CA1 area that correlates with spatial memory impairment. Neuroscience 91: 203–210.

de la Torre JC (2010a) Alzheimer's disease is incurable but preventable. J Alzheimer's Dis 20: 861–870.

de la Torre JC (2010b) The vascular hypothesis of Alzheimer's disease: bench to bedside and beyond. Neuro-Deg Dis 7: 116–121.

de la Torre JC (2010c) Vascular risk factor detection and control may prevent Alzheimer's disease. Ageing Res Rev 9: 218–225.

de la Torre JC, Aliev G (2005) Inhibition of vascular nitric oxide after rat chronic brain hypoperfusion: spatial memory and immunocytochemical changes. J Cereb Blood Flow Metab 25: 663–672.

de Wilde MC, Farkas E, Gerrits M, Kiliaan AJ, Luiten PGM (2002) The effect of n-3 polyunsaturated fatty acid-rich diets on cognitive and cerebrovascular parameters in chronic cerebral hypoperfusion. Brain Res 947: 166–173.

El-Naggar T, Gomez-Serranillos MP, Palomino OM, Arce C, Carretero ME (2010) Nigella sativa L. seed extract modulates the neurotransmitter amino acids release in cultured neurons in vitro. J Biomed Biotechnol 2010: 398312.

Erkan N, Ayranci G, Ayranci E (2008) Antioxidant activities of rosemary (Rosmarinus Officinalis L.) extract, black-seed (Nigella sativa L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chem 110: 76–82.

Farkas E, Luiten PGM, Bari F (2007) Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. Brain Res Rev 54: 162–180.

Giannakopoulos P, Herrmann F, Bussiere T, Bouras C, Kövari E, Perl D, Morrison J, Gold G, Hof P (2003) Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. Neurology 60: 1495–1500.

Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JW (2008) Long-term effects of A [beta] 42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 372: 216–223.

Hosseinzadeh H, Parvardeh S, Asl MN, Sadeghnia HR, Ziaee T (2007) Effect of thymoquinone and Nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. Phytomedicine 14: 621–627.

Ismail N, Ismail M, Latiff LA, Mazlan M, Mariod AA (2008) Black Cumin Seed (Nigella Sativa Linn.) Oil and Its Fractions Protect Against Beta Amyloid Peptide Induced Toxicity in Primary Cerebellar Granule Neurons. J Food Lipids 15: 519–533.

Kanter M, Coskun O, Korkmaz A, Oter S (2004) Effects of Nigella sativa on oxidative stress and cell damage in streptozotocin induced diabetic rats. The Anat Rec A Discov Mol Cell Evol Biol 279: 685–691.

Kanter M (2008a) Nigella sativa and derived thymoquinone prevents hippocampal neurodegeneration after chronic toluene exposure in rats. Neurochem Res 33: 579–588.

Kanter M (2008b) Protective effects of Nigella sativa on the neuronal injury in frontal cortex and brain stem after chronic toluene exposure. Neurochem Res 33: 2241–2249.

Kim SK, Cho KO, Kim SY (2008) White matter damage and hippocampal neurodegeneration induced by permanent bilateral occlusion of common carotid artery in the rat: comparison between wistar and sprague-dawley strain. Korean J Physiol Pharmacol 12: 89–94.

Liu HX, Zhang JJ, Zheng P, Zhang Y (2005) Altered expression of MAP-2, GAP-43, and synaptophysin in the hippocampus of rats with chronic cerebral hypoperfusion correlates with cognitive impairment. Brain Res Mol Brain Res 139: 169–177.

Maei HR, Zaslavsky K, Teixeira CM, Frankland PW (2009) What is the most sensitive measure of water maze probe test performance? Front Integr Neurosci 3: 33.

Marosi M, Rákos G, Robotka H, Németh H, Sas K (2006) Hippocampal (CA1) activities in Wistar rats from different vendors:: Fundamental differences in acute ischemia. J Neurosci Methods 156: 231–235.

McGaugh JL (2000) Memory – a century of consolidation. Science 287: 248.

Milionis HJ, Florentin M, Giannopoulos S (2008) Metabolic syndrome and Alzheimer's disease: a link to a vascular hypothesis. CNS Spectr 13: 606–613.

Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11: 47–60.

Nakazawa K, McHugh TJ, Wilson MA, Tonegawa S (2004) NMDA receptors, place cells and hippocampal spatial memory. Nat Rev Neurosci 5: 361–372.

Nunez J (2008) Morris water maze experiment. J Vis Exp 19: 897. (doi: 10.3791/897)

Obrenovich ME, Smith MA, Siedlak SL, Chen SG, De La Torre JC, Perry G, Aliev G (2006) Overexpression of GRK2 in Alzheimer disease and in a chronic hypoperfusion rat model is an early marker of brain mitochondrial lesions. Neurotox Res 10: 43–56.

Obrenovich ME, Palacios HH, Gasimov E, Leszek J, Aliev G (2009) The GRK2 overexpression is a primary hallmark of mitochondrial lesions during early Alzheimer disease. Cardiovasc Psychiatry Neurol 2009: 327360. (doi: 10.1155/2009/327360)

Pappas B, De La Torre J, Davidson C, Keyes M, Fortin T (1996) Chronic reduction of cerebral blood flow in the adult rat: late-emerging CA1 cell loss and memory dysfunction. Brain Res 708: 50–58.

Peng Y, Xu S, Chen G, Wang L, Feng Y, Wang X (2007) l-3-n-Butylphthalide improves cognitive impairment induced by chronic cerebral hypoperfusion in rats. J Pharmacol Exp Ther 321: 902–910.

Randhawa MA (2008) Black seed, Nigella sativa, deserves more attention. J Ayub Med Coll Abbottabad 20: 1–2.

Reisberg B, Franssen E, Shah MA, Weigel J, Bobinski M, Wisniewski HM (2002) Clinical diagnosis of dementia. In: Dementia (2nd ed.) (Maj M, Sartorius N, Eds). John Wiley, Chichester, UK, p. 69–74.

Salem ML (2005) Immunomodulatory and therapeutic properties of the Nigella sativa L. seed. International Immunopharmacol 5: 1749–1770.

Sivilia S, Giuliani A, Fernández M, Turba M, Forni M, Massella A, De Sordi N, Giardino L, Calzà L (2009) Intravitreal NGF administration counteracts retina degeneration after permanent carotid artery occlusion in rat. BMC Neurosci 10: 52.

Stevens WD, Fortin T, Pappas BA (2002) Retinal and optic nerve degeneration after chronic carotid ligation. Stroke 33: 1107–1112.

Takizawa S, Fukuyama N, Hirabayashi H, Kohara S, Kazahari S, Shinohara Y, Nakazawa H (2003) Quercetin, a natural flavonoid, attenuates vacuolar formation in the optic tract in rat chronic cerebral hypoperfusion model. Brain Res 980: 156–160.

Vorhees CV, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Prot 1: 848–858.

Wakita H, Tomimoto H, Akiguchi I, Lin JX, Ihara M, Ohtani R, Shibata M (2003) Ibudilast, a phosphodiesterase inhibitor, protects against white matter damage under chronic cerebral hypoperfusion in the rat. Brain Res 992: 53–59.

Weinstock M, Shoham S (2004) Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. J Neural Transm 111: 347–366.

Wu ZL, Thomas SA, Villacres EC, Xia Z, Simmons ML, Chavkin C, Palmiter RD, Storm DR (1995) Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. Proc Natl Acad Sci U S A 92: 220–224.

Yildiz F, Coban S, Terzi A, Ates M, Aksoy N, Cakir H, Ocak AR, Bitiren M (2008) Nigella sativa relieves the deleterious effects of ischemia reperfusion injury on liver. World J Gastroenterol 14: 5204–5209.

Zaoui A, Cherrah Y, Mahassini N, Alaoui K, Amarouch H, Hassar M (2002) Acute and chronic toxicity of Nigella sativa sativa fixed oil. Phytomedicine 9: 69–74.

Zhang M, Moon C, Chan GCK, Yang L, Zheng F, Conti AC, Muglia L, Muglia LJ, Storm DR, Wang H (2008) Ca-stimulated type 8 adenylyl cyclase is required for rapid acquisition of novel spatial information and for working/episodic-like memory. J Neurosci 28: 4736–4744.