The Neuroprotective Effects of Flaxseed Oil Supplementation on Functional Motor Recovery in a Model of Ischemic Brain Stroke: Upregulation of BDNF and GDNF

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Abstract- Cerebral ischemic stroke is a common leading cause of disability. Flaxseed is a richest plant-based source of antioxidants. In this study, the effects of flaxseed oil (FSO) pretreatment on functional motor recovery and gene expression and protein content of neurotrophic factors in motor cortex area in rat model of brain ischemia/reperfusion (I/R) were assessed. Transient middle cerebral artery occlusion (tMCAo) in rats was used as model brain I/R. Rats (6 in each group) were randomly divided into four groups of Control (Co+normal saline [NS]), Sham (Sh+NS), tMCAo+NS and tMCAo+FSO. After three weeks of pretreatment with vehicle or FSO (0.2 ml~800 mg/kg body weight), the rats were operated in sham and ischemic groups. Ischemia was induced for 1 h and then reperfused. After 24 h of reperfusion, neurological examination was performed, and animals were sacrificed, and their brains were used for molecular and histopathological studies. FSO significantly improved the functional motor recovery compared with tMCAo+NS group *(P*<0.05*)*. A significant reduction in brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) mRNAs and protein levels were observed in the tMCAo+NS group compared with Co+NS and Sh+NS group *(P*<0.05*)*. A significant increase of BDNF and GDNF mRNAs and proteins was recorded in the tMCAo+FSO group compared with Co+NS, Sh+NS and tMCAO+NS groups *(P*<0.05*)*. The results of the current study demonstrated that pretreatment with FSO had neuroprotective effects on motor cortex area following cerebral ischemic stroke by increasing the neurotrophic factors (BDNF, GDNF).

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Keywords: Flaxseed oil; BDNF; GDNF; Motor cortex; Brain stroke

Introduction

Cerebral ischemic stroke is a major leading cause of death around the world (1). The cerebral ischemic stroke occurs in about 80% of all stroke cases by obstruction of the cerebral blood supply (2). This condition can damage different functions such as sensory and motor functions, cognition and communication depending on the regions where the ischemic stroke involve (3).

No long-term and effective clinical therapeutics have been introduced for motor impairment following the stroke (4,5). Several studies have shown that natural and herbal medicines can be applied as a protective agent to treat a wide range of disease categories (6-10). Flaxseed (FS, *Linum usitatissimum*) meal and flaxseed oil (FSO) have been used as food in Asian, European and African centuries. Recently, FS was introduced as the richest plant-based source of omega-3 (*ω*-3/*n*-3) polyunsaturated fatty acids (PUFAs), specifically α-linolenic acid (ALA) (11,12). Different animal studies reported that the dietary supplementation of FS/FSO has protective effects during ischemia-reperfusion and hypercholesterolemic

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conditions (13-15). These healing effects are associated with the activity of ALA or its longer-chain PUFA derivatives such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (16). Moreover, FS is a dietary source of fiber (17) and phytoestrogenic lignans that are suggested to have free radical scavenging and/or antioxidant characteristics (18-21). Further, it was shown that deprivation of n-3 decreases the neurotrophic factor content in animal models (17,22).

Brain-derived neurotrophic factor (BDNF), identified as one of the critical growth factors promotes the neuronal survival and regulate the different neuronal functions such as differentiation, migration and synaptic function in the central nervous system (CNS). It induces these effects through the tropomyosin receptor kinase B (TrkB) signaling (23,24). It has been suggested that BDNF decrease the infarct size following brain ischemia (25,26), induce widespread neuronal remodeling and improve motor function (27). Glial cell line-derived neurotrophic factor (GDNF) also has been shown to have neuroprotective effects. This can decrease the brain edema and size of infarct following ischemic brain injury (27-29). GDNF promotes the function of various types of peripheral neurons and regulates their differentiation and migration (29-32).

As FS is one of the richest dietary sources of PUFAs and antioxidants and discussed above, 3 weeks pretreatment with FSO was used, and after 24 h of ischemia-reperfusion, its effects on neurotrophic factors were evaluated following transient middle cerebral artery occlusion (tMCAo) model of ischemia.

Materials and Methods

Animals

In this study, 24 male Wistar rats with 26 to 28 months aged (classified as old) and weighing 280 to 320 gr were kept in a standard animal room with 12 h light/dark cycle. They kept in the cages with free access to food and water. The animals cared in accordance with the guidelines of Tehran University of medical sciences on animal care. The experiment was approved by Animal Ethics Committee of this university.

Transient middle cerebral artery occlusion (tMCAo)

Focal cerebral ischemia/reperfusion was induced in the left hemisphere tMCAo. Under the anesthesia induced with isoflurane (5.0%, Baxter International) and spontaneously inhaled with 1.0-2.0% isoflurane, the left common carotid artery (CCA) was exposed through a midline neck incision. Adjacent vagus nerve was isolated, and the bifurcation of CCA was found. Then, internal carotid artery (ICA) was carefully dissected to conduct an intraluminal 4-0 nylon monofilament (Doccol Co., USA) to block the origin of the medial carotid artery (MCA). In this way, the monofilament was inserted into the CCA and conducted into ICA to occlude the MCA. In the sham-operated group, the surgery was applied by inserting the monofilament into the ICA and immediately withdrew.

Animal groups and treatments

Rats were randomly allocated to the following three groups (n=8 for each group):

- **Group I: Co+NS.** Rats served as vehicle control and received saline (0.2 ml saline/rat) by oral administration (gavage) for 3 weeks without any procedure.
- **Group II: Sham+NS.** Rats received normal saline (0.2 ml saline/rat) by gavage (orally) administration for 3 weeks before the procedure and operated without ischemia induction.
- **Group III: tMCAo+NS.** Rats received normal saline (0.2 ml saline/rat) by gavage (orally) administration for 3 weeks before the procedure and subjected to occlusion for 1 h followed by 24 h reperfusion.
- **Group IV: tMCAo+FSO.** Rats received 0.2 ml (~800 mg/kg body weight) of flaxseed oil (FSO) by gavage (orally) administration for 3 weeks before the procedure and induced brain ischemia.

Neurologic examination

The neurologic examination was performed for each rat 24 h after procedure according to Bederson *et al*., study (33). This neurological assessment included a grading System of 0-3. In this system, the flexion of forelimb contralateral to the injured hemisphere after suspending each rat by the tail and circling behavior of rats toward the paretic side were the criteria for evaluation. Rats with normal neurological function extend both forelimbs toward the floor. Grade 0 (Normal): no observable deficit; grade 1 (Moderate): forelimb flexion; grade 2 (Severe): decreased resistance to lateral push (and forelimb flexion) without circling; grade 3 (Severe): the same behavior as grade 2 with circling.

Total RNA extraction and quantitative real-time PCR

After 24 h, gene expression of BDNF and GDNF were evaluated (n=3 in each group). Under anesthesia, the brain rapidly removed and placed in ice-cold saline (0.9%). Then, the coronal sections (1 mm) were prepared,

and the left motor cortex area was isolated (34). The expression of BDNF and GDNF genes in motor cortex area was measured using quantitative real time PCR (ABI PRISM 7500 real-time PCR system, Roche Diagnostics, Germany). The total RNA was extracted, and cDNA was synthesized from 1 μg total RNA using PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan). Quantitative real-time PCR was carried out in a Cycler (Light Cycler 2.0, Roche) using SYBR Green (Takara Bio Inc., Otsu, Japan). The primers were designed using Allele ID software (version 6) (Table 1). The b-actin gene considered as the internal control standard.

Table 1. The list of primers and expected length of products

Genes	Forward primer	Reverse primer	Amplicon size (bp)
BDNF	AATAATGTCTGACCCCAGTGCC	CTGAGGGAACCCGGTCTCAT	196
GDNF	GCGCTGACCAGTGACTCCAA	GCGACCTTTCCCTCTGGAAT	189
b-actin	ACAACCTTCTTGCAGCTCCTC	CTGACCCATACCCACCATCAC	200

Concentration of BDNF and GDNF proteins in motor cortex area

The protein concentrations of BDNF and GDNF in motor cortex area rats (three in each group) were evaluated using ABCAM ELISA kits in accordance with their manufacturer's guidelines. Motor cortex area was harvested and homogenized using lysis. Then it was centrifuged at 14,000 rpm at 4° C for 3 min, and the supernatant was diluted using sample buffer. Subsequently, it was incubated in 96-well flat-bottom plates previously coated with anti-BDNF and anti-GDNF monoclonal antibodies. Then, the plates were incubated with polyclonal anti-rabbit antibody for 2 h. For calculating the protein concentration, the color reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm.

Histopathological study for determination of neuronal damage

Three rats in each group were selected for light microscopy study. Under deep anesthesia, the brains were prefixed by a transcardial perfusion 2500 mL normal saline (NS) followed by 250 mL of 4% paraformaldehyde (PFA, Sigma) in 0.1 M phosphate buffer pre-fixation). The brains were cut coronally into 3-5 mm-thick sections including coronal sections (1.7-2.8 mm posterior to the bregma) (34). The post-fixed was performed using 10% formalin at 4° C for 72 h. For histopathological studies, the brain samples were embedded in paraffin, and 5 μm coronal sections (one from every five sections) were prepared using a rotary microtome (Leica Biosystems, Milan, Italy). For light microscopy observation, the tissue sections stained with Hematoxylin and eosin (H and E) according to the standard protocol (35) and analyzed using a light field microscope (Olympus, CX31, Tokyo, Japan). The shrunken and ischemic cells with dark appearance (dark neurons) in the motor cortex were counted in at least three ×400 images prepared using a camera connected to the microscope (36). The number of dark neurons was expressed as a percentage of the total number of neurons.

Statistical analysis

Quantitative data were expressed as mean±standard error of the mean (SEM). The data were analyzed statistically by one-way analysis of variance (ANOVA) with post hoc of Tukey's tests. Non-parametric test (Kruskal-Wallis Test) were used to analyzing the data with abnormal distribution. The significant level was considered as $P<0.05$.

Results

Effects of FSO pretreatment on functional motor recovery in rats with tMCAo

According to the figure 1, there were significant differences in the mean grade of the neurological score. A significant decrease was observed in the mean grade of the neurological score in the tMCAo+NS group compared to Co+NS and Sh+NS groups ($P<0.05$, figure 1*)*. There was significant increase in the mean grade of the neurological score in the tMCAo+FSO group compared with tMCAo+NS groups ($P<0.05$, figure 1).

Effects of FSO pretreatment on BDNF and GDNF gene expression in motor cortex area in rats with tMCAo

According to the figure 1a, there were significant differences in the gene expression of BDNF and GDNF between study groups. A significant decrease was observed in the gene expression of BDNF in the tMCAo group compared with Co and Sh groups ($P<0.05$, figure 2a*)*. There was significant increase in the gene expression of BDNF in the tMCAo+FSO group compared to Co+NS, Sh+NS and tMCAo+NS groups ($P<0.05$, figure 2a). Furthermore, a significant increase was recorded in the

gene expression of GDNF in the tMCAo+FSO group compared to Co+NS, Sh+NS and tMCAo+NS groups (P<0.05, figure 2b).

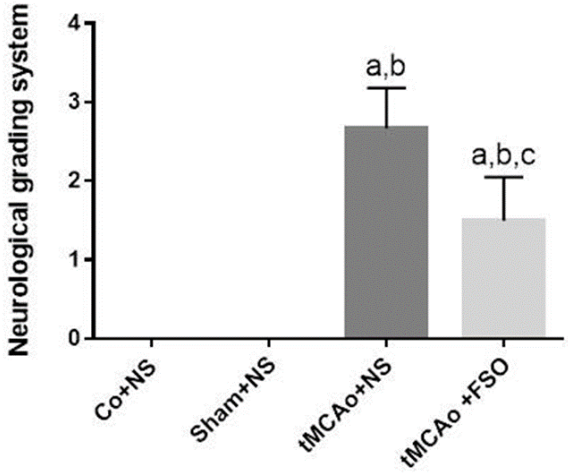

Figure 1. Effects of FSO on functional motor recovery following brain ischemia in rat. a:P < 0.05 compared to Co+NS group; b:P<0.05 compared to Sham+NS groups, c:P<0.05 compared to the tMCAo+NS group. Co+NS: normal group with normal saline pretreatment, Sham+NS: sham-operated group with normal saline pretreatment; tMCAo+NS: Ischemia induction group with normal saline pretreatment, tMCAo+FSO: Ischemia induction group with flaxseed oil pretreatment

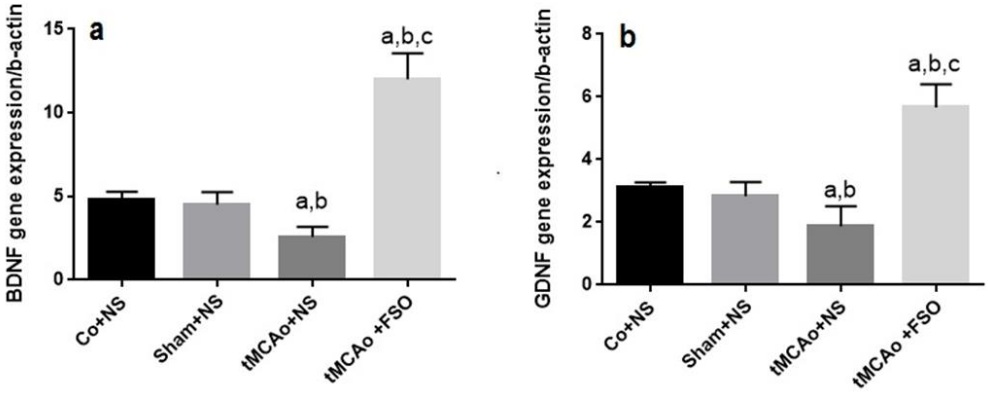

Figure 2. Effects of FSO on neurotrophic factor gene expression of motor cortex area following brain ischemia in rat. a) BDNF gene expression, b) GDNF gene expression. a:P< 0.05 compared to Co+NS group; b:P < 0.05 compared to Sham+NS groups, c:P < 0.05 compared to the tMCAo+NS group. Co+NS: normal group with normal saline pretreatment, Sham+NS: sham-operated group with normal saline pretreatment; tMCAo+NS: Ischemia induction group with normal saline pretreatment, tMCAo+FSO: Ischemia induction group with flaxseed oil pretreatment

Effects of FSO pretreatment on BDNF and GDNF protein concentration in motor cortex area in rats with tMCAo

According to ELISA results, the protein concentration of BDNF was reduced in the tMCAo group compared with Co+NS and Sh+NS groups (P<0.05, figure 3a). There was significant increase in the concentration of BDNF protein in the tMCAo+FSO group compared with Co+NS, Sh+NS and tMCAo+NS groups (P<0.05, figure 3a). The protein concentration of GDNF significantly was reduced in the tMCAo group compared to Co+NS and Sh+NS groups (P<0.05, figure 3b). Moreover, a significant increase was observed in the protein concentration of GDNF in the tMCAo+FSO group compared to Co+NS, Sh+NS and tMCAo+NS groups (p<0.05, figure 3b).

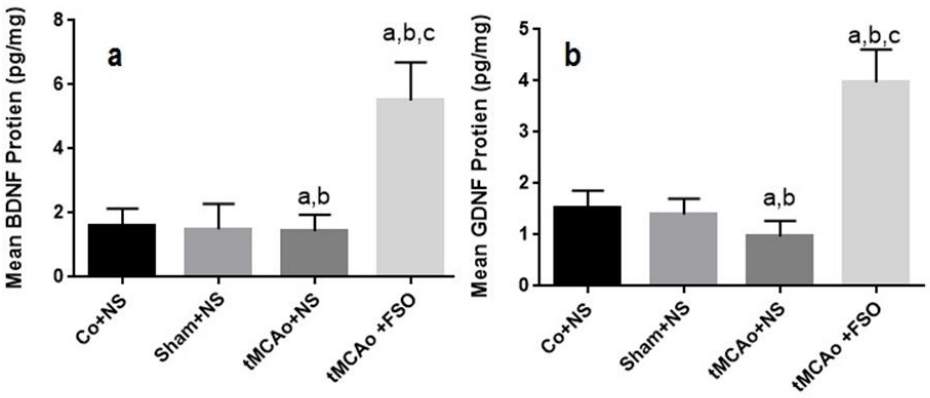

Figure 3. Effects of FSO on neurotrophic factor protein concentration of motor cortex area following brain ischemia in rat. a) BDNF protein concentration, b) GDNF protein concentration. a:$P<0.05$ compared to Co+NS group; b:$P<0.05$ compared to Sham+NS groups, c:$P<0.05$ compared to the tMCAo+NS group. Co+NS: normal group with normal saline pretreatment, Sham+NS: sham-operated group with normal saline pretreatment; tMCAo+NS: Ischemia induction group with normal saline pretreatment, tMCAo+FSO: Ischemia induction group with flaxseed oil pretreatment

Effects of FSO pretreatment on the number of dark neurons in motor cortex area of in rats with tMCAo

The percentage of dark neurons was calculated in the cortex area of hippocampus in the study groups. There was significant increase in the mean percentage of dark neurons in tMCAo+NS and tMCAo+FSO groups compared with Co+NS, Sh+NS ($P<0.05$, figure 4). A significant decrease was reported in the mean number of dark neurons in tMCAo+FSO compared to tMCAo+NS group ($P<0.05$, figure 4).

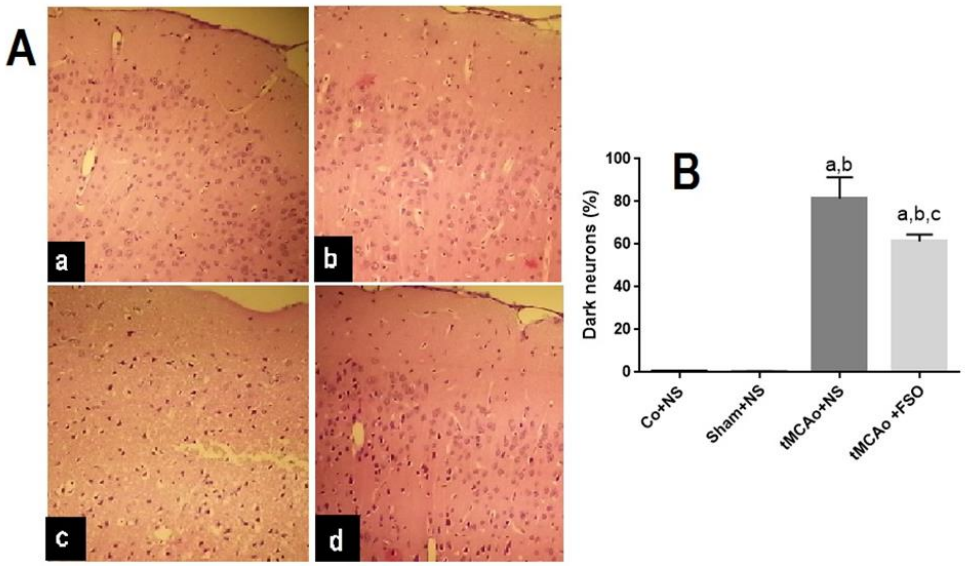

Figure 4. Effects of FSO on the percentage of dark neurons of cortex area following brain ischemia in rat. A-a) Co+NS group, A-b) Sham+NS group, A-c) tMCAo+NS group, A-d) tMCAo+FSO group (×400),. B) Comparing the dark neuron percentage in different groups a:$P<0.05$ compared to Co+NS group; b: a:$P<0.05$ compared to Sham+NS group, c: $P<0.05$ compared to the tMCAo+NS group. Co+NS: normal group with normal saline pretreatment, Sham+NS: sham-operated group with normal saline pretreatment; tMCAo+NS: Ischemia induction group with normal saline pretreatment, tMCAo+FSO: Ischemia induction group with flaxseed oil pretreatment

Discussion

A cascade of pathological events following the brain ischemia is related to a complex process involving

metabolic dysfunction, neuronal loss and neurological deficit symptoms (37,38). The most common affected areas are cerebral cortex and striatum after transient brain ischemia (39,40). The brain I/R occurs with high incidence of disabilities and mortalities which were confirmed in tMCAo model of brain ischemic stroke (41,42). To mimic these clinical conditions, we used tMCAo as a model of cerebral I/R for experimental evaluations in rats.

As the mechanisms of the brain, I/R were investigated in different studies in recent years, the new neuroprotective strategies have been developed to protect the brain from cerebral ischemia. The impacts of different agents with natural sources have been tried on various types of cerebral I/R models (43-46). FSO supplementation with high concentrations of omega-3 fatty acids and lignans and antioxidant and anti-inflammatory properties were used in different studies to reduce the injuries from I/R condition in different tissues (14,47,48).

The finding of the present study demonstrated that the pattern of the neurotrophic factors (BDNF and GDNF) expression was disrupted in motor cortex area after tMCAo. Further, pretreatment with FSO could upregulate the expression of neurotrophic factors (BDFN and GDNF) in the cerebral motor cortex area following brain I/R.

Different mechanisms have been suggested for beneficial effects of FSO against neurological defects. It was shown that FS might ameliorate I/R injury by increasing the reactive oxygen species (ROS) detoxification and/or decreasing ROS generation (47). Moreover, the results of studies revealed that oral consumption of ALA enhances the serum level of BDNF in healthy human adults (49). In a study by Luo *et al.*, (2014), it was stated that fat-1 mice (*fat-1* gene encoding for ω-3 fatty acid desaturase) with high endogenous ω-3 PUFAs exhibit protective effects on hippocampal CA1 neurons and cognitive functions in a global ischemia injury model.

Ma *et al.*, (2013) demonstrated that secoisolariciresinoldiglycoside (SDG) as a predominant lignan in flaxseed, could enhance the BDNF level in the frontal cortex of in ovariectomized mice subjected to unpredictable chronic stress (50). It should be considered that administration of neurotrophic factors can promote the neuronal survival following the brain ischemia based on literature. In a review article, Chen *et al.*, (2013) demonstrated that BDNF is a safe and potential agent with neuroprotective characteristics against brain IR injury (51). In addition, Duarte *et al.*, (2012) showed the neuroprotective effects of GDNF following the ischemic brain (28). According to the results, pretreatment with FSO can protect the neurons of motor cortex area against the IR injury by increasing the BDNF and GDNF levels.

Based on the findings, the percentage of dark neurons in the motor cortex area increased in the tMCAo group, and the neurological score showed the severe disabilities in this group. Among the different animal models for brain ischemia, tMCAo has been introduced as a rodent model of ischemia which is performed to study the mechanisms triggered following the ischemia condition and evaluate the potential therapies (52,53). In the current study, pretreatment with FSO could effectively enhance the functional motor recovery following the ischemia. Moreover, the percentage of dark neurons in motor cortex area reduced in this group compared to the tMCAo group. Same results which showed that FS could improve memory and motor activity in animal models were obtained from different investigations. Fernandes *et al*., (2011) showed that maternal intake of FS-based diet improved spatial memory and locomotor activity by increasing the on the fatty acid profile of hippocampus (54). Moreover, de Barros Mucci *et al*., (2015) found that maternal FS intake improved motor hyperactivity and spatial memory in a rat model of neonatal hypoxic-ischemic encephalopathy (55).

According to these findings, it can be concluded that pretreatment with FSO exhibit neuroprotective effects on neurons of motor cortex area and enhance the functional motor recovery following the cerebral I/R injury by increasing the BDNF and GDNF levels

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