Phospho-p38 Mitogen Activated Protein Kinase (Phospho-p38 MAPK) and Oxidative Stress in Cerebral Ischemia/Reperfusion in Rats and the Neuroprotective Potential of Omega-3 Fatty Acids

Gihan M. Sharara[1], Hala M. Abou Heif[2], and Yasmine A. Ashram[2]

Departments of [1]Medical Biochemistry and [2] Medical Physiology, Faculty of Medicine, Alexandria University
gihansharara@hotmail.com

Abstract: Background: Cerebral ischemia activates several intricate cell signaling cascades that are critical for cell survival/damage triggered by a multitude of stimuli. The stress-activated mitogen activated protein kinases (MAPKs) (p-38 and c-Jun N-terminal kinase [JNK]) mainly function as mediators of cellular stress by phosphorylating intracellular enzymes, transcription factors and cytosolic proteins involved in cell survival, inflammatory cytokine production and apoptosis. The brain is particularly sensitive to oxidative damage. **Aim of the work**: Was to study oxidative stress and phospho-p38 MAPK in the brain of rats after cerebral ischemia / reperfusion (I/R) and to associate this biochemical changes with the neurological motor function of ischemic rats. The effects of pretreatment with omega-3 fatty acids present in fish oil on cerebral I/R injury were also evaluated. **Material & Methods:** The study was carried out on 56 adult male albino rats divided into 2 sections A&B. Section A included thirty two rats that were divided into 2 main groups: Group I: eight-sham operated rats as controls and Group II: twenty four rats that were subjected to cerebral ischemia induced by 30 minutes of left common carotid artery (CCA) occlusion followed by variable durations of reperfusion. Group II was subdivided into 3 subgroups (IIa, IIb and IIc) eight rats/each group that were reperfused for 6, 24 and 72 hours respectively. Section B: included twenty four rats subdivided into 3 groups (8 rats/each). Group1: Sham operated rats, Group 2: rats were kept on ordinary diet before left CCA occlusion that was followed by 24 hours of reperfusion, Group 3: rats were kept on fish oil supplemented diet 0.4 g/kg/day , 14 days before cerebral ischemia and 24 hours of reperfusion. Neurological evaluation of motor sensory functions was performed. At the end of the experiment, brain phospho-p38 MAPK was measured by Western blot and ELISA, malondialdehyde (MDA) as an index of lipid peroxidation of brain tissue, glutathione peroxidase (GSHPx) antioxidant enzyme activity and caspase-3 activity as an index of apoptosis in brain tissue. **Results:** Brain tissues of ischemic reperfused rats showed significant increase of phospho-p38 MAPK, MDA and caspase-3 activity and significant decrease of GSHPx at 6, 24 and 72 hours of reperfusion as compared with control rats. The biochemical changes were maximal by 24 hours of reperfusion. Values of phospho-p38 MAPK, caspase-3 activity and MDA level in brain tissue of ischemic reperfused rats by 72 hours were significantly lower than these values in rats reperfused for 24 hours, but were still significantly higher than control rats. The mean neurological score of the ischemic rats reperfused for 6, 24 and 72 hours was significantly lower compared with sham operated rats. The lowest value of neurological score was observed in rats reperfused for 24 hours coincident with maximal increase of phospho-p38 MAPK, MDA and caspase-3 activity in brain tissue and the maximal reduction of GSHPx activity. The neurologic score of rats assessed at 72 hours of reperfusion was significantly higher than that of rats reperfused for 24 hours, but was still significantly lower than control values, which indicated some functional improvement that correspond with attenuation of ischemic brain injury. Rats kept on fish oil supplemented diet has significantly higher neurological score compared with that of ischemic perfused rats on ordinary diet when assessed after 24 hours of cerebral I/R. This significant neuroprotection from ischemic injury was associated with lower levels of phospho-p38 MAP kinase, MDA and caspase-3 activity and higher level of GSHPx in ischemic brain tissue of rats on fish oil supplemented diet compared to those of rats on ordinary diet. **Conclusion:** Increased oxidative stress and activation of p38 MAPK signaling pathway represent an important mechanism of brain injury following cerebral I/R in rats. Fish oil pretreatment attenuated this injury by reducing oxidative stress, p38 MAPK activation and apoptosis. Targeting MAPK signaling pathways by inhibitors represents an important promising mechanism of neuroprotection. The use of fish oil supplement may be beneficial to prevent or ameliorate ischemic cerebral vascular disease.

[Gihan M. Sharara, Hala M. Abou Heif, and Yasmine A. Ashram. **Phospho-p38 Mitogen Activated Protein Kinase (Phospho-p38 MAPK) and Oxidative Stress in Cerebral Ischemia/Reperfusion in Rats and the Neuroprotective Potential of Omega-3 Fatty Acids**. *Journal of American Science*. 2012; 8(3): 723-733]. (ISSN: 1545-1003). http://www.americanscience.org. 97

Key words: Cerebral ischemia/reperfusion, p38 MAPK, Oxidative stress, Apoptosis.

Abbreviations: Ischemia/reperfusion (I/R), phospho-p38 mitogen activated protein kinase (phospho-p38 MAPK), malondialdehyde (MDA), glutathione peroxidase (GSHPx).

1. Introduction

The mitogen-activated protein (MAP) kinase cascades have crucial roles in signal translocation from the cell surface to the nucleus by regulating cell death and survival. Three MAP kinase cascades that converge on extracellular signal regulating kinases (ERKs), c-Jun IV-terminal kinases (JNKs), and p38 MAP kinases have already been characterized [1] . Each cascade consists of three types of kinases, MAP kinase, MAP kinase kinase (MKK) and MKK kinase (MKKK) [2]. MKKK phosphorylates and thereby activates MKK, which then caused an activation of MAP kinase by site specific phosphorylation. Two among those three MAP kinase cascades that converge on JNK and p38 MAP kinases are preferentially activated by stresses such as oxidative stress, cytokines and osmotic shock [3]. Activated MAPKs can directly phosphorylate their substrate in the cytoplasm or the nucleus to regulate transcription [4].

Cerebral ischemia activates several intricate cell signaling cascades that are critical for cell survival/damage triggered by a multitude of stimuli [5] . The stress-activated MAPKs (p38 and JNK) mainly function as mediators of cellular stress by phosphorylating intracellular enzymes, transcription factors and cytosolic proteins involved in cell survival, inflammatory cytokine production and apoptosis [6,7] . Accordingly, the study of MAPK activation in ischemic brain may provide fertile ground for the discovery of novel therapeutic agents for stroke [8] .

Cell death following cerebral ischemia was traditionally considered to be necrotic in nature. However, neurons in the ischemic penumbra or peri-infarct zone, suffer transiently reversible damage and then ultimately undergo death by apoptosis, an energy dependent process that contributes equally to the pathogenesis [9] .

Caspases play an essential role in apoptosis. Although three major apoptotic pathways have been proposed; mitochondrial, death receptors mediated and endoplasmic reticulum (ER) initiated pathways; all these mechanisms converge to caspase-3 activation and nuclear fragmentation as the final step [10] .

Oxidative stress is one of the most important factors that exacerbate brain damage upon reperfusion that follows ischemic insult to the brain [11] . The brain is particularly vulnerable to oxidative injury because of its high rate of oxidative metabolic activity, relatively low antioxidant capacity and non replicating nature of its neuronal cells [12] . Several lines of evidence indicate that reactive oxygen species (ROS) formation may be involved in a series of molecular events that link ischemic injury to neuronal cell death [13] .

Fish oil contains n-3 essential fatty acids (EFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are important for the histological, anatomical and biochemical integrity of the brain [14]. DHA is a major component of brain membrane phospholipids and is necessary for continuity of neuronal functions.[14] Loss of brain DHA results in impairment of sensory, behavioral and cognitive functions, both in animals and humans.[15,16] Fish oil (DHA/EPA) was reported to be protective against several neurological and neuropsychiatric disorders.[17]

The aim of the present work was to study oxidative stress and phospho-p38 mitogen activated protein kinase (phospho-p38 MAPK) in the brain of rats after cerebral ischemia reperfusion (I/R) and to associate this biochemical changes with the neurological motor function of ischemic rats. The effects of pretreatment with omega-3 fatty acids present in fish oil on cerebral I/R injury were also evaluated.

2. MATERIAL AND METHODS

The study was performed on 56 adult male albino rats weighing between 190-220 grams. The animals were kept under standardized temperature, humidity and light conditions with free access to standard laboratory diet and water *ad libitum*. Animal care and use followed the Ethics Committee of Faculty of Medicine, Alexandria University. Cerebral ischemia was induced by unilateral (left) common carotid artery (CCA) occlusion[18] followed by reperfusion, while control rats were sham operated. The study included 2 sections:

Section A: Effect of cerebral I/R on biochemical data measured in ischemic brain and neurologic score of ischemic rats.

This section consisted of 32 rats divided into:

Group I: Eight sham operated rats served as control.

Group II: Twenty four rats were subjected to cerebral I/R by occluding left CCA for 30 minutes followed by varying durations of reperfusion. They were subdivided into 3 subgroups (8 rats/each).

- **Group IIa**: Rats were reperfused for 6 hours following 30 minutes CCA occlusion.[19]
- **Group IIb:** Rats were reperfused for 24 hours following CCA occlusion.[19]
- **Group IIc:** Rats were reperfused for 72 hours following CCA occlusion.[19]

Section B: Effect of pretreatment with omega 3 fatty acids on biochemical and neurologic evaluation of ischemic rats after 24 hours of reperfusion.

This section included 24 adult rats divided into 3 groups (8 rats/group).

Group 1: Sham operated.

Group 2: Ischemic rats reperfused for 24 hours. They were kept on standard diet.

Group 3: Rats kept on standard diet plus fish oil including omega 3 essential fatty acids supplement for 14 days before CCA occlusion and reperfusion (SEDICO, 0.4 g/kg/day by gavage) [20]. The fatty acid composition of SEDICO pharmaceutical CO_1 capsule (1000 mg) is formed of EPA/DHA 30%. Rats in groups 1&2 were given saline in the same way for 14 days.

Experimental procedure

I-Cerebral ischemia reperfusion model

Animals in the experimental groups were subjected to transient left CCA occlusion. They were fasted overnight prior to surgery with free access to tap water. Anaesthesia was induced by ether inhalation and was maintained by thiopental sodium (50 mg/kg intraperitoneal).[21] Body temperature was kept constant at 36.5±0.5°C using heating bad. After complete anaesthesia, rats were placed on their back and a vertical midline cervical incision was made, CCA was carefully dissected. Ischemia was induced by placing non traumatic microvascular clip on left CCA just proximal to its bifurcation.[18] During ischemia rats were monitored for body temperature and respiration pattern. After 30 minutes of occlusion, the clips were removed to allow for reperfusion of carotid artery for 6, 24, 72 hours according to the studied groups. Restoration of blood flow within the carotid artery was confirmed by careful observation. Thereafter, neck skin incision was closed in layers and animals were allowed to recover from anaesthesia. In sham operated rats, all surgical procedures were done but without vascular occlusion.

II-Neurological tests:

Neurological evaluation of motor sensory functions was carried out once a day, starting the day before surgery and continuing until the end of each experiment. Evaluations were always performed between 10 and 11 am to exclude behavioral changes based on circadian rhythm and to ensure that rats operated on during early morning were recovered from anaesthesia.

The neurological evaluation consisted of 6 tests according to Garcia *et al.*[22]:

1- Spontaneous activity, 2. Symmetry in movement of 4 limbs, 3.Forepaw outstretching; 4.Climbing, 5.Body propioception and 6.Response to vibrissae touch.

The score assigned to each rat at completion of the evaluation equals the sum of all six tests score: The final minimum score was 3 and maximum score was 18.

At the end of reperfusion, rats were killed under ether anaesthesia by decapitation after aortic exsanguination. The brain was dissected out of the skull and the affected hemisphere was immediately stored at -80°C for further analysis.

III- Biochemical tests:

1- Determination of phospho-p38 MAP kinase [23] .

- Preparation of brain tissue homogenate

Brain tissues were homogenized in lysis buffer (RIPA cell lysis buffer: 50 mM Tris HCL, pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% triton x-100, 1% sodium deoxycholate and 0.1% SDS). The samples lysed with the provided RIPA Cell Lysis Buffer were modified by the addition of protease inhibitors (protease inhibitor cocktail [PIC] and phenylmethyl sulfonyl fluoride [PMSF]) and also by the addition of phosphatase inhibitors (sodium pyrophosphate and sodium orthovanadate) immediately before running the assay. All chemicals were purchased from Sigma Chemicals, USA.

Principle of the assay of p38 MAPK:

Assay Design phospho-p38 Titerzyme Enzyme Immunometric Assay (EIA) kit was used for quantitative determination of phospho-p38 in cell lysate.[23]

2-Colorimetric assay of caspase-3 activity as indicator of apoptosis in brain tissue.

Protein extract were prepared by homogenization of tissues in a lysis buffer (Tris [hydroxymethyl] aminomethane, NaCl and triton X-100). The cell lysate was incubated on ice for 10 minutes and then centrifuged. The supernatant was used for determination of protein by Lowery method[24] and caspase 3-activity.[25]

Principle of the assay:

Assay design caspase-3 colorimetric assay kit involves the conversion of a specific chromogenic substrate for caspase 3 enzyme (ASP-Glu-Val-ASP *p*-nitroanilide, DEVD-*p*NA) followed by colorimetric detection of the product at 405 nm. The standard protocol was followed as detailed by the manufacturer.

3-Determination of glutathione peroxidase (GSHPx) antioxidant-enzyme activity in brain tissue.

Briefly, an aliquot of brain homogenate (0.4 mg of protein) in 0.05 MPBS containing 1.15% (w/v) KCl was mixed with 230 µL of coupling solution (containing 33.6 mg of disodium EDTA, 6.5 mg of NaN_3, 30.7 mg of reduced glutathione, 16.7 mg of NADPH and 100 units of glutathione reductase in 100ml of 50mM Tris-HCl pH 7.6). The volume was adjusted to 260 µl with 0.05 MPBS. Kinetic decay of NADPH fluorescence (Ex. 355 nm/Em.465 nm) was measured after the addition of 40 µl of 1mM H_2O_2 as the substrate using spectro-photometer. The results were expressed as nmol NADPH oxidized per minute per mg protein.[26]

4-Assay of malondialdehyde (MDA) as index of lipid peroxidation in brain tissue.[27]

This was done by measuring thiobarbituric acid reactive substances in tissue homogenate. The absorbance was measured spectrophotometrically at 532 nm. The results were expressed as nmol malodialdehyde or MDA/mg protein.

5- Western Blot Analysis for p-38 and phospho-p-38 [28]

For Western Blot analysis part of the cortex of the affected side was quickly removed at 6 hours , 24 hours, and 72 hours of reperfusion and was homogenized and sonicated in homogenizing buffer (250 mM sucrose, 20 mM HEPES, pH 7.4 with KOH, 100 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]). The homogenate was centrifuged (1000 × g, 15 min, 4°C) and the resulting supernatant was used for quantitation. Protein concentrations were determined and equal amounts of protein were loaded per lane after adding the same volume of Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA). Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis was performed on a Tris-glycine gel (Invitrogen) and then transferred to a polyvinylidene difluoride membrane (Invitrogen). The primary antibodies were 1:1000 dilution of the anti-phospho-p38 (Thr180/Tyr182) antibody, 1:2000 dilution of the anti-p38 antibody. Western blots were performed with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Cell Signaling Technology) using enhanced chemiluminescence Western blotting detection reagents (Amersham International, Buckinghamshire, UK). The film was scanned with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and the results were quantified using Multi-Analyst software (Bio-Rad).

Statistical method:

After data entry and careful meticulous revision, the file was transferred to SPSS format (Statistical Package for Social Sciences) version 10 and the following testes were performed: descriptive analysis (mean ± standard deviation (SD), minimum and maximum) and analysis of the variance (ANOVA) and least significant difference (LSD) for post-hoc comparison. A level of $P< 0.05$ was defined as statistically significant.

3. Results

Section A: Effect of cerebral I/R on biochemical data measured in ischemic brain and on neurologic score of ischemic rats. (Table I).

Phospho-p38 MAPK in ischemic brain showed a significant increase in all the studied groups after 6, 24, 72 reperfusion hours compared with sham operated rats. This increase was maximal by 24 hours (group IIb) and declined significantly by 72 hours (group IIc) compared with (group IIb). However, level of p38 MAPK in ischemic brain tissue in (group IIc) was still significantly higher than its level in either sham operated control (group I) or rats reperfused for 6 hours (group IIa) ($F= 197.4$, $P= 0.0001$*).

Activation and Phosphorylation of p38 After I/R in Rats (Figure 1).

To investigate the activation and phosphorylation of p38 after I/R we performed Western blot analysis. For the Western blot analysis, we used a specific antibody against phosphop38

(Thr180/Tyr182) and p38. The protein level of p38 phosphorylation was also significantly increased from 6 hours to 72 hours after reperfusion and peaked at 1 day ($P < 0.05$).**(Figure 1).**

Caspase 3 activity in brain tissue was increased in ischemic reperfused rats in groups IIa, IIb and IIc compared with sham operated rats with maximal activity at 24 hours of reperfusion (group IIb). Caspase 3 activity in brain tissue of ischemic reperfused rats for 72 hours (group IIc) was significantly lower than that of ischemic reperfused rats for 24 hours (group IIb), but this activity was still significantly higher compared with that of rats reperfused for 6 hours (group IIa) or control rats (group 1) ($F= 60.5$, $P= 0.0001$*).**(Table I).**

Glutathione peroxidase activity (GSHPx) was significantly lower in brain tissue of ischemic rats after 6, 24, 72 hours of reperfusion (in groups IIa, IIb & IIc respectively) compared with sham operated rats. Maximal reduction of GSHPx was observed by 24 hours of reperfusion compared with other groups. GSHPx activity in brain tissue of ischemic rats reperfused for 72 hours was significantly higher than that of group IIb but still significantly lower than groups IIa & group I control rats ($F= 82.9$, $P= 0.0001$*).**(Table I).**

Level of malondialdehyde (MDA) which indicate lipid peroxidation in ischemic brain tissue was significantly higher after 6, 24 and 72 hours of reperfusion with maximal increase being after 24 hours of reperfusion compared with sham operated rats. The level of MDA in ischemic brain declined at 72 hours of reperfusion when compared with its level in the ischemic brain in rats reperfused for 24 hours, but it was still significantly increased as compared with its level in the brain after 6 hours of reperfusion and also with control level ($F 416.22$, $P= 0.0001$*).**(Table I).**

The mean neurological score of ischemic reprefused rats was significantly lower after 6, 24 and 72 hours of reperfusion compared with sham operated rats. The lowest value being after 24 hours of reperfusion compared with other groups. Significant improvement was observed as regards the neurologic score of rats reperfused for 72 hours (group IIc) which

was significantly higher than that of (group IIb) reperfused for 24 hours, although its value in group IIc was still below the control level (group I) (F= 65.5, *P*= 0.0001*).**(Table I).**

Section B: **Effect of pretreatment with fish oil on biochemical and neurological data of ischemic rats after 24 hours of reperfusion. (Table II).**

Group 2:

Ischemic rats on ordinary diet reperfused for 24 hours showed significant increase of phospho-p38, increase of caspase-3 activity and increase of MDA but a decrease of GSHPx in ischemic brain tissue compared with control values of sham operated rats. The neurological score was significantly lower than sham operated controls.

Western blot analysis showed that phospho-p38 values were significantly increased in the I/R rats after 24 hours reperfusion on ordinary diet (Group 2) compared with the control values of sham operated rats (Figure 2, $P < 0.05$, respectively).

Group 3:

Ischemic rats kept on fish oil supplemented diet showed significantly lower phospho-p38 MAPK, lower caspase-3 activity, lower MDA and significantly higher GSHPx in brain tissue after 24 hour of reperfusion compared with their values in the brain of ischemic rats of group 2. The neurological score was significantly higher in group 3 than group 2.

Western blot analysis showed that phospho-p38 values were significantly decreased in the I/R rats after 24 hours reperfusion on fish oil supplement (Group 3) compared with the group 2 rats (Figure 2, $P < 0.05$).

Table (I): Comparison of biochemical data in the brain and mean neurologic score of rats after cerebral ischemia/reperfusion (I/R): 30 min ischemia with varying durations of reperfusion for 6, 24 and 72 hours.

Parameter studied	Sham operated Group I	I/R for 6 hours Group IIa	I/R for 24 hours Group IIb	I/R for 72 hours Group IIc	F P
Phospho-p38 MAPK pg/gram tissue Min-Max Mean±SD	240-273 258.88±11.61[a]	500-630 574.0±53.43[b]	1890-2005 1953.13±51.05[c]	810-963 884.88±56.42[d]	197.4 (0.0001)*
Caspase 3 activity units/mg protein Min-Max Mean±SD	2.9-4.7 3.93±0.57[a]	8.7-10.5 9.55±0.72[b]	21.5-25.9 23.21±1.46[c]	13.9-16.2 14.79±0.76[d]	60.5 (0.0001)*
GSHPx activity nmol/min/mg protein Min-Max Mean±SD	10.62-13.2 11.84±0.81[a]	7.45-9.5 8.41±0.73[b]	4.19-5.35 4.71±0.46[c]	6.22-6.97 6.53±0.23[d]	82.9 (0.0001)*
MDA nmol/mg protein Min-Max Mean±SD	1.4-2.6 2.05±0.38[a]	4-5.2 4.71±0.46[b]	8.2-9.9 9.01±0.47[c]	6.99-7.9 7.32±0.37[d]	416.22 0.0001*
Mean neurological score Min-Max Mean±SD	16-18 17.25±0.71[a]	13.75-15.5 14.59±0.62[b]	9.75-11.75 10.55±0.72[c]	12.5-13.9 12.99±0.49[d]	65.5 0.0001*

Same letters has no significant difference.

Table (II): Comparison of biochemical data in the brain tissue and mean neurologic score of rats on ordinary diet and those on fish oil supplement after cerebral ischemia/reperfusion (I/R): 30 min ischemia and 24 hours reperfusion.

Parameter studied	Sham operated Group I	I/R on ordinary diet Group 2	I/R on fish oil supplement Group 3	F P
Phospho-p38 MAPK pg/gram tissue Min-Max Mean±SD	 235-270 256.8±11.3 [a]	 1890-2059 1979.75±73.67 [b]	 809-920 854.88±52.1 [c]	 185.6 (0.0001)*
Caspase 3 activity units/mg protein Min-Max Mean±SD	 3.2-4.2 3.83±0.33 [a]	 18.9 -22 19.70±1.04 [b]	 12.8-14.6 13.76[c]±0.66	 94.2 (0.0001)*
GSHPx activity nmol/min/mg protein Min-Max Mean±SD	 10.5-12.6 11.65±0.85 [a]	 4.19-5.23 4.65±0.45 [b]	 6.59-7.82 7.20±0.48 [c]	 58.67 (0.0001)*
MDA nmol/mg protein Min-Max Mean±SD	 1.9-2.5 2.21±0.25 [a]	 7-9.5 8.44±0.85 [b]	 4.2-5.3 4.70±0.41 [c]	 46.6 (0.0001)*
Mean neurological score Min-Max Mean±SD	 16-18 17.13±0.83 [a]	 10-12.5 10.81±0.87 [b]	 12.75-14.99 13.96±0.62 [c]	 34.5 (0.001)*

Same letters has no significant difference.

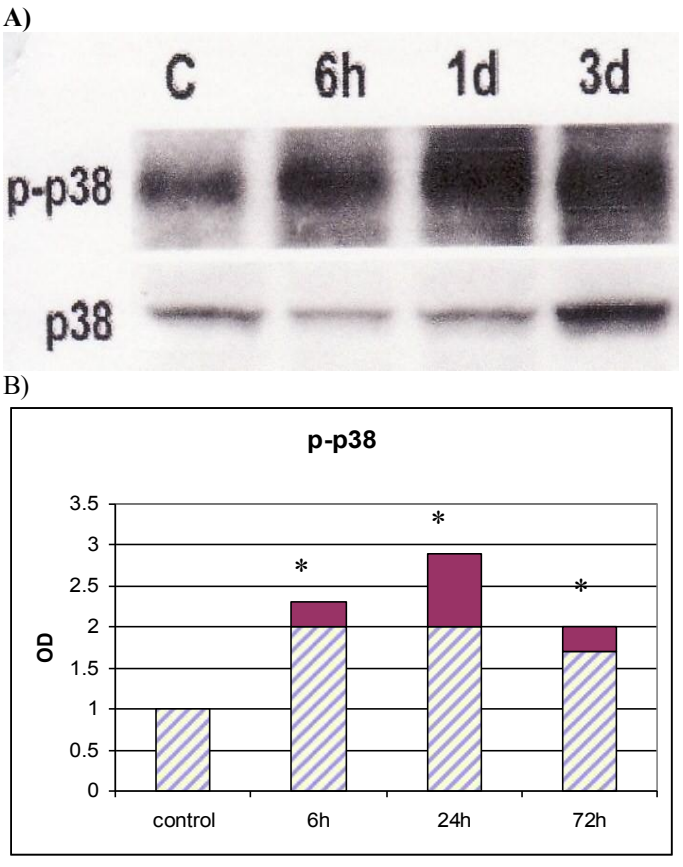

Figure 1. A) Time course of Immunoblots illustrating phospho-p38 and p38 MAPK protein.
B) Time course of expression of phospho-p38 MAPK (p-p38) by optical density (OD) after ischemia- reperfusion in rats, compared with non-ischemic controls (C). *$P < 0.05$, O.D., optical density.

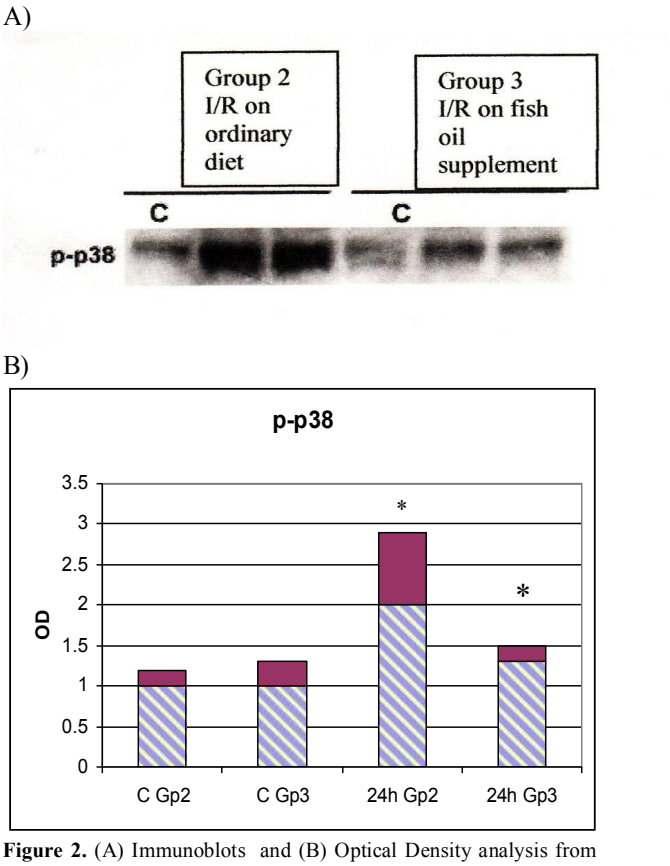

Figure 2. (A) Immunoblots and (B) Optical Density analysis from Western blot of brain phospho-p38 MAPK (p-p38) 24 hours after I/R rats on ordinary diet (group 2) and I/R rats on fish oil supplement (group 3).
*$P < 0.05$, compared with non-treated ischemic group. C, control; O.D., optical density.

4. Discussion

The mitogen-activated protein (MAP) kinase cascades are multifunctional signaling pathways that are one of the most important intracellular signal transduction pathways transmitting extracellular stimuli to the nuclei of cells and respond to a variety of stimuli including oxidative stress, cytokines and initiators of cell death.[2] Extracellular signal-regulated kinase is activated in response to several kinds of growth factors, intracellular calcium increase, and glutamate receptor stimulation. In contrast, c-Jun N-terminal kinase and p38 MAPK are activated in response to a variety of cellular stresses, such as DNA damage, heat shock protein, or inflammatory cytokines, and they modulate expression of several transcription factors. These three kinds of signals are involved in oxidative stress [3].Free radicals have been shown to be associated with both ischemic and postischemic injury to the brain [12] . Neuronal damage is enhanced after I/R injury and may be manifested as either apoptosis or necrosis of the neurons [9] .

In the present study, oxidative stress following cerebral ischemia reperfusion is evident by significant increase of brain tissue MDA and significant decrease of the antioxidant enzyme GSH-Px activity after 6, 24, 72 hours of reperfusion compared to sham operated rats. This was associated with significant increase of phospho-p38 MAPK in the affected hemisphere of the brain of ischemic reperfused rats after 6, 24 and 72 hours of reperfusion compared with sham operated rats. The level of phospho-p38 in the affected hemisphere was maximal by 24 hours of reperfusion and declined after 72 hours of reperfusion but remained significantly higher than sham operated rats. A significant increase of caspase-3 activity was observed in the affected hemisphere of ischemic perfused rats at the indicated time points which was maximal in group IIb reperfused for 24 hours indicating significant enhancement of neuronal apoptosis.

Our data by Western blotting demonstrated a significant increase in phospho- p38 in the brain and decrease in non-phosphorylated p-38 starting at 6 hours sustained until 72 hours and reaching a peak at 24 hours after I/R. This is coincident with more oxidative stress and neurological deficits. These results suggest that activation and phosphorylation of p38 MAPK promotes damage in the brain after ischemia-reperfusion injury. It is also coincident with the time of activation of astrocytes or microglia, which are implicated in BBB breakdown or inflammation after focal ischemia. Moreover, those cells are also the main types in p38 MAPK signaling [19].

The neurological response of experimental rats was also affected by ischemia. The neurological score was significantly lower in rats of groups IIa, IIb and IIc reperfused at 6, 24 and 72 hours respectively, compared with sham operated rats. The lowest score was present in ischemic rats reperfused for 24 hours. This was coincident with maximal increase of phospho-p38 MAPK, caspase 3 activity and reduction of GSH-Px. However, the neurological score of rats at 72 hours of reperfusion was higher than that of rats reperfused for 24 hours indicating some degree of functional improvement although still below the control values.

The sensory motor score developed by Garcia *et al.*[22] Showed a strong correlation with the number of necrotic neurons in rats subjected to permanent or transient focal cerebral ischemia. The most affected rats were those of permanent ischemia group. The rats subjected to 30 minutes of arterial occlusion followed by 7 days of survival had the least-affection of contralateral limb movement according to Garcia *et al.*[22] scoring. Bolay & Dalkara[29] demonstrated that pyramidal motor function is rapidly lost on occlusion of middle cerebral artery (MCA) as a result of loss of excitability of the cortex and blockade of axonal conduction in subcortical region. Axonal conduction readily recover following reperfusion after 1-3 hours of ischemia which could explain the improvement observed in the neurological score of rats after transient ischemia. However, motor dysfunction continue after reperfusion because of persistent synaptic transmission defect within the motor cortex. The delayed and incomplete recovery of synaptic transmission is a major problem causing electrical dysfunction after reperfusion [30] .

Menzies *et al.*[30] attributed the recovery to the numerous anastomoses between cerebral arteries which allow for secondary recirculation phase after removal of the clip. This occurs only in young aged rats. The functional recovery could be attributed to the contralateral hemisphere which can take over functions previously occupied by the lesioned hemisphere.[30]

The role of phospho-p38 MAPK in ischemia-induced cell death has been evaluated previously in animal models of global cerebral ischemia induced by bilateral common carotid artery (CCA) occlusion by using western blotting and immunohistochemistry techniques [31-34] . Sugino *et al.*[31] found enhanced phospho-p38 immunoreactivities in CA1 & CA3 regions of hippocampus that started 15 minutes of transient global ischemia in gerbils and reached a maximum by 6 hours of reperfusion. After that, phospho-p38 immunoreactivities were reduced in CA1 region of hippocampus and disappeared by 18 hours of reperfusion, but persistant immunoreactivities for phospho-p38 were still observed in CA3 region of hippocampus after 72 hour of reperfusion. Inhibition of p38 activation by pretreating ischemic rats with selective p38 inhibitor (SB20358), protected against neuronal death in CA1 & CA3 pyramidal cells of hippocampus.[31] On the other hand, Walton *et al.* [32] reported a delayed activation of p38 MAPK that started

to be increased in microglia four days after global cerebral ischemia. Other investigators[33] reported increased p38 MAPK activity that was detected in CA1 region of the hippocampus within 3 to 6 hours of injury onset in the global ischemia model in rats. Hu *et al.* [34] found increased phosphorylation of activating transcription factor 2 (ATF-2) by p38 MAPK in dying neurons of CA1 region of hippocampus which was maximal by 24 hours after transient forebrain ischemia. This might reflect damaging signals in these neurons.

Moreover, increased phospho-p38 MAPK level was also detected in different brain regions following transient focal cerebral ischemia induced in rats by middle cerebral artery occlusion (MCAO) [19,35]. Tian *et al.* [19] reported that the increase of phospho-p38 MAPK detected by immuno blotting technique after transient MCAO in rats was biphasic. The first phase occurred within one hour in cortical neurons whereas a striking delayed induction of phospho-p38 was detected in microglia around the area of necrotic infarction after 24-72 hours from the start of reperfusion. Irving *et al.* [35] found increased phospho-p38 immunostaining in cells with astrocyte like morphology in both core and penumbral-like regions up to 24 hours following transient MCAO (90 minutes) and 6 hours of permanent MCAO in rats.

The main role of microglia is to respond to disruption of brain homeostasis induced by trauma or stroke injury. Activated microglia produces a series of inflammatory mediators (cytokines, chemokines and adhesion molecules) that expand brain damage following stroke or trauma or other stresses. [36] The mitogen activated protein kinase p38 activation has been linked to inflammatory cytokine production and cell death following cellular stress.[36] Stroke induced p38 MAPK activation in the brain has been demonstrated especially in astrocytes and activated microglia adjacent to dying neurons.[36] Treatment with p38 MAPK inhibitors provided significant reduction in infarction size, neurological deficit and reduced the inflammatory cytokines protein expression produced by focal stroke.[37,38] The neuroprotection offered by inhibition of p38 MAPK signaling emphasizes a significant opportunity for targeting MAPK pathways in CNS injury.[37,38]

Cerebral ischemia and reperfusion in particular are responsible for oxidative stress by generation of free radicals which culminate into deleterious effects during pathogenesis[39] During oxidative stress rapid overproduction of free radicals overwhelms the detoxication and scavenging capacity of cellular antioxidant enzymes viz superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and non enzymatic antioxidants resulting in severe immediate damage to DNA proteins and lipids.[40] The reactive oxygen species (ROS) that are responsible for oxidative stress include O_2^-, OH^-, $ONOO^-$.[39] ROS are not only toxicant to the cell but also second messengers that control several signaling pathways including MAP kinases.[40]

Strong experimental evidences suggest that lipid peroxidation plays a widespread role in neuronal cell death. Lipid peroxidation results in loss of membrane integrity, impairment of the function of membrane transport proteins and ion channels, disruption of cellular ion homeostasis and concomitantly increases neuronal vulnerability to excitotoxicty.[40,41]

In the present work, significant increase of MDA and decrease of GSHPx was evident at 6, 24, 72 hours of cerebral ischemia reperfusion which was maximal after 24 hours coincident with maximal increase of caspase-3-activity in brain tissue of affected hemisphere of ischemic rats compared with sham operated rats.

Candelario-Jalil *et al.* [42] studied the time course of lipid peroxidation after 5 minutes of bilateral CCA occlusion in gerbils. MDA level was maximal in the hippocampus at 48, 72 and 96 hours of reperfusion. The cortical level of MDA was significantly higher by 12 and 24 hour of reperfusion while striatal lipid peroxidation was maximal by 2 and 6 hours of reperfusion. They also demonstrated the effect of cerebral ischemia on the antioxidant defenses. In the hippocampus, maximal reduction of GSHPx occurred in late period of reperfusion (72 hours after ischemia), associated with marked depletion of glutathione (GSH). Loss of GSH and oxidative damage have been suggested to constitute an early possibly signaling events in apoptotic cell death. The results of this study showed that transient ischemia-induced oxidative injury evolves temporally and spatially in the brain and provided evidence that oxidative stress may be involved in delayed neuronal death following ischemia in gerbils.[42] Other researches, Lefkowitz *et al.* [43] and Ishibashi *et al.* [44] found that transgenic mice overexpressing GSHPx antioxidant enzyme when subjected to one hour of focal cerebral ischemia followed by 24 hrs of reperfusion, had lower incidence of brain edema and apoptosis, reduced infarction size and lower incidence of behavioral and motor disturbances compared with wild type nontransgenic littermares.

The association between oxidative stress, MAPK activation and apoptosis was explained by several authors.[45-47] Apoptosis signal regulating kinase 1 (ASK1) is a MAP kinase of the C jun N-terminal kinase (JNK) and p38 MAPK kinase pathway, which is preferentially activated in response to oxidative stress.[45,48] In resting cells, ASK1 constantly forms an inactive complex with thioredoxin (Trx), whereas upon exposure of the cells to ROS donor or TNFα, ASK1 is dissociated from Trx and thereby becomes fully activated.[46] Trx is a readox regulatory protein that has two redox sensitive cystiene residues within the active

center. Only a reduced form of Trx is associated with N-terminal regulatory domain of ASK1 and silences the activity of ASK1. Dissociation of ASK1 from oxidized Trx switches an inactive form of ASK1 to active kinase.[47,49] ASK1 activation drives the cell towards apoptosis when it is exposed to prolonged excessive oxidative stress through MAPK activation.[49,50]

MAPK pathways are known to be influenced not only by receptor ligand interactions, but also by different stressors placed on the cell. One type of stress that induces potential activation of MAPK pathways is the oxidative stress caused by reactive oxygen species (ROS). Generally, increased ROS production in a cell leads to the activation of ERKs, JNKs, or p38 MAPKs, but the mechanisms by which ROS can activate these kinases are unclear. Oxidative modifications of MAPK signaling proteins and inactivation and/or degradation of MKPs may provide the plausible mechanisms for activation of MAPK pathways by ROS [48]

Our study was also designed to examine the omega 3 polyunsaturated fatty acids (Omega-3 PUFA) present in fish oil (DHA & EPA essential fatty acids) on biochemical parameters and neurologic function of ischemic brain after 24 hours of reperfusion. We found that rats on fish oil supplemented diet had significantly lower MDA level, lower phospho-p38 MAPK concentration, lower caspase-3 and higher GSHPx activities in ischemic brain, 24h after reperfusion compared with rats on ordinary diet. Neurological score of ischemic rats on fish oil supplemented diet was significantly higher than that of ischemic rats on ordinary diet, however, it is still below the values obtained in sham operated rats.

The administration of fish liver oil significantly suppressed activation and phosphorylation p38 MAPK which led to a reduction in edema, and infarct volume. Blockade of the p38 pathway contributed to reduced injury in cerebral tissue, which led to improved neurological status. In the present study, omega 3 fatty acids intake after 24hours I/R showed significantly decreased activation and expression of p-p38, concomitant with decrease in oxidative stress and smaller infarction and less edema in both the brain and fewer neurological deficits. These results support our hypothesis that omega 3 fatty acids could be neuroprotective against I/R brain injury. The studies related to the beneficial effects of fish oil on ischemic brain had significantly increased.[51-54] Choi-kwon *et al.* [51] found that rats on fish oil supplemented diet for 6 weeks prior to MCAO had reduced brain infarction volume, reduced lipid peroxidation while GSHPx activity was increased in the brain tissue after 2 hours of ischemia compared to rats kept on ordinary diet before cerebral I/R. Cao *et al.* [52] found that administration of fish oil for 10 weeks prior to transient cerebral ischemia in gerbils, significantly decreased lipid peroxidation, increased antioxidant capacity and decreased hippocampal neuronal loss as compared with ischemic rats on ordinary diet. Berman et al confirmed that Docosahexaenoic acid confers neuroprotection in a rat model of perinatal hypoxia-ischemia.[54] Bas *et al.* [20] recently reported that fish oil pretreatment (0.4 g/kg/day for 14 days) decreased oxidative stress and apoptosis of hippocampal formation after bilateral CCA occlusion in rats. Michael-titus[53] study reported a therapeutic role for fish oil in the management of neurologic injury. They found that 6 weeks of fish oil supplemented diet after induction of spinal cord lesion by trauma or compression resulted in better neurologic outcome than rats with spinal cord lesion and received no dietary supplement by fish oil. The improvement was related to antioxidant anti-inflammatory properties of fish oil.

Conclusion & Recommendations:

Increased oxidative stress and activation of p38 MAPK signaling pathway represent an important mechanism of brain injury following cerebral ischemia/reperfusion in rats. Fish oil pretreatment attenuated this injury by reducing oxidative stress, phospho-p38 MAPK and apoptosis in ischemic rat brain. Fish oil supplement also improved the functional neurological outcome of ischemic rats after reperfusion. Targeting MAPK signaling pathways by inhibitors represents an important promising mechanism of neuroprotection. This study may provide a new therapeutic strategy for preventing brain dysfunction induced by ischemia.The use of fish oil supplement may be neuroprotective against ischemia/reperfusion brain injury and beneficial to prevent or ameliorate ischemic cerebral vascular disease.

Corresponding author
Gihan M. Sharara
Departments of [1]Medical Biochemistry and [2] Medical Physiology, Faculty of Medicine, Alexandria University

References

1. Chang L, Karin M. Mammalian MAP kinase signaling cascades. Nature, 2001; 410: 37-40.
2. Kyriakis M, Avnuch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev., 2001; 81: 807-69.
3. Morrison DK, Davis RJ. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev cell Dev Biol., 2003; 19: 91-118.
4. Yang SH, Sharrocks AD, Whitmarsh AJ. Transcriptional regulation by the MAP kinase signaling cascades. Gene, 2003; 320: 3-21.

5. Vikman P, Asar S, Henriksson M, Stenman E. Cerebral ischemia induces transcription of inflammatory and extracellular matrix related genes in rat cerebral arteries. Exp Brain Res., 2007; 183: 499-510.
6. Sumbayev VV, Yasinska TM. Regulation of MAP kinase-dependent apoptotic pathway: implications of reactive oxygen species and nitrogen species. Arch Biochem Biophys, 2005; 436: 406-12.
7. Baron FC, Kilgore KS. Role of inflammation and cellular stress in brain injury and central nervous system diseases. Clin Neurosci Res., 2006; 6: 329-56.
8. Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev., 2007; 54: 34-66.
9. Ferrer I, Planas AM. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. J Neuropathol Exp Neurol., 2003; 62(4): 329-39.
10. Danial NN, Korsmeyer SJ. Cell death: Critical control points. Cell, 2004; 116(2): 205-19.
11. Abramoy AY, Scorziello A, Duchen MR. Three distinct mechanisms generate reactive oxygen free radicals in neurons and contributes to cell death during anoxia and reoxygenation. J Neurosci., 2007; 27(5): 1129-38.
12. Blomgren K, Hagberg H. Free radicals, mitochondria and hypoxia-ischemia in developing brain. Free Radic Biol Med., 2006; 40(3): 388-97.
13. Zhang Q, Zhang G. Activation and autophosphorylation of apoptosis signal-regulating kinase 1 (ASK-1) following cerebral ischemia in rat hippocampus. Neurosci let., 2002; 329: 232-6.
14. Bourre JM. Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. J Nutr Health Aging, 2004; 8: 163-74.
15. Calon F, Lim GP, Yang F, Morihara T, Teter B, Ubeda O. Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. Neuron, 2004; 43(5): 633-45.
16. Catalan J, Moriguchi T, Slotnick B, Murthy M, Greiner RS, Salem Jr. Cognitive deficit in docosahexaenoic acid-deficient rats. Behav Neurosci., 2002; 116(6): 1022-31.
17. Salvati S, Attoi L, Benedetto R, Di Biase A, Leonardi F. Polyunsaturated fatty acids and neurological diseases. Mini Rev Med Chem., 2006; 6(11): 1201-11.
18. Collino M, Aragno M, Mastrocola R, Benetti E, Gallicctio M, Dianzani C, Danni O. Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: Effects of the PPAR-α agonist WY 14643. Free Radic Biol Med., 2006; 41: 579-89.
19. Tian D, Litvak V, Lev S. Cerebral ischemia and seizures induce tyrosine phosphorylation of PYK2 in neurons & microglia cells. The J Neurosci., 2000; 20 (17): 6478-87.
20. Bas O, Sogur A, Sahin O, Mollaoglu H, Ozen OA, Yaman M. The protective effect of fish n-3 fatty acids on cerebral ischemia in rat hippocampus. Neurochem Int., 2007; 50: 548-54.
21. Chen I, Gong Q, Xiao C. Effects of propofol, midazolam and thiopental sodium on outcome and aminoacids accumulation in focal cerebral ischemia-reperfusion in rats. Chin Med J., 2003; 116(2):292-6.
22. Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Stroke, 1995; 26: 627-34.
23. Chard T. An introduction to radioimmunoassay and related techniques. 4th ed. Amsterdam: Elsevier; 1990: 546.
24. Lowery OH, Rosenbrough NJ, Randal RJ. Protein measurement with Folin phenol reagent. J Biol Chem., 1951; 193: 265-75.
25. Cohen GM. Caspases: the executioners of apoptosis. Biochem J., 1997; 326(pt1): 1-16.
26. Holmes B, Park BH, Malawista SE, Quie PG, Nelson DL, Good RA. Chronic granulomatous disease in female: A deficiency of leukocyte glutathione peroxidase. NEngl J Med., 1970; 285: 217-21.
27. OhKawa H, Ohish N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid. Anal Biochem., 1979; 95: 351-8.
28. Milano G, Von Segesser LK, Morel S, Joncic A, Bianciardi P, Vassalli G, and Samaja M. Phosphorylation of phosphatidylinositol-3-kinase-protein kinase B and extracellular signal-regulated kinases 1/2 mediate reoxygenation-induced cardioprotection during hypoxia. Experimental Biology and Medicine, 2010, 235:401-410.
29. Bolay H, Dalkara T. Mechanisms of motor dysfunction after transient MCA occlusion: Persistent transmission failure in cortical synapses is a major determinant. Stroke, 1998; 29: 1988-94.
30. Menzies S, Hoff J, Betz A. Middle cerebral artery occlusion in rats: neurological and pathlogical evaluation. Neurosurg, 1992; 31: 100-7.
31. Sugino T, Nozaki K, Takagi Y, Hattori H, Hashimoto N, Nishida E. Activation of mitogen activated protein kinase after transient forebrain ischemia in gerbil hippocampus. J Neurosci., 2000; 20(12): 4506-14.
32. Walton KM, Dirocco R, Bartlett BA, Koury E, Marcy VR. Activation of p38 MAPK in microglia after ischemia. J Neurochem., 1998; 70: 1764-7.
33. Shioda S, Ozawa H, Dohi K, Mizushima H, Matsumoto K, Nakajo S, Takaki A. PACAP

protects hippocampal neurons against apoptosis: involvement of JNK/SAPK signaling pathway. Ann NY Acad Sci., 1998; 865(11): 111-7.
34. Hu BR, Liu Cl, Park DJ. Alteration of MAP kinase pathways after transient forebrain ischemia. J Cereb Blood Flow Metab., 2000; 20(7): 1089-95.
35. Irving EA, Barone FC, Reith AD, Hadingham SJ, Parsons AA. Differential activation of MAPK/ERK and p38/SAPK in neurons and glia following focal cerebral ischemia in the rat. Mol Brain Res., 2000; 77: 65-75.
36. Lee SJ, Drabik K, Van Wagoner NJ, Less S, Choi C, Dong Y. ICAM-1- induced expression of proinflammatory cytokines in astrocytes: involvement extracellular signal regulated kinase and p38 mitogen activated protein kinase pathway. J Immunol., 2000; 165(8): 4658-66.
37. Legos JJ, Erhardt JA, White RF, Lenhard SC. SB239063, a novel p38 inhibitor, attenuates early neuronal injury following ischemia. Brain Res., 2001; 892: 70-7.
38. Barone FC, Irving EA, Ray AM, Lee JC, Kumar S. SB 239063, a second generation p38 mitrogen activated protein kinase inhibitor, reduces brain injury and neurological deficits in focal cerebral ischemia. J Pharmacol Exp Ther., 2001; 296(2): 312-21.
39. Lewen A, Matz P, Chan PH. Free radical pathways in CNS injury. J Neurotrauma., 2000; 17: 871-90.
40. Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab., 2001; 21: 2-14.
41. Adibhatala RM, Hatcher JF. Phospholipase A2, reactive oxygen species and lipid peroxidation in cerebral ischemia. Free Radic Biol Med., 2006; 40: 376-87.
42. Canderlario-Jalil E, Mhadu NH, Al-Dalain SM, Martinez G, Leon OS. Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. Neurosci Res., 2001; 41: 233-41.
43. Lefkowitz MW, Reuhl K, Perry B, Chan PH, Inoye M, Mirochnitchenko O. Overexpression of human glutathione peroxidase protects transgenic mice against focal cerebral ischemia/reperfusion damage. Mol Brain Res., 1998; 53: 333-8.
44. Ishibashi N, Prokopenko O, Lefkowitz MW, Reutil KR, Mirochnitchenko O. Glutathione peroxidase inhibits cell death and glial activation following experimental stroke. Mol Brain Res., 2002; 109: 34-44.
45. Fujisawa T, Takeda K, Ichijo H. ASK family of proteins in stress response and disease. Mol Biotechnol., 2007; 37(1): 13-28.
46. Fujino G, Noguchi T, Takeda K, Ichijo H. Thioredoxin and protein kinases in redox signaling. Semin Cancer Biol., 2006; 16(6): 427-35.
47. Nagai H, Nuguchi T, Takeda K. Ichijo H. Pathophysiological role of ASK1-MAP kinase signaling pathways. J Biochem Mol Biol., 2007; 40(1): 1-6.
48. Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, and Pae HO. Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? Journal of Signal Transduction 2011; 48(11):1338-43. Article ID 792639.
49. Wu D, Cederbaum A. Activation of ASK-1 and downstream MAP kinases in cytochrome P4502E1 potentiated tumor necrosis factor alpha liver injury. Free Radic Biol Med. 2010; 49(3):348-60.
50. Van Laethem A, NYSK, Van Kelst S. Apoptosis signal regulating kinase-1 connects reactive oxygen species to p38 MAPK-induced mitochondrial apoptosis in UVB irradiated keratinocytes. Free Radic Biol Med., 2006; 41(9): 1361-71.
51. Choi-Kwon A, Park K, Lee H, Park M, Lee J, Jeon S, Choe M, Park K. Temporal changes in cerebral antioxidant enzymes activities after ischemia and reperfusion in a rat focal brain ischemia model: effect of dietary fish oil. Dev Brain Res., 2004; 152: 11-18.
52. Cao D, Xu J, Xue R, Zheng W, Liliu Z. Protective effect of chronic ethyl docosahexaenoate administration on brain injury in ischemic gerbils. Pharmacol Biochem Behav., 2004; 79: 651-9.
53. Michael-titus AT. Omega-3 fatty acids and neurological injury. Prostaglandins Leukot Essent Fatty Acids. 2007; 77: 295-300.
54. Berman DR, Liu YQ, Barks J and Mozurkewich E, . Docosahexaenoic acid Confers Neuroprotection in a Rat Model of Perinatal Hypoxia-ischemia potentiated by *E. coli* lipopolysaccharide-induced systemic inflammation Am J Obstet Gynecol. 2009; 200(3): 305.e1–305.e6.