Zagazig J. Pharm. Sci. Dec., 2013
Vol. 22, Issue. 2, pp, 39-49

ISSN 1110-5089

Docosahexanoic acid (DHA) reduces brain damage induced by reversible middle cerebral artery occlusion (MCAO) in mice.

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ABSTRACT

Stroke is a leading cause of death and permanent disability in adults worldwide. The only FDA-approved treatment for acute ischemic stroke is the intravenous recombinant tissue plasminogen activator (rt-PA) within 3 hours of onset.

Among the mechanisms involved in stroke is the activation of phospholipases with subsequent release of arachidonic acid (AA) and docosahexanoic acid (DHA). Arachidonic acid (AA) yields eicosanoids implicated in the induction and maintenance of the acute inflammatory responses while docosahexanoic acid (DHA) is substrate for the biosynthesis of resolvins and protectins that have anti-inflammatory activity.

The effect of delayed administration of IV DHA (30 mg/kg) was investigated against brain damage induced by reversible middle cerebral artery occlusion (MCAO) in mice. DHA demonstrated a neuroprotective effect against brain damage induced by reversible MCAO as evidenced by the reduction in the infarct area, neurological dysfunction and NF-kB activity.

These results suggest the possible use of DHA against brain damage following stroke as late as 5 hours of onset

Key words: docosahexanoic acid, MCAO, reversible, mice.

INTRODUCTION

Stroke is the third leading cause of death and the most frequent cause of permanent disability worldwide (Lo *et al.*, 2003). Stroke generally refers to a local interruption of blood flow to the brain due to blockage of a cerebral artery. Approximately 12% of strokes are hemorrhagic (rupture of a cerebral blood vessel; 9% intracranial, 3% subarachnoid), whereas the remaining 88% are ischemic and result from occlusion of a cerebral artery (either thrombolic or embolic) (Adibhatla *et al.*, 2008).

Within seconds to minutes after the loss of blood flow to a region of the brain, the energy failure rabidly initiates the ischemic cascade which comprises a series of subsequent biochemical events that eventually lead to disintegration of cell membranes and neuronal death at the center/core of the infarction (Dirnagl *et al.*, 1999).

Focal ischemia is characterized by an ischemic core surrounded by a "penumbra" region which is hypoperfused tissue surrounding the ischemic core. The penumbra area is subjected to a wave of deleterious metabolic processes propagated from the core to the neighboring tissue, including excitotoxicity, spreading depression, oxidative stress, and inflammatory response (Ramos-Cabrer *et al.*, 2011). The ischemic core is generally considered unsalvageable, whereas the penumbra may be rescued by timely intervention and poses a target for the development of therapeutic treatment (Adibhatla *et al.*, 2008). Penumbra can be protected either by reflow, or by administration of a neuroprotectant. Without such intervention the cells in the penumbra

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will also die and the core will expand (Green and Shuaib, 2006).

The only FDA approved treatment for ischemic strokes is tissue plasminogen activator (tPA) which works by dissolving the clot and improving blood flow to the part of the brain being deprived of blood flow provided that it was given within a period not exceeding 3 hours after ischemia onset (Roth, 2011). However, a significant number of stroke victims don't get to the hospital in time for tPA treatment (Khosravi *et al.*, 2013).

Three phases can be characterized in infarct progression; the acute phase is characterized by increased intracellular calcium concentrations and stimulation of glutamate release, causing excitotoxicity and a spreading depression throughout the ischemic region (Barone and Feuerstein, 1999; Chu et al., 2006; Dirnagl et al., 1999), vasogenic edema (Barone and Feuerstein, 1999) and generation of reactive oxygen species (ROS) especially if reperfusion takes place (Barone and Feuerstein, 1999). In the second, subacute phase, an apoptotic and neuroinflammatory response develops as a result of the stimulatory influences of the acute phase (Barone and Feuerstein, 1999; Dirnagl et al., 1999; Hossmann, 2006). Finally, in the chronic phase, repair and regeneration takes place which will determine the ultimate extent of damage (Chu et al., 2006).

Excess glutamate release and stimulation of its receptors in addition to Ca+2 overload results in activation of phospholipases (Adibhatla et al., 2006; Lipton, 1999) leading to the release of rachidonic acid (AA) by cytosolic phospholipase A2 (cPLA2) (Balsinde et al., 2002; Capper and Marshall, 2001; Kudo and Murakami, 2002) and docosahexanoic acid (DHA) (Bazan, 2003; 2009). The enzymatic peroxidation of AA produces eicosanoids which play important roles in regulating signal transduction, gene transcription processes and in inducing and maintaining the acute inflammatory responses (Phillis *et al.*, 2006). Series of compounds derived from docosahexaenoic acid (DHA) have been discovered in resolving murine inflammatory exudates (Serhan *et al.*, 2008; Serhan et al., 2000; Serhan *et al.*, 2002). These naturally occurring bioactive lipid mediators are termed resolvins (Rvs) (derived from 'resolution phase interaction products') and protectins (PDs) (Hong *et al.*, 2003; Serhan and Chiang, 2008; Serhan *et al.*, 2006).

Inflammation plays an important role in the pathogenesis of ischemic stroke and other forms of ischemic brain injury (Jin *et al.*, 2013). Inflammation exacerbates ischemic injury, but also provides the necessary environment for regeneration and repair (Kerschensteiner *et al.*, 1999). Resolution of inflammation is accompanied by an active switch in the mediators that predominate in exudates from prostaglandins and leukotrienes of arachidonic acid to resolvins and protectins from docosahexanoic acid (Hong *et al.*, 2003; Serhan *et al.*, 2000; Serhan *et al.*, 2002).

The current study is designed to investigate the possible activity of docosahexaenoic acid (DHA) against brain damage induced by reversible middle cerebral artery occlusion (MCAO).

MATERIALS and METHODS

Adult male C57BL/6 mice aged 8 - 12 weeks weighing 19-30 gm were purchased from the Theodore Bilharz Research Institute, Cairo, Egypt. All experimental procedures were approved by the Ethical Committee for

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Animal Handling at Zagazig University (ECAHZU).

In the current study, reversible middle cerebral artery (MCA) occlusion was induced as described previously (Engel et al., 2011). Briefly, animals were anesthetized with intraperitoneal injection of 250 mg/kg avertin (Aldrich, Germany) then a midline neck incision was made to expose the common carotid artery (CCA). A small cut was made at the base of CCA and a standard silicon rubber-coated 6.0 nylon monofilament (Doccol Corp., USA) was inserted into the CCA and propagated upwards into the internal carotid artery (ICA) for a distance of 10 mm from the bifurcation to occlude MCA at its origin for 1 hr followed by reperfusion for 24 h. Animal body temperature was adjusted to 37 oC ± 1 0C during and after the operation until they regained consciousness.

DHA (Sigma, Germany) was administered through the tail vein 5 hrs after ischemia at a dose of 30 mg/kg. Twenty four hours after MCAO, animals were deeply anesthetized, perfused with 50 ml Ringer's solution through the aorta and brains were isolated and stored at – 80 oC.

The influence of ischemia on behavior of the animals was assessed by determination of any signs of neurological dysfunction using a 3 point scoring system: 0-normal neurological function, 1- moving in a circular mode (to the right), 2- inability to walk spontaneously and performance in the footprint test (Barlow *et al.*, 1996; Paylor and Crawley, 1997; Jaworski et al., 2006).

Twenty µm cryosections were prepared using cryostat (SLEE, MEV, Mainz, Germany) and were used to detect infarct size using silver staining (Vogel *et al.*, 1999) and NF-kB activity.

Infarct area calculated according to the following equation: Infarct area (%) = [(right hemisphere area – left hemisphere noninfarct area)/right hemisphere area] × 100%, corrected for brain swelling. Infarct area was measured in the sections at the level of the pregma.

NF-kB activity were determined by immunohistochemistry as follows: following fixation in methanol (10 min at -20 oC) and permeabilization in Triton, Tween (5 min in 1% in PBS), non-specific binding was blocked using normal goat serum (NGS, Millipore, USA) 10 % in PBS for 1 hr at 4 oC. Anti -NF-kB P65 primary antibody (Mouse Monoclonal Antibody, Milipore, USA) was added to the sections at a concentration of 1:100 in NGS for 2 hr. After washing 3 times in PBS, secondary antibody (FITC-conjugated goat anti-mouse) was added at a concentration of 1:100 in NGS for 1 hr in the dark. The slides were rinsed in PBS 3 times. Nuclei were stained by DAPI (4', 6-diamidino-2-phenylindole, Sigma, Germany) 1:10000 in d H2O for 5 min. before the slides were washed in d H2O twice then air-dried and coverslipped using fluoromount mounting media (Sigma, Germany).

Sections were examined under a fluorescent microscope (Leica DM 500, Germany) and at least 10 photos were taken for each section. The CD45 or P65 fluorescence was quantified using Image J software ®.

Statistical analysis:

Data are expressed as mean ± standard error of mean. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukeys post Hoc test at P

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< 0.05 using Graphpad Prism software version 5.

RESULTS

Figure (1a) shows that one day after reversible MCAO, mice showed significant sensorimotor deficit as evidenced by an elevation in the neurological score reaching 1.6 while control animals, before ischemia and reversible sham animals had normal sensorimotor function (score 0).

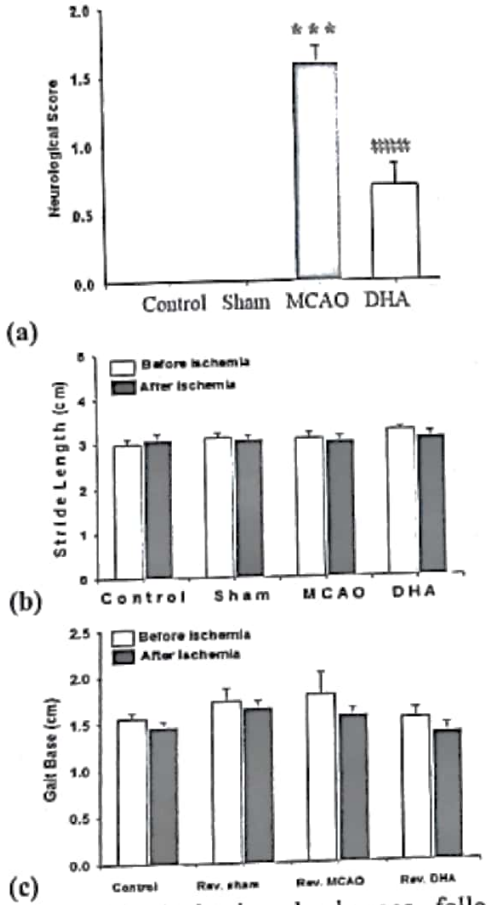

Figure (1): Behavioural changes following treatment with docosahexanoic acid (DHA) 5 hours after reversible MACO on: Neurological scores (a), stride length (b) and gait base in footprint test (c). Data are expressed as mean ± SEM, n = 10.

*** significantly different from the corresponding sham group at $P<0.0001$. ### significantly different from the corresponding MCAO group at $P<0.0001$.

Treatment with DHA 5 hrs after reversible MCAO showed significant improvement in sensorimotor function reaching 0.7 in comparison to 1.6 in reversible MCAO.

In footprint test, no changes were observed in stride length and gait base in control group or reversible sham group using paired t test. In addition, no significant changes in stride length and gait base were observed in the reversible MCAO group 24 hrs after the ischemic injury when compared to before ischemia using paired t-test and when compared to reversible sham stride length and gait base 24 hr after the ischemic injury.

Treatment with intravenous DHA (30 mg/kg at 5 hrs following reversible MCAO) didn't produce any significant change in the stride length and gait base respectively 24 hr after ischemic injury when compared with reversible MCAO group stride length and gait base 24 hr after ischemic injury (figure 1b and c).

One day after reversible MCAO, mice showed significant infarct area reaching 47 % of ibsilateral and 55 % of contralateral hemisphere area at pregma in comparison to no infarct with the reversible sham and control. Treatment with DHA 5 hrs showed significant reduction in infarct area in comparison with reversible MCAO reaching 14.7 % vs 47 % of ibsilateral hemisphere and 15.8 % vs 55 % of contralateral hemisphere (figure 2).

One day after reversible MCAO, mice showed significant NF-κB activation in the ibsilateral hemisphere measured as fluorescence mean gray value reaching 4.7 in comparison to 0.54 and 0.44 in reversible sham and control animals respectively.

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Vol. 22, Issue. 2, pp, 39-49

ISSN 1110-5089

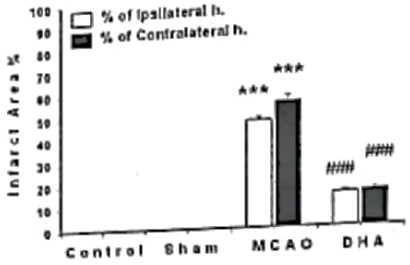

Figure (2): Effect of MCAO and treatment with docosahexanoic acid (DHA) 5 hours after reversible MACO on infarct volume. Data are expressed as mean ± SEM, n = 10.
*** Significantly different from the corresponding sham group at $P<0.0001$. ### significantly different from the corresponding MCAO group at $P<0.0001$

Treatment with DHA 5 hrs after reversible MCAO revealed significant reduction in NF-κB activity fluorescence reaching 2 in comparison to 4.7 in reversible MCAO (figure 3).

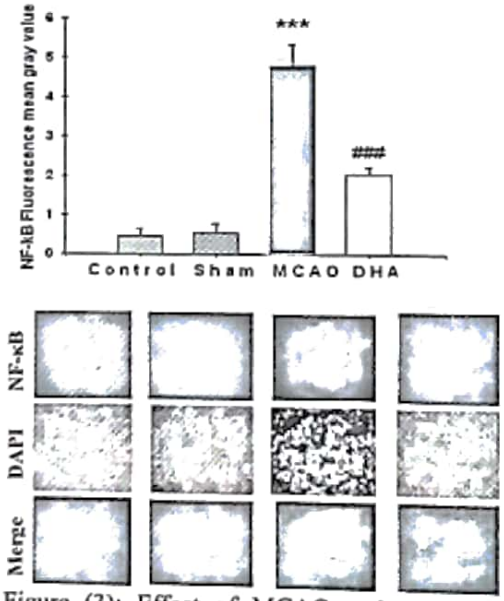

Figure (3): Effect of MCAO and treatment with docosahexanoic acid (DHA) 5 hours after reversible MACO on nuclear factor kappa B activity. Data are expressed as mean of fluorescence mean grey value ± SEM, n = 10.
*** significantly different from the corresponding sham group at $P<0.0001$. ### significantly different from the corresponding MCAO group at $P<0.0001$.

DISCUSSION

Stroke is the third leading cause of death but it is also the leading cause of adult disability, 50% of stroke survivors have a hemiparesis, 26% are dependent in activities of daily living, and 26% are forced into a nursing home (Go *et al.*, 2013). For this, stroke is a lethal disease, but it disables more than it kills. This fact has led a recent effort to develop strategies for neural repair after stroke (Carmichael, 2003).

Stroke generally refers to a local interruption of blood flow to the brain due to blockage of a cerebral artery (Adibhatla *et al.*, 2008). In humans, ischemic stroke occurs most often in the area perfused by the MCA (Mhairi Macrae, 1992). Consequently, experimental focal cerebral ischemia models of MCAO have been developed to mimic human stroke (Durukan and Tatlisumak, 2007).

In the current study, MCAO ischemia was experimentally induced in C57 BL/6 mice by reversible MCAO to investigate the role of reperfusion in the brain injury and treatment with DHA (IV at a dose of 30 mg/kg) at 5 hrs after ischemia onset in an attempt to increase the current restricted and narrow therapeutic window which is limited to 3-h after ischemia onset with t-PA.

Exposure of C57BL/6 mice to reversible MCAO for 1 hr followed by 24 hrs reperfusion showed significant infarct area. Similar results were previously reported following transient MCAO for 60 minute in C57BL/6 mice (Dziennis *et al.*, 2011; Leung *et al.*, 2012) or for 120 min MCAO (Yang *et al.*, 2012). Also Sprague-Dawley rats subjected to 2-h of MCAO followed by reperfusion for 24 h showed comparable infarct area (Dejda *et al.*, 2011).

Treatment with DHA showed significant reduction in infarct area following reversible MCAO. These findings keep pace with previous studies in Sprague-Dawley rats

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(Belayev *et al.*, 2009) and mice (Lalancette-Hebert *et al.*, 2011).

In the present study, mice subjected to reversible MCAO showed a significant grade of sensorimotor dysfunction on neurological score while mice subjected to sham operation and control mice didn't show any sensorimotor dysfunction. Similar results were observed in C57/B6 mice after exposure to transient focal cerebral ischemia by intraluminal occlusion of MCA for 60 minutes followed by reperfusion for 24-hr (Yin *et al.*, 2010), (Gelderblom *et al.*, 2013; Leung *et al.*, 2012) or in Sprague-Dawley rats exposed to transient MCAO for 2-h followed by reperfusion for 1-d, 3-d and 7 days the study of (Choi et al., 2010). Treatment with DHA 5-h after reversible ischemia showed significant improvement in the sensorimotor functions at 24-h after ischemia onset. These results are in accordance with previous studies on Sprague–Dawley rats which exposed to transient MCAO for 2-h followed by reperfusion with I.V. administration of DHA after 3 hrs of ischemia (Belayev *et al.*, 2011; Eady *et al.*, 2012).

Foot print test is one of the measurements that target the impact of stroke on locomotion. Gait impairment often occurs as a result of an ischemic stroke. Post-stroke gait is characterized by temporal asymmetry, reduced walking velocity, reduced gait base and reduced stride length (Mah et al., 1999). Impaired gait function not only reduces ambulation but can also lead to imbalance and falls, especially in elderly patients experiencing a stroke (Scherder et al., 2007).

In the current study, mice subjected to reversible MCAO didn't reveal significant change in the stride length or gait base 1 day after ischemia from before ischemia or reversible sham mice. These results are compatible with a previous study in C57BL/6 mice exposed to transient middle cerebral artery occlusion for 30-min followed by reperfusion for 1, 4, 7, 14 and 30 days and revealed no significant changes at 1 d while a progressive decrease in the stride length and gait base, with the greatest deficits at 7 d after ischemia was observed (Qin *et al.*, 2011)). In addition, mice exposed to transient focal MCAO showed significant reduction in stride length at 10 days after ischemia onset compared to sham operated mice (Hetze *et al.*, 2012). Moreover, rats undergoing focal cerebral MCAO ischemia for 60 minute followed by reperfusion for 1, 6, 21 and 42 days after ischemia showed generally shorter stride length at all days after ischemia but the difference was significant only at 6 and 42 days (Parkkinen *et al.*, 2013).

Treatment with DHA at 5-h after ischemia in mice subjected to reversible MCAO didn't show significant changes in the stride length and gait base compared to before ischemia or MCAO. No previous studies examined the effect of DHA treatment on stride length and gait base in footprint test after exposure to reversible experimentally induced ischemia.

NF-κB plays an important role in inflammation and immune regulation (Ghosh *et al.*, 1998). NF-κB activation is thought to play an important role in cerebral ischemic injury. Some reports have demonstrated that activation of NF-κB is able to prevent cerebral ischemic injury (Nurmi *et al.*, 2004; Schneider *et al.*, 1999), whereas other reports have shown that NF-κB activation promotes ischemic brain damage (Barakat *et al.*, 2009; Ridder and Schwaninger, 2009).

In the current study, C57BL/6 mice subjected to focal reversible cerebral ischemia showed significant activation of NF-κB in ipsilateral hemisphere while no normal NF-κB activity was observed in the contralateral hemisphere or in sham operated brain. These results are in accordance with a previous study in C57BL/6 mice after 30-min MCAO followed by reperfusion, which revealed marked activation of NF-κB at 4-h after ischemia onset (Kunz *et al.*, 2008).

In addition, rats subjected to 90-min MCAO followed by 24-h reperfusion showed NF-κB p65 positive cells in both sham and reversible MCAO animals but with significant elevated value in reversible MCAO (3 fold increase)(Li *et al.*, 2012). The increased activity of NF-κB may be attributed to the extra release of cytokines and reactive oxygen and nitrogen species, during reperfusion and leukocyte infiltration, which are known stimulant of NF-κB.

Treatment with DHA in mice subjected to reversible MCAO mediated significant reduction in NF-κB activation. These results are in accordance with a previous which reported that exposure of mice to transient MCAO with intraluminal filament for 60 min with DHA infusion directly after MCAO showed significant inhibition of ischemia-reperfusion-induced NF-κB (Marcheselli *et al.*, 2003).

In addition, DHA administration before transient focal cerebral ischemia and daily administration for 6 weeks in mice showed suppressed NF-κB DNA binding activity at all DHA administrations (Pan *et al.*, 2009).

The ability of DHA to reduce the infarct area, improve behavioural dysfunction implies a reduction in neuronal death and inflammation which might be attributed to its ability to reduce NF-κB activation and enhancement of resolution of inflammatio.

The present study revealed the efficacy of DHA against brain damage following stroke when administered 5 hours after the onset of MCAO which makes a possible drug candidate for treatment of stroke.

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حامض الدوكوساهيكسانويك (دي اتش ايه) يقلل من تلف الدماغ المستحدث بانسداد الشريان الدماغي الاوسط العكسي في الفئران.

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السكتة الدماغية هي سبب رئيسي للوفاة والعجز الدائم لدى البالغين في جميع أنحاء العالم .العلاج الوحيد للسكتة الدماغية الحادة المصرح به من ادارة الاغذية والعقاقير هو الحقن الوريدي بمنشط الأنسجة البلازمينوجيني المؤتلف في غضون ٣ ساعات من ظهورها.

من بين الآليات التي تشارك في السكتة الدماغية هي تنشيط انزيم الفوسفوليباز وما يترتب عليه من إطلاق حامض الأراشيدونيك و حامض الدوكوساهيكسانويك .حامض الأراشيدونيك ينتج الايكوسانويدز المتورطة في احداث واستمرار الاستجابات الالتهابية الحادة بينما حامض الدوكوساهيكسانويك ينتج الريسولفينز والبروتيكتينز التي لها أنشطة مضادة للالتهاب.

تم دراسة تأثير التعاطي المتأخر لحامض الدوكوساهيكسانويك (٣٠ مجم/كجم) ضد تلف الدماغ المستحدث بانسداد الشريان الدماغي الاوسط العكسي في الفئران. أظهر حامض الدوكوساهيكسانويك تأثير وقائي للاعصاب ضد تلف الدماغ كما اتضح من تقليل مساحة الاحتشاء، اختلال الوظائف العصبية ونشاط ان اف كابا بي.

هذه النتائج تشير إلى إمكانية استخدام حامض الدوكوساهيكسانويك ضد تلف الدماغ التالي للسكتة الدماغية في وقت متأخر حتي ٥ ساعات.