

Expression of Bcl-2 and Bax after hippocampal ischemia in DHA + EPA treated rats

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Abstract To determine the impact of ω 3 fatty acids on post-ischemic expression of pro- and anti-apoptotic proteins in hippocampus, male rats were received 10 or 100 mg/kg [Docosahexaenoic acid (DHA) + Ecosapentaenoic acid (EPA); gavage; 21 days before ischemia to 2–10 days after ischemia]. Global cerebral ischemia reperfusion (IR) was performed using the four-vessel occlusion model; ischemia 8 min and reperfusion 6, 48 h and 10 days. IR increased Bcl-2 and Bax expression after 48 h ($p < 0.05$ and $p < 0.01$ vs. sham) and 10 days (only Bax; $p < 0.05$), without significant difference with

DHA + EPA groups after 6 h. But after 48 h expression of Bcl-2 increased ($p < 0.05$ vs. IR) and Bax decreased ($p < 0.05$). At day 10 after ischemia expression of Bax in DHA + EPA acid groups was less than IR ($p < 0.05$) and in 100 mg/kg DHA + EPA group Bcl-2 expression was more than IR ($p < 0.05$). These data suggested that long-term gavage with DHA + EPA increase hippocampal neurons survival for days after ischemia, revealed by increased Bcl-2 and decreased Bax expressions.

Keywords Bcl-2 · Bax · Ischemia reperfusion · Hippocampus · Omega 3 fatty acid

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Introduction

Omega-3 (ω 3) polyunsaturated fatty acids including docosahexaenoic acid (DHA) have crucial roles in normal physiology of central nervous system (CNS) [1, 2] and memory [3, 4]. ω 3 fatty acids deficiency during pre- and postnatal period cause variety of cognitive and behavioral disorders and neurodegenerative diseases [5, 6]. Beyond their requirement for normal brain functions, there are evidences that ω 3 fatty acids supplementation have beneficial effects in the treatment of psychiatric disorders [6, 7] and other pathological conditions, such as ischemic cerebral vascular disease and stroke [8, 9].

Most previous studies have demonstrated the effect of DHA treatment on attenuation of oxidative stress and apoptotic changes after hippocampus ischemia reperfusion [9, 10]. Administration of DHA before ischemia was also effective in reducing ischemic damage, as showed by 21 days treatment before ischemia and reduced learning and memory deficits besides significant preservation of hippocampus cells [11]. In the case of cerebral ischemia, oral

administration of DHA for 28 days before ischemia reduced brain damage and cerebral edema in gerbils [12, 13]. Although, some previous studies showed that long-term treatment with fish oil, a rich dietary source of DHA, can improve simply functional impairments without protective effects on neuronal death [14, 15], the results of recent in vitro study in hippocampal slices emphasized the importance of ω 3 fatty acids for cell protection and also suggested the involvement of anti-apoptotic signaling pathways [16].

In spite the crucial role of apoptosis in the development of normal brain [17], it contributes to ischemia induced neuronal loss [18, 19]. After global cerebral ischemia marked deletion of the CA1 neurons initiates during 2–4 days and reaches greatest effect within 1–2 weeks [20, 21]. Programmed cell death involves complex interactions among the key regulators of the apoptotic response; Bcl-2 family of proteins that includes anti-apoptotic Bcl-2 and pro-apoptotic Bax [22]. Many in vitro and in vivo studies that have applied defective herpes simplex viral (HSV) vectors to induce Bcl-2 over-expression suggests that over-expression of Bcl-2 can reduce ischemic brain injury in animal models of stroke [23–25]. Injection of Bcl-2 expressing plasmid into the lateral ventricle of rat brain and immediately following middle cerebral artery occlusion enhanced neurogenesis and survival of newborn neurons [26].

To our knowledge, the effect of long-term gavage with ω 3 fatty acids on the expression of Bcl-2 family of proteins in hippocampus after ischemia has not been studied yet. In the study by Pan et al. [27], long-term DHA administration elevated Bcl-2 expression in brain samples prepared 24 h after 90 min of ischemia. ω 3 fatty acids supplementation significantly reduced DNA fragmentation and caspase-3 activation in developing cerebellum of hypothyroid pups. The effect, that was associated with their ability to decrease the level of Bax protein and increase anti-apoptotic proteins like Bcl-2 and Bcl-x_L [28]. In other tissues like gastric epithelial cells and pancreatic acinar cells, ω 3 fatty acids inhibited oxidative stress-induced cell death, DNA fragmentation, and induction of pro-apoptotic p53 and Bax proteins [29, 30].

Because DHA could be a safe, relatively inexpensive, and orally available neuroprotective strategy for preventing or treating cerebral ischemia, especially in populations with high-risk factors [15], the main objective of the present study was to locate its effect on apoptotic proteins expression that have a role on neuronal cells destiny after ischemia.

Methods and materials

Animals

Male Wistar rats (230–300 g) were housed at a controlled temperature ($22 \pm 2^\circ\text{C}$) on a 12-h alternating light–dark

cycle and free access to water and a standard commercial diet with fixed value of carbohydrate, protein, fat, fiber and vitamins plus minerals (660, 230, 40, 60 and 10 g/kg, respectively; Nuvital Nutrients, Curitiba, Parana, Brazil). Three groups (each group $n = 7$) underwent sham operation (as controls), and six other groups (each group $n = 7$) were subjected to global cerebral ischemia. All experimental procedures used in the present study were approved by the Ethics Review Committee for Animal Experimentation in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Treatments and groups

A standardized formulation contained 250 mg of DHA and 50 mg of EPA (EPA ethyl ester (98%) and DHA ethyl ester (>95%) without any other fatty acids component, Biotik-do-Brasil Industria-e-Comercio Ltda, Brazil) was diluted in accordance to the group of study [10 mg/kg (DHA + EPA = 8.3 + 2.7 mg/kg) or 100 mg/kg (DHA + EPA = 83 + 27 mg/kg)] and a constant volume of 1 ml containing 10 or 100 mg/kg was administered by gavage. Sham operation or control groups received diluted solution without DHA or EPA for 21 days before sham operation or ischemia. DHA + EPA gavage started 21 days before ischemia and sustained until end of reperfusion (6, 48 h or 10 days after ischemia).

Ischemia

Transient, global cerebral ischemia was performed using the four-vessel occlusion model as previously described by Pulsinelli and Brierley [31]. Briefly, rats were anesthetized with a halothane (2%) and oxygen (flow = 1.5–2 L/min) mixture. After fixing animals in a stereotaxy instrument, electrocautery needle inserted through each alar foramen and the vertebral arteries were bilaterally electro-coagulated at the level of the first cervical vertebrae. Common carotid arteries were exposed by a midline cervical incision, and were loosely tied with a silk string. The wound was then sutured and anesthesia discontinued. After 24 h recovery period, carotid arteries were occluded by tightening the silk string for 8 min in awake and spontaneously ventilating animals. Animals were kept warm during ischemia and 2 h of reperfusion, and rectal temperature was monitored using a rectal probe (BIO-BRET2-ISO for Rats, BIOSEB, France). Same surgical procedures were conducted in sham-operated animals except occlusion of vertebral and carotid arteries. After 6, 24 h and 10 days of reperfusion, animals were anesthetized (Ketamine Hydrochloride; Parke-Davis, Cambridge, UK) and killed by decapitation and brains were prepared for Western blotting.

Western blotting

Brains were quickly removed and dissected on ice. Half of the hippocampus from each hemisphere were placed in solubilizing buffer [sodium dodecyl sulfate (2.5%), glycerol (10%), Tris-HCl (62.5 mmol/L), pH (6.8), and 2-mercaptoethanol (5%)] and boiled for 10 min. Then the whole tissue extract was frozen at -70°C . Protein concentration was measured using a bicinchoninic acid protein assay (absorbance at 560 nm, Pierce). For electrophoresis, the same amount of protein per sample (50 μg) was applied on a 12% sodium dodecyl sulfate polyacrylamide gels with 4.5% stacking gel. After cleaning with deionized water, membrane was incubated at 4°C in 0.1 mol/L sodium phosphate buffer (pH = 7.4), then incubated for 2 h with a 1:3,500 dilution of rabbit polyclonal anti-rat Bcl-2 or Bax antibody (Abcam). After three washes, membrane was incubated with HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) that followed by visualizing with the ECL chemiluminescence system (Amersham). The film was developed and used for the measurement of optic density.

Light microscopy and cell counting

Dependent to the reperfusion period; 6, 48 h or 10 days, brain was removed from the skull and post fixed by immersion in the formalin 5% for at least 2 days before histological processing. Then the samples were dehydrated and embedded in paraffin. A series of 10 μm thick were cut and stained with cresyl violet and sent to cell counting. A blind histopathologist assessed coronal sections obtained at 120 μm intervals through the prepared tissue of hippocampus. The number of all neural cells, including those in which the nucleus could not be recognized, and the number of neurons with distinct cytoplasmic and nuclear outlines were counted in four squares within an area of 2,500 μm^2 , using analysis imaging software (Soft Imaging System) at a total magnification of 400 \times . Neurons touching the bottom or right borders were included and those touching the upper or left borders were rejected.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analysis was performed in SPSS 14.0 software (SPSS, Inc., Chicago, IL). Between groups analysis, to compare the effects of ischemia and treatments, was performed by one-way ANOVA, followed by post-hoc analysis (Tukey test). Student's *t* test was used to compare the effects between different time scales of reperfusion periods after ischemia (6, 48 h and 10 days). $p < 0.05$ was taken to indicate a statistically significant difference.

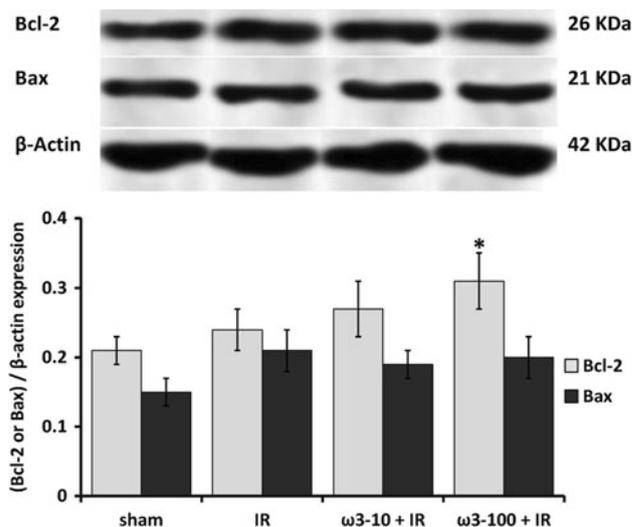


Fig. 1 Expression of Bcl-2 and Bax proteins in different groups 6 h after ischemia or sham operation. *Upper panel* illustrates the expression of Bcl-2, Bax and β -actin in a sample from each group. *Lower panel* shows the mean value of Bcl-2/ β -actin or Bax/ β -actin (ratio of expression of each protein to the expression of the β -actin). Data are shown as Mean \pm SEM. Sham: control group without ischemia reperfusion, IR: control group of ischemia (8 min) and reperfusion (6 h). ω 3-10 + IR and ω 3-100 + IR: Ischemia (8 min) and reperfusion 6 h at DHA + EPA (10 and 100 mg/kg) treated groups. * $p < 0.05$ versus sham-operated group. # $p < 0.05$ versus IR group

Results

All rats were alive to the end of the study. Body weight was measured at the beginning of gavage and also at the end of the study before killing the animals. The mean of body weight for DHA + EPA 10 mg/kg and DHA + EPA 100 mg/kg groups at the beginning of study was 262.51 ± 21.34 and 252.64 ± 19.47 which reached to 284.01 ± 33.04 and 301.77 ± 34.37 at the end of the study ($p = 0.14$ and $p = 0.29$; paired sample *t* test).

Effects of ischemia and reperfusion on expression of Bcl-2 and Bax

In comparison to the samples prepared 6 h after sham operation, 8 min global cerebral ischemia (IR) could not change the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in hippocampal samples, which prepared after 6 h of reperfusion (Fig. 1). However, after 48 h of reperfusion, expression of both Bcl-2 and Bax were increased compared to the samples prepared from sham-operated animals ($p < 0.05$ and $p < 0.01$, respectively; Fig. 2). After 10 days of reperfusion period, expression of Bax was also more than samples prepared 10 days after sham operation ($p < 0.05$, Fig. 3). Although the mean of

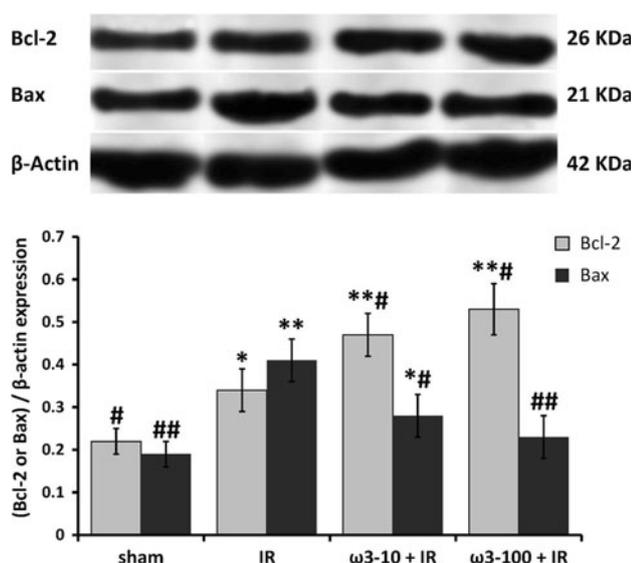


Fig. 2 Expression of Bcl-2 and Bax proteins in different groups 48 h after ischemia or sham operation. *Upper panel* illustrates expression of Bcl-2, Bax and β -actin in a sample from each group 48 h after operation. *Lower panel* shows the mean value of Bcl-2/ β -actin or Bax/ β -actin (ratio of expression of each protein to the expression of the β -actin). Data are shown as Mean \pm SEM. Sham: control group without ischemia reperfusion, IR: control group of ischemia (8 min) and reperfusion (48 h). ω 3-10 + IR and ω 3-100 + IR: Ischemia (8 min) and reperfusion 48 h at DHA + EPA (10 mg/kg and 100 mg/kg) treated groups. * p < 0.05 and ** p < 0.01 versus sham-operated group. # p < 0.05 and ## p < 0.05 versus IR group

Bcl-2 expression at tenth post-ischemic day was more than sham samples, the difference between two groups was not statistically significant (Fig. 3).

Effects of gavage with DHA + EPA on expression of Bcl-2 and Bax after ischemia

After 6 h of reperfusion

A 21-day gavage with 10 mg/kg DHA + EPA could not change the expression of Bcl-2 or Bax 6 h after ischemia when compared to the sham-operated animals or IR group (Fig. 1). At dose of 100 mg/kg, DHA + EPA induced a significant Bcl-2 protein expression in comparison with sham (p < 0.05, Fig. 1), which was not significant when compared with IR group. The effect of DHA + EPA 100 mg/kg gavage on the expression of Bax was not significant when compared with sham or IR treated rats.

After 48 h of reperfusion

Expression of Bcl-2 was significantly increased 48 h after ischemia in rats which were pretreated with 10 or 100 mg/kg of DHA + EPA in comparison to the sham-operated rats (p < 0.01, Fig. 2) and IR group (p < 0.05). Conversely,

expression of Bax protein in the 10 and 100 mg/kg DHA + EPA group decreased after 48 h of reperfusion compared to IR (p < 0.05 and p < 0.01, respectively; Fig. 2). Expression of Bax in 10 mg/kg DHA + EPA groups was more than sham-operated rats (p < 0.05).

10 days after ischemia

Expression of Bcl-2 remained higher 10 days after ischemia in 10 and 100 mg/kg DHA + EPA groups (Fig. 3) and was more than sham-operated rats (p < 0.05 and p < 0.01, respectively). Also, Bcl-2 expression in 100 mg/kg DHA + EPA group was more than IR group (p < 0.05). At day 10 after ischemia expression of Bax in 10 and 100 mg/kg DHA + EPA groups was less than IR group (p < 0.05), with no significant difference with sham-operated group (Fig. 3).

Variations in Bcl-2 and Bax expression in different time points of reperfusion period

In IR group 48 h after ischemia expression of Bcl-2 and Bax was significantly more than the expression at 6 h after ischemia (p < 0.05), while increased expression of Bcl-2 at day 10 after ischemia was not significant when compared to 6 h after ischemia (Figs. 1, 2, 3). Expression of Bax at day 10 after ischemia was also more than 6 h after ischemia (p < 0.05) with no significant difference when compared to 48 h after ischemia.

At 48 h after ischemia, expression of Bcl-2 was significantly more than the 6 h after ischemia in groups which had pre-ischemic gavage with 10 or 100 mg/kg DHA + EPA groups (p < 0.01). At day 10 after ischemia, the expression of Bcl-2 was also high compared with 6 h after ischemia (p < 0.05), without significant difference in comparison with 48 h after ischemia. In DHA + EPA groups, expression of Bax remained unchanged during 48 h or 10 days after ischemia when compared with the value of 6 h after ischemia.

Cell count

After 48 h and 10 days of reperfusion, a significant reduction was seen in the total number of normal pyramidal neuron population compared to the sham groups (p < 0.01 and p < 0.05; Table 1; Figs. 4, 5).

A 21-day gavage with 10 mg/kg DHA + EPA had no significant effect on number of normal cell population compared to IR or sham groups (Table 1; Fig. 6a). However, after 48 h and 10 days of reperfusion, there was significant difference between DHA + EPA 10 mg/kg treated group and IR group with more population of pyramidal neurons in DHA + EPA pretreated rats (p < 0.01 and p < 0.05, Table 1; Fig. 6b, c). The results

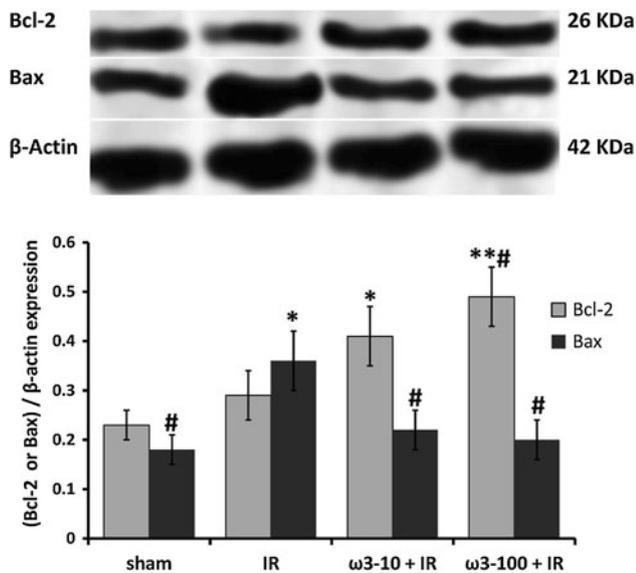


Fig. 3 Expression of Bcl-2 and Bax proteins in different groups 10 days after ischemia or sham operation. *Upper panel* illustrates expression of Bcl-2, Bax and β -actin in a sample from each group 10 days after operation. *Lower panel* shows the mean value of Bcl-2/ β -actin or Bax/ β -actin (ratio of expression of each protein to the expression of the β -actin). Data are shown as Mean \pm SEM. Sham: control group without ischemia reperfusion, IR: control group of ischemia (8 min) and reperfusion (10 days). ω 3-10 + IR and ω 3-100 + IR: Ischemia (8 min) and reperfusion 10 days at DHA + EPA (10 and 100 mg/kg) treated groups. * p < 0.05 and ** p < 0.01 versus sham-operated group. # p < 0.05 versus IR group

for DHA + EPA 100 mg/kg were similar to lower dose 10 mg/kg and significantly preserved the number of normal neurons in values more than IR group after 48 h or 10 days (p < 0.01 and p < 0.001; Table 1; Fig. 7b, c).

Discussion

Previously, a large body of evidence had suggested that ω 3 fatty acids supplements before ischemia is effective in

reducing ischemic damage to the hippocampus function and histology [19, 32]. However, the results of two other studies showed that the protective effects of ω 3 fatty acids supplements is limited on the improvement of functional parameters without effect on neuronal cell death [14, 15]. Following the recent study by Moreira et al. [16] that suggested the importance of ω 3 fatty acids for cell protection after ischemia and probable role of anti-apoptotic signaling pathways in this effect, we designed the present study to bring the molecular evidences for the role of gavage with DHA + EPA in apoptotic or anti-apoptotic pathways leading to cell protection or death.

Our findings showed that 8 min of ischemia increased the expression of both apoptotic and anti-apoptotic proteins Bax and Bcl-2 after 48 h of reperfusion, while the expression of Bax remained higher 10 days after ischemia. DHA + EPA supplements for 21 days before ischemia increased the expression of Bcl-2 protein and decreased Bax expression 48 h after ischemia, the effect that has persisted 10 days after ischemia and was concomitant with decreased number of neuronal cell loss in hippocamp. Although two doses of DHA + EPA (10 and 100 mg/kg) acted similar, it seems that at higher doses the effect starts early (6 h after ischemia) and last strongly after 10 days of reperfusion.

Studies carried out to determine the maturation rate of neuronal cell death and loss after a brief period of ischemia (4–15 min) in the CA1 region of rodents have shown that it is starting from 2 to 4 days after ischemia and reaches to the maximal level of 1–2 weeks later, while the extent of neuronal death was highly correlated to the duration of ischemia [20, 21]. In the present study expression of pro-apoptotic protein Bax increased 48 h after ischemia and was high 10 days later, which was in accordance to the time period of neuronal loss at hippocampus CA1 area. After 48 h of reperfusion, increased expression of anti-apoptotic protein Bcl-2 may reflect a compensatory response for counteracting the cell death process [33, 34].

Table 1 Normal cell population of pyramidal neurons in 2,500 μ m² area of hippocampal tissue of rats in different time points of reperfusion

Groups	Reperfusion period		
	6 h	48 h	10 days
Sham	19.64 \pm 3.33	21.08 \pm 3.51	19.9 \pm 3.62
IR	15.33 \pm 2.81	11.73 \pm 1.29**	12.75 \pm 1.36*
DHA + EPA 10 mg + IR	17.7 \pm 2.45	17.82 \pm 2.14##	17.49 \pm 2.10#
DHA + EPA 100 mg + IR	16.28 \pm 2.72	19.16 \pm 2.80##	19.51 \pm 2.23###

Data are shown as Mean \pm SEM. Data for each group in any time point is the mean of cell counts in two sides of hippocampus each included 3 sections. The total number of samples for each group includes $n = 2$ (two sides of hippocampus) \times 3 (three section from each side) \times 7 (number of rats per group) = 42

* p < 0.05 and ** p < 0.01 versus sham group

p < 0.05, ## p < 0.01 and ### p < 0.001 versus IR group

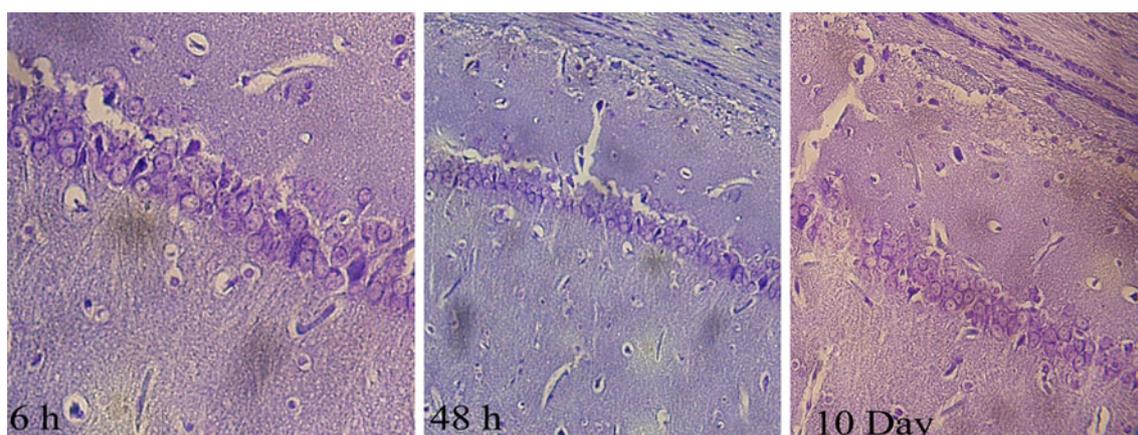
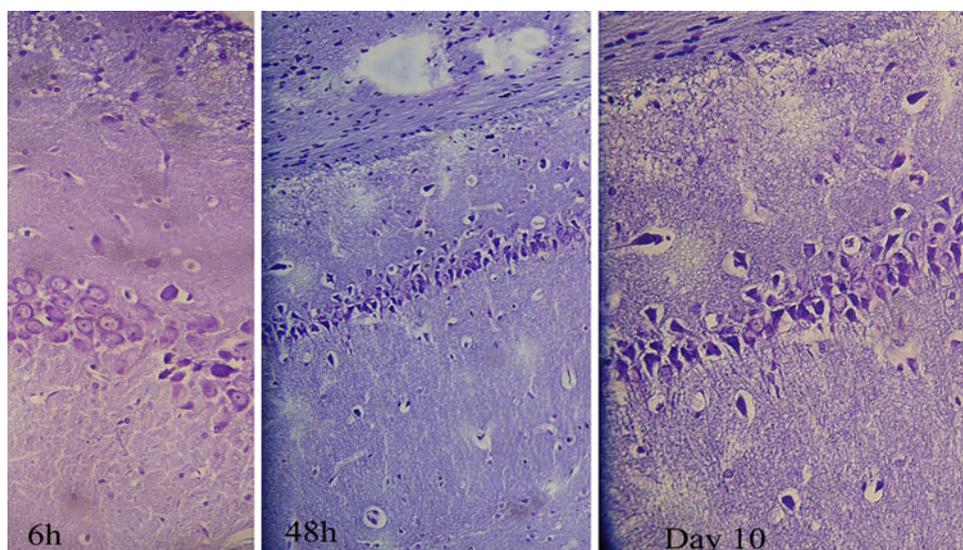


Fig. 4 Normal cell population of pyramidal cells of hippocampus in sham groups after 6 h (a), 48 h (b) and 10 days (c) of operation, $\times 400$ cresyl violets

Fig. 5 Normal cell population of pyramidal cells of hippocampus in IR groups after 8 min of ischemia in all groups followed by 6 h (a), 48 h (b) and 10 days (c) of reperfusion period. The population of normal cells was decreased after 48 h and 10 days of reperfusion, $\times 400$ cresyl violets



The effect of increased expression of Bcl-2 on blocking both apoptosis and necrosis has been demonstrated previously [35]. In vitro and in vivo studies using defective herpes simplex viral (HSV) vectors to over-express Bcl-2 protein showed protection against ischemic injury [36, 37]. Expression of Bcl-2 protein was increased 48 h after ischemia and remained high up to day 10 in both DHA + EPA doses that was concomitant with decreased expression of Bax protein in these groups (Figs. 2, 3). The over-expression of Bcl-2 not only protected against the ischemic injury to the neural cells, but also enhanced survival of newborn neurons in primary hippocampal cultures and in adult rat brain and enhanced neurogenesis under normal and ischemic conditions and seems to be a useful strategy in repair of brain after stroke [26, 38].

The impact of $\omega 3$ fatty acids on the expression of Bcl-2 and Bax proteins has been evaluated in other organs and

brain [27–30]. In the human gastric epithelial cells $\omega 3$ fatty acids inhibited oxidative stress-induced cell death, DNA fragmentation, and induction of p53 and Bax of the cells [29]. Other study evaluated the effect of $\omega 3$ fatty acids on suppression of apoptotic cell death in pancreatic acinar cells exposed to hydrogen peroxide, and showed that DHA inhibited DNA fragmentation and expression of apoptotic genes p53 and Bax [30]. DHA + EPA supplemented to the pregnant and lactating rats to evaluate its effect on apoptosis in developing brain model (hypothyroidism-induced neuronal apoptosis) and increased the levels of anti-apoptotic proteins like Bcl-2 and Bcl-x_L at cerebellum [28]. In study by Pan et al. DHA were administered intraperitoneally for 3 days or 6 weeks before 90 min focal cerebral ischemia in rats, and increased Bcl-2 expression in brain samples prepared after 24 h of reperfusion that was accompanied by decreased

Fig. 6 Normal cell population of pyramidal cells of hippocampus in DHA + EPA 10 mg/kg pretreated rats after IR. IR: Ischemia (8 min) and reperfusion (6, 48 h and 10 days). Samples prepared 6 h (a), 48 h (b) and 10 days (c) after reperfusion period. The population of normal cells was preserved over 48 h and 10 days of reperfusion period in these group. $\times 400$ cresyl violets

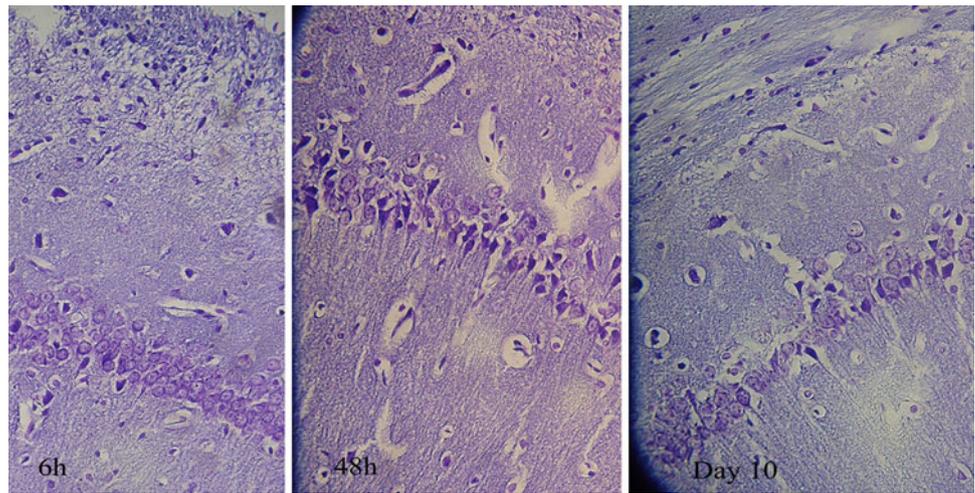
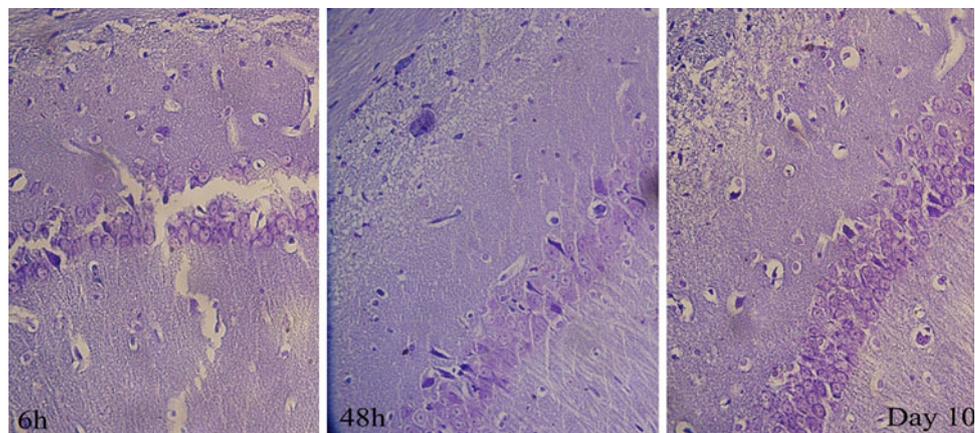


Fig. 7 Normal cell population of pyramidal cells of hippocampus in DHA + EPA 100 mg/kg pretreated rats after IR. IR: Ischemia (8 min) and reperfusion (6, 48 h and 10 days). Samples prepared 6 h (a), 48 h (b) and 10 days (c) after reperfusion period. The population of normal cells was preserved over 48 h and 10 days of reperfusion period in these groups, $\times 400$ cresyl violets



brain edema, malondialdehyde production and caspase-3 activity [22].

The histological findings confirmed the protective effects of gavage with DHA + EPA before ischemia by preserving neuronal populations in numbers close to sham group and preventing from significant neuronal loss which was observed 48 h and 10 days after ischemia in IR group. Study by Blondeau et al. [39], revealed that injection of 500 nmol/kg linolenic acid for 3 days (interavenous) prior to 6 min global brain ischemia preserved the normal population of neurons which was concomitant with increased expression of neuroprotective HSP70 heat shock protein (anti-apoptotic protein) and decreased expression of Bax protein during 3 days after ischemia. These findings confirm the long-term effects of $\omega 3$ fatty acids on protection against ischemia induced apoptosis and neuronal cell loss.

After the studies that showed the protective effects of $\omega 3$ fatty acids on improvement of hippocampus function after ischemia [14, 15], the present study brought molecular evidences that confirm its effect on decreasing neuronal loss at hippocampal tissue 48 h and 10 days after

ischemia. Therefore, we suggest that long-term gavage with DHA + EPA can preserve hippocampal tissue against ischemic injury by induction of Bcl-2 protein expression and suppression of Bax protein expression and reducing neuronal loss that last for days after ischemia.

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