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Evaluation of Brain Monoamines in Experimental Brain Injury: Impact of Polyunsaturated Fatty Acids

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ABSTRACT

Objective: This study aimed to compare the anti-inflammatory and antioxidant properties of flaxseed and fish oils as sources of omega-3 fatty acids on lipopolysaccharide (LPS) induced brain injury (BI) in rats. Methods: sixty male albino rats were used in this study and classified into six groups including control, flaxseed oil, fish oil, lipopolysaccharide, treated flaxseed oil and treated fish oil groups. Serum tumour necrosis factor- α (TNF- α) and interleukin - 1 α (IL- 1 α) were determined by ELISA. Brain malondialdehyde (MDA), superoxide dismutase (SOD) activity, reduced glutathione (GSH) and total antioxidant capacity (TAC) were estimated by colorimetric methods. Brain neurotransmitters were carried out by reversed phase HPLC and UV detection was performed at 270 nm. Immunohistochemistry of cyclooxygenases (COX-1, COX-2) was also performed. Results: The data showed that LPS significantly increased brain MDA, neurotransmitters and COX-2 concomitant with a reduction in brain SOD, GSH, TAC and COX-1. Contrarily, flaxseed and fish oils supplementation improved these values in treated groups. Conclusion: Omega-3 fatty acids in the form of flaxseed and fish oils are considered promising agents in attenuating brain injury and reducing the elevation of brain neurotransmitters due to their antioxidant and anti-inflammatory effects.

Key words: Brain injury, Neurotransmitters, Fatty acids, Cytokines, COX.

INTRODUCTION

Brain injury (BI) induces a local inflammatory response and the activation of microglia and cytokine production in addition to mobilization and permeation of immuno-inflammatory cells; this response may participate in neuronal damage and cell death [1].

In particular, IL -1 α , IL -1 β , IL - 6 and TNF - α have been found to be involved in the acute inflammatory response that results from BI and may contribute to the secondary damage that results from the BI induced inflammatory response [2].

Generation of oxygen free radicals following BI is one of the most confirmed aspects of secondary injury to brain tissues [3].

Lipopolysaccharide (LPS), the major component of the outer membrane of Gram negative bacteria is known as endotoxins that elicit strong immune responses in experimental animals [4]. LPS is used widely in experimental animals to study the effect of inflammatory stimuli on brain functions. Systemic administration of LPS has been shown to impair antioxidant mechanisms, induce lipid peroxidation, impair mitochondrial redox activity [5, 6] and increase different pro-inflammatory cytokines [7] leading to neuronal damage [8] and hence impairment of neurotransmitters production.

Omega-3 fatty acids (n-3) comprise a family of unsaturated fatty acids that consists of α -linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); which are essential fatty acids because they must be obtained from the food due to the inability of mammals to synthesize these fatty acids *de novo* [9]. These fatty acids serve as energy substrates and integral membrane components; therefore they play a fateful role in regulation of cell membrane fluidity, dopaminergic and serotonergic transmission, membrane-bound enzymes and cellular signal transduction [10].

Flaxseed oil contains high amount of polyunsaturated fatty acids [11], especially ALA [12, 13]. However, fish oil contains EPA and DHA that are known to reduce body inflammation and have other health benefits [14].

From this point of view, we planned this study to compare the role of flaxseed oil and fish oil as different sources of omega-3 fatty acids in attenuation of oxidative stress and inflammation induced by LPS and to evaluate the role of supplemented oils in preventing the impairment of brain monoamines in experimental brain injury induced by LPS.

MATERIALS AND METHODS

Materials

Norepinephrine, dopamine and serotonin (HPLC standards), and lipopolysaccharide were purchased from Sigma Aldrich Chemicals Company (St Louis, Missouri, USA).

Flaxseed and fish oils were purchased from local market (Cairo, Egypt).

Experimental Animals

Sixty male albino rats (180-200 g) were obtained from the animal house of the National Research Centre - Giza - Egypt and were allowed a standard rodent chow diet and water *ad libitum*. They were housed in standard environmental conditions. The ambient temperature was 25 ± 2 °C and the light / dark cycle was 12 / 12 hours. All animals received human care in compliance with guidelines of the Ethical Committee of National Research Centre, Egypt, and followed the recommendations of National Institutes of Health Guide for Care and Use of Laboratory Animals.

Methods

Induction of brain injury

LPS was dissolved in normal saline, pH was adjusted at 7.4 and intraperitoneal (IP) injected in rats (200 μ g /kg b.w.); after 4 hours, blood was withdrawn from the retro-orbital venous and brain was removed from each rat to confirm induction of BI [15].

Experimental design

Sixty male albino rats were divided into six groups (10 rats in each group) as follow:

Group I (control group): healthy rats received a vehicle.

Group II (flaxseed oil group): healthy rats received 1.2 ml flaxseed oil / kg b.w. / day orally for 30 days [16].

Group III (fish oil group): healthy rats received 1.2 ml fish oil / kg b.w. / day orally for 30 days [16].

Group IV (LPS group): healthy rats received a vehicle before injection of LPS at the end of the experiment.

Group V (flaxseed oil- LPS group): healthy rats received 1.2 ml flaxseed oil / kg b.w. / day orally for 30 days followed by LPS injection.

Group VI (fish oil – LPS group): healthy rats received 1.2 ml fish oil / kg b.w. / day orally for 30 days followed by LPS injection.

After the experimental period, rats were kept fasting for 12 hours before blood sampling. Blood was withdrawn from the retro-orbital venous plexus of the eye using a capillary tube and was collected into tubes to separate serum by

centrifugation at 3000 rpm for 15 min using cooling centrifuge (Laborcentrifuge, 2K15 centrifuge, Sigma, Germany), then divided into aliquots and stored at -20°C for biochemical assays.

Brain was removed quickly and washed with ice-cold saline solution (0.9% NaCl) and divided into two parts; the first part was homogenized and prepared for biochemical estimation and the other part was prepared for immunohistochemical studies.

Preparation of tissue homogenate

Tissues were cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na₂HPO₄ and 0.7 g of NaH₂PO₄ per 500 ml deionized water [pH 7.4]) per gram tissue, then centrifuged at 4000 rpm for 15 minutes at 4°C using. Supernatant was then separated and used for estimation of biochemical parameters [17].

Biochemical assays

Determination of serum tumor necrosis factor- α (TNF- α) and interleukin – 1 α (IL- 1 α)

Serum TNF- α and IL- 1 α were measured using enzyme-linked immunosorbent assay (ELISA) according to the method of Taylor [18] and Barland et al. [19] respectively. These assays employ a quantitative sandwich enzyme immunoassay technique, which measures serum TNF- α and IL- 1 α levels.

Determination of brain MDA

Lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA). Thiobarbituric acid (TBA) reacts with malondialdehyde in acidic medium at temperature of 95°C for 30 minutes to form thiobarbituric acid reactive product. The absorbance of the resultant pink product can be measured at 534nm [20].

Determination of brain superoxide dismutase (SOD) activity

Brain SOD was determined using kinetic kit (from Biodiagnostic, Egypt) according to Nishikimi et al. [21]. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

Determination of brain reduced glutathione (GSH)

Reduced glutathione (GSH) was determined according to Beutler et al. [22]; this method is based on the reduction of 5,5' dithiobis (2- nitrobenzoic acid) (DTNB) with GSH to produce a yellow compound, its absorbance can be measured spectrophotometrically at 405nm.

Determination of brain total antioxidant capacity (TAC)

TAC was determined according to Koracevic et al. [23]. This method is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H₂O₂). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide; the residual H₂O₂ is determined colorimetrically by an enzymatic reaction which involves the conversion of 3, 5, dichloro-2- hydroxyl benzenesulphonate to a colored product.

Determination of brain neurotransmitters by HPLC

Determination of brain norepinephrine, dopamine and serotonin was carried out as described previously by Hussein et al. [24] using high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (G131A model).

HPLC condition

Separation was achieved on ODS-reversed phase column (C18, 25 x 0.46 cm i.d. 5 μ m). The mobile phase consists of potassium phosphate buffer/methanol 97/3 (v/v) and was delivered at a flow rate of 1.5 ml/min. UV detection was performed at 270 nm, and the injection volume was 20 μ l from different dilutions of standards and from each sample.

Calculation

The concentration of norepinephrine, dopamine and serotonin in samples were determined using standard curve of each standard.

Immunohistochemistry of cyclooxygenases

From 10% formalin fixed paraffin embedded samples, 5 μ m-thin sections were prepared on positive charged slides. The sections were deparaffinized and treated with 0.2% saponin (Thermo Fisher Scientific, Fremont Blvd, USA) at room temperature for 30 minutes. After the sections were treated with methanol containing 3% hydrogen peroxide for 15 minutes to eliminate endogenous peroxidase, the sections were reacted with 10% normal rabbit serum for 10 minutes to block nonspecific reactions. As the primary antibody, each of anti-cyclooxygenase-1 and anticyclooxygenase-2 polyclonal antibodies (Thermo Fisher Scientific, Fremont Blvd, USA) were diluted 100 times and reacted with the sections at 4 °C for 15 hours. After the streptavidin–biotin complex method (Thermo Fisher Scientific, Fremont Blvd, USA), biotin-labeled anti-goat immunoglobulin G antibody as the secondary antibody was reacted with the sections at room temperature for 15 minutes, and the peroxidase-labeled streptavidin was reacted at room temperature for 10 minutes, followed by color development using diaminobenzidine (DAB) reagent. After counterstaining with hematoxylin, the sections were observed under a microscope.

Statistical analysis

All data were expressed as mean \pm standard error. Data were analyzed using one-way ANOVA using SPSS (Version 16). Duncan's new multiple-range test was used to assess differences between means. A significant difference was considered at the level of $P < 0.05$.

RESULTS

In this study, the mean values of brain GSH, SOD and TAC were significantly decreased in LPS group whereas a significant increase in brain malondialdehyde was observed in the same group compared to control (Fig 1-4).

Flaxseed and fish oils supplementation, significantly increased brain GSH, SOD and TAC in treated group compared to LPS group (Fig 1-3). In addition, the mean value levels of SOD were significantly increased in treated fish oil compared to treated flaxseed oil group (Fig 2). On the other hand, administration of flaxseed and fish oils in this study significantly decreased brain malondialdehyde in treated groups compared to LPS group (Fig.4).

As shown in figure (5), serum TNF- α was significantly increased in LPS group compared to control while it significantly decreased by supplemented oils in treated groups compared to LPS group.

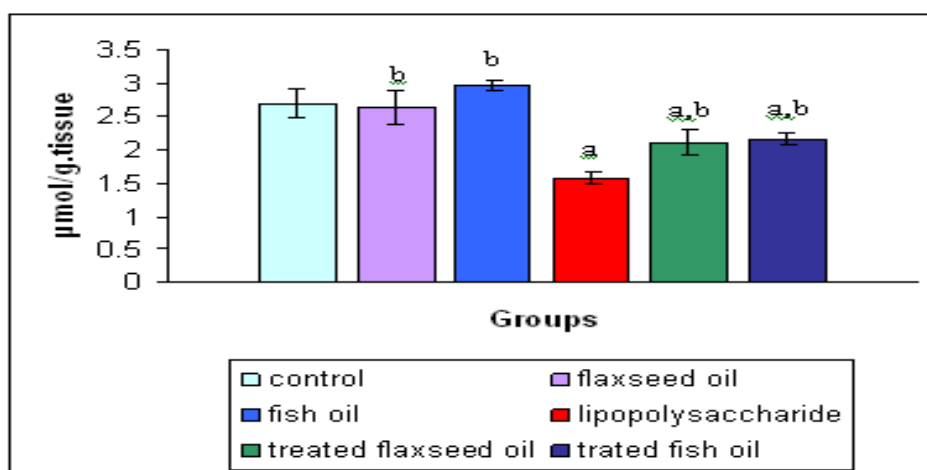


Figure (1): Mean value levels of brain GSH in different studied groups

P^a: Significant difference compared to control group.

P^b: Significant difference compared to LPS group.

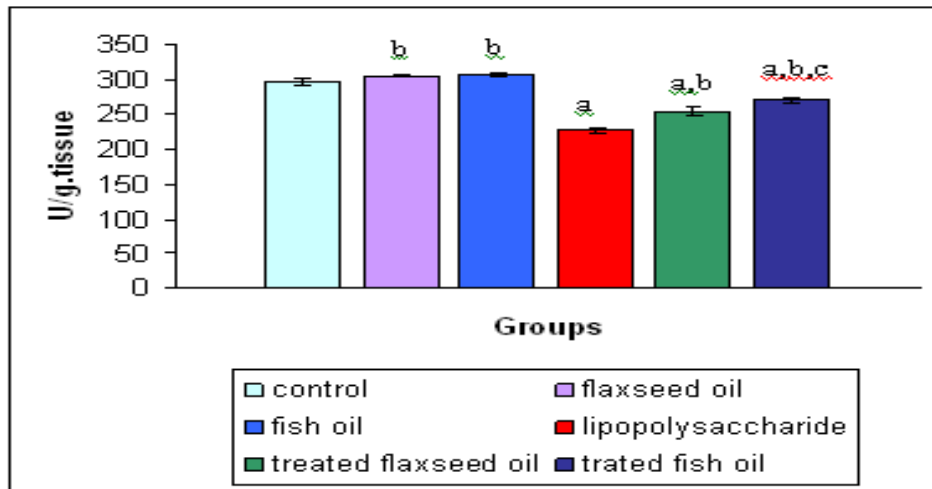


Figure (2): Mean value levels of brain SOD in different studied groups

P^a: Significant difference compared to control group.

P^b: Significant difference compared to LPS group.

P^c: Significant difference between treated fish oil and treated flaxseed oil group.

In the present study, the mean value level of interleukin – 1 α in lipopolysaccharide group was increased (p=0.06) compared to control group but this elevation was not statistically significant. Flaxseed and fish oils supplementation decreased these values in treated groups although these reduction was statistically insignificant (p=0.58 and p=0.31) respectively compared to lipopolysaccharide group (Fig 6).

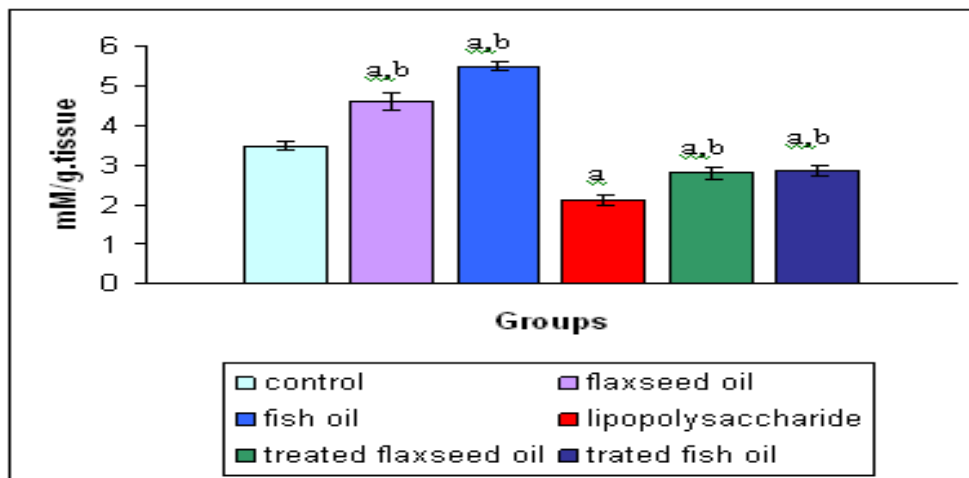


Figure (3): Mean value levels of TAC in different studied groups

P^a: Significant difference compared to control group

P^b: Significant difference compared to LPS group.

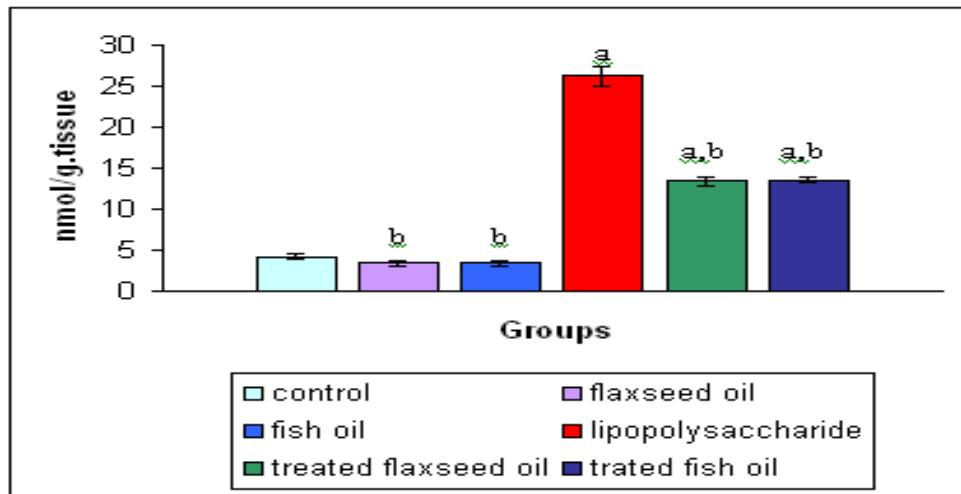


Figure (4): Mean value levels of MDA in different studied groups

P^a : Significant difference compared to control group.

P^b : Significant difference compared to LPS group.

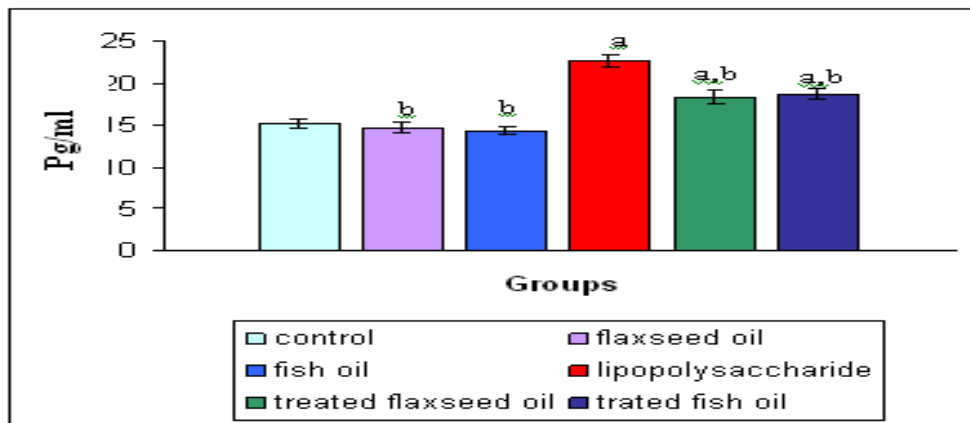


Figure (5): Mean value levels of tumor necrosis factor-α in different studied groups

P^a : Significant difference compared to control group.

P^b : Significant difference compared to LPS group.

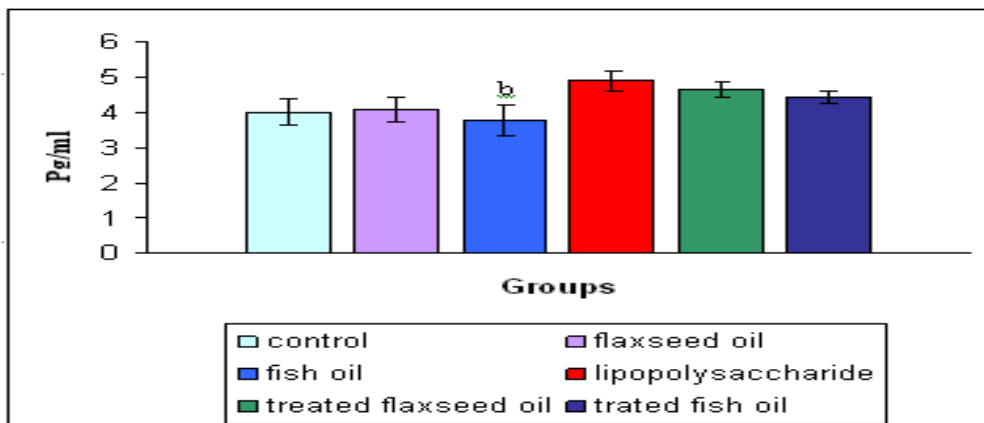


Figure (6): Mean value levels of interleukin-1α in different studied groups

P^b : Significant difference compared to LPS group.

Brain monoamines (dopamine, norepinephrine and serotonin) levels were significantly increased in LPS group compared to control, however, flaxseed and fish oils supplementation significantly decreased these values ($P \leq 0.05$) compared to LPS group (table 1).

Table (1): Brain neurotransmitters in different studied groups

Parameters		Norepinephrine (µg/g.tissue)	Dopamine (µg/g.tissue)	Serotonin (µg/g.tissue)
Groups				
Control	Mean±SE	4.31 ± 0.06	3.38 ± 0.03	3.55 ± 0.17
Flaxseed oil	Mean±SE	4.37 ± 0.03	3.15 ± 0.02	2.65 ± 0.17
	P^a Value	0.472	0.000*	0.00*
	P^b Value	0.000*	0.000*	0.00*
	% change ^a	1.2%	-6.8%	-25%
Fish oil	Mean±SE	4.47 ± 0.08	3.21 ± 0.03	3.03 ± 0.06
	P^a Value	0.144	0.002*	0.01*
	P^b Value	0.000*	0.000*	0.00*
	% change ^a	3.6%	-5.0%	-15%
LPS	Mean±SE	6.63 ± 0.10	5.26 ± 0.11	4.58 ± 0.08
	P^a Value	0.000*	0.000*	0.00*
	% change ^a	53.8%	55.7%	29%
Treated flaxseed oil	Mean±SE	5.45 ± 0.10	4.23 ± 0.12	3.79 ± 0.10
	P^a Value	0.000*	0.000*	0.20
	P^b Value	0.000*	0.000*	0.00*
	% change ^a	26.3%	25.4%	7%
	% change ^b	-17.9%	-19.5%	-17%
Treated fish oil	Mean±SE	5.27 ± 0.06	4.27 ± 0.13	3.84 ± 0.15
	P^a Value	0.000*	0.000*	0.12
	P^b Value	0.000*	0.000*	0.00*
	P^c Value	0.150	0.838	0.79
	% change ^a	22.2%	26.5%	8%
	% change ^b	-20.5%	-18.8%	-16%

P^a : Significant difference compared to control group.

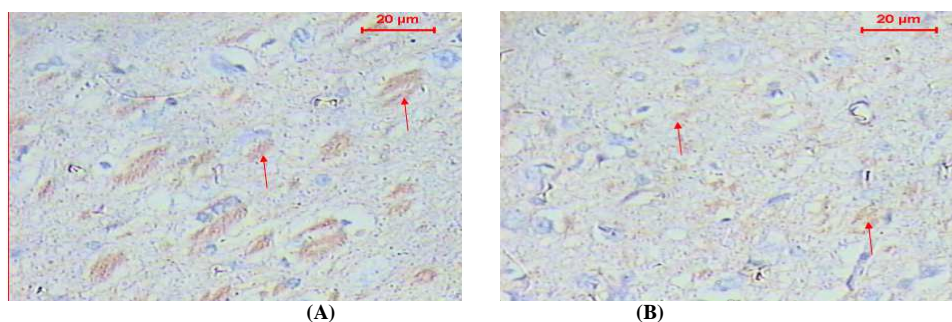
P^b : Significant difference compared to LPS group.

P^c : Significant difference between treated fish oil and treated flaxseed oil group.

% change^a: % of change from control group.

% change^b: % of change from LPS group.

In this study, examination of sections in brain cerebrum of control, flaxseed oil group and fish oil group showed positive reaction of Cox-1 as indicated by the presence of the brown color (Figures 10(A), (B) and (C) respectively), while the brain cerebrum of rats of LPS group showed negative reaction of Cox- 1 represent by absence of brown color (Figure 10 (D)). On the other hand, brain cerebrum of rats of treated groups (flaxseed and fish oils) followed by intraperitoneal (i.p.) injection of lipopolysaccharide showed positive reaction of Cox-1 as indicated by the presence of the brown color (Figures 10(E), (F) respectively).



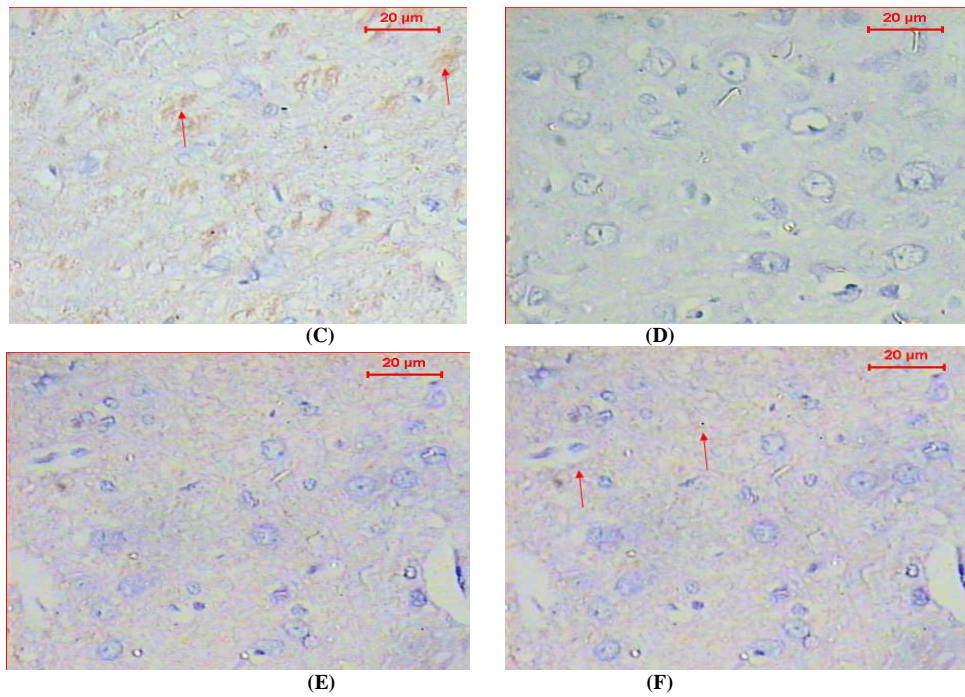
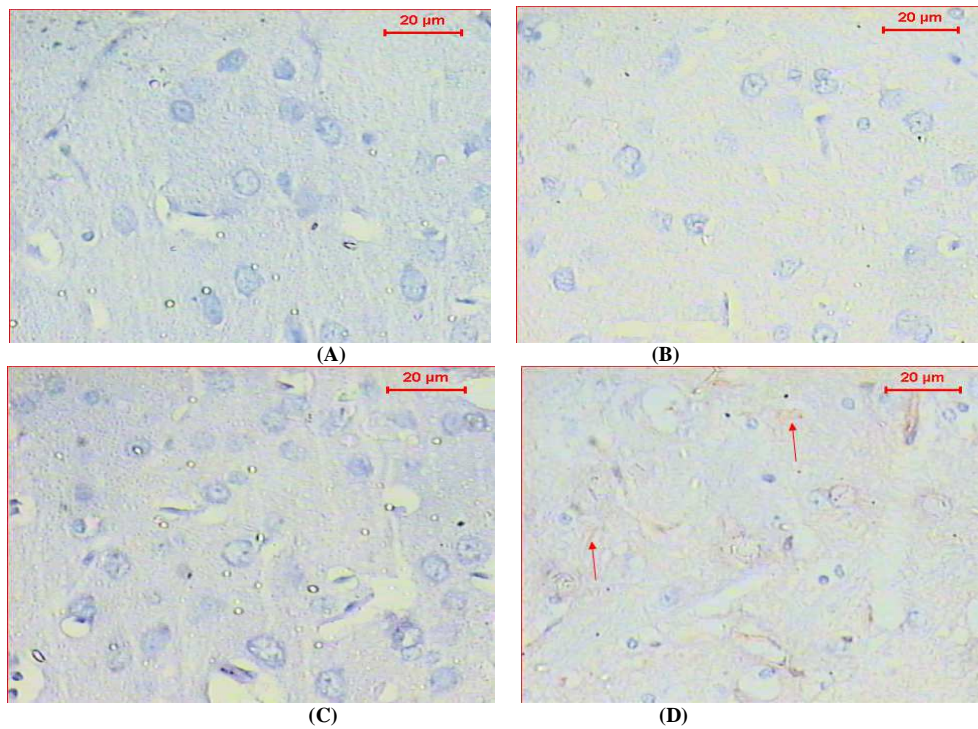


Figure (10): A micrograph of section in brain cerebrum of (A) control group, (B) flaxseed oil group, (C) fish oil group, (D) LPS group, (E) treated flaxseed oil and (F) treated fish oil . (A), (B) and (C) show positive reaction of Cox-1 as indicated by the presence of the brown color (arrows), however (D) shows negative reaction of Cox -1 as indicated by the absence of the brown color. (E) and (F) show positive reaction of Cox-1 as indicated by the presence of the brown color (arrows). (Cox-1 immunostaining, Scale bar 20 µm)



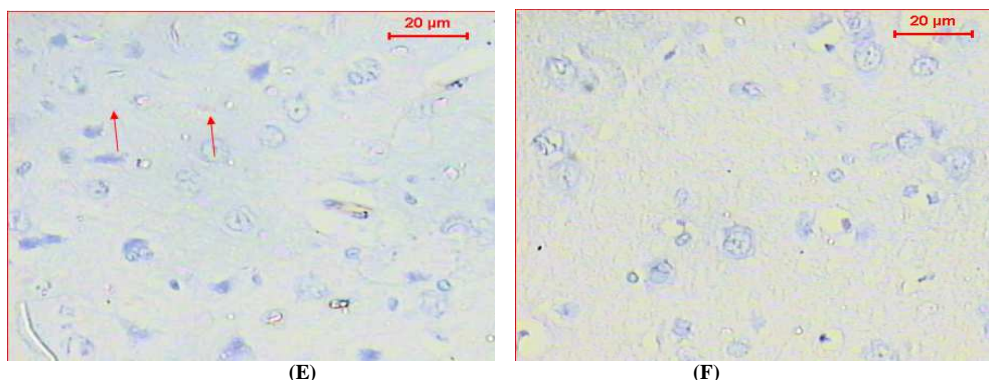


Figure 11): A micrograph of section in brain cerebrum of (A) control group, (B) flaxseed oil group, (C) fish oil group, (D) LPS group and (E) treated flaxseed oil group and (F) treated fish oil group. (A), (B) and (C) show negative reaction of Cox-2 as indicated by the absence of the brown color. (D) shows positive reaction of Cox-2 as indicated by the presence of the brown color (arrows). (E) shows negative reaction of Cox-2 as indicated by absences of brown color except in some neurons (arrows) and (F) shows negative reaction of Cox-2 as indicated by the absence of the brown color. (Cox-2 immunostaining, Scale bar 20 µm).

Contrarily, examination of sections in brain cerebrum of control, flaxseed oil and fish oil groups showed negative reaction of Cox-2 as indicated by the absence of the brown color (Figures 11(A), (B) and (C) respectively), while the brain cerebrum of rats of LPS group showed positive reaction of Cox-2 represented by brown color (Figure 11(D)). On the other hand, brain cerebrum of rats of treated groups showed negative reaction of Cox-2 as indicated by absence of the brown color (Figures 11(E),(F) respectively).

DISCUSSION

Brain injury (BI) is a big health problem, including a major cause of death and disability all over the world. Biochemical cascades are considered important factors that lead to primary and secondary injury; these mechanisms result in imbalance between oxidant and antioxidant agents lead to elevation of oxidative stress, neural dysfunction and death [25].

Lipopolysaccharide is a chemical agent and it induces a well-documented model of experimental BI [26].

In the present study, intraperitoneal injection of LPS significantly increased MDA concomitant with a reduction in GSH, SOD and TAC levels compared to control group, reflecting an oxidative stress status. These results were in agreement with Abdel-Salam *et al.* [27] who indicated that LPS induced oxidative stress in the brain, resulting in a marked increase in lipid peroxidation, that caused by free radicals and reactive oxygen species (ROS) released due to impairment of the balance between the various antioxidant mechanisms occurring after BI. This effect leads to an increase in the MDA concentration and decrease in both SOD and GSH [28]. The increase in ROS production leads to a depletion of antioxidant parameters appeared in a significant reduction in TAC [29].

Our study appeared a significant increase in NE, DA and SE levels in LPS group compared to control group. This result was in agreement with Dunn [30] who indicated that administration of endotoxin (LPS) stimulated the secretion of proinflammatory cytokines which induced hypothalamus (HPA) activation leading to elevation of norepinephrine (NE) and serotonin (5-HT) levels released in the brain.

Thus, LPS stimulates the biosynthesis of norepinephrine and dopamine in all brain regions [31] and increases brain oxidative stress which is well associated with the development of neurodegenerative diseases [32].

Other important result in our study is the significant increase in cytokines (IL-1 α and TNF- α) levels in lipopolysaccharide group compared to control, these results were in agreement with Choi *et al.* [33] who indicated that LPS stimulated activation of microglial that released pro-inflammatory and neurotoxic factors including TNF- α , IL-1, IL-6, and NO to cause neuronal damage.

LPS binds to immune cells and initiates the inflammatory cytokines including tissue TNF- α which stimulates the production of reactive oxygen species (ROS) by activation of macrophages leading to brain injury [34]. In addition,

LPS can affect monocytes, macrophages and fibroblasts resulting in the production of pro-inflammatory cytokines [35].

Cyclooxygenase is an enzyme which is capable to convert arachidonic acid (the abundant fatty acid in the cell membrane) to prostaglandin (PG) G₂ and PGH₂ under numerous physiological conditions. COX is responsible for inflammatory phenomena. The two isoforms of COX (COX-1 and COX-2) are almost identical in their structure but they have important differences in their intracellular locations and inhibitor selectivity [36].

In our results, the immunohistochemical technique appeared a negative reaction of COX-1 in LPS group that appears as absence of brown color while control showed positive reaction that appears as brown color indicating the increase of COX-1 reaction; these results were in agreement with Teeling *et al.* [37] who reported that COX-1 is not responsible for the induction of brain IL-6, IL-1 β and TNF- α synthesis, so COX-1 has a role in regulating brain inflammatory responses.

Contrarily, LPS group showed a positive reaction of COX-2 that appeared as a brown color while control group showed negative reaction that appears as absence of brown color indicating the reduction of COX-2 reaction. These results were in agreement with Zendehdel *et al.* [38] who indicated that LPS injection increased cyclooxygenase-2 (COX-2) expression in all brain regions.

Several studies confirmed that, long chain polyunsaturated fatty acids (LC-PUFAs) have anti-inflammatory, anti-excitotoxic and anti-apoptotic properties [39]. So, PUFAs may help when administered prior to or following BI. In the current study, MDA was significantly decreased while SOD, TAC and GSH were significantly increased in treated flaxseed and fish oils groups compared to LPS group; these results were in agreement with Saada *et al.* [40] who indicated that, the administration of fish oil (EPA+DHA) inhibited oxidative stress, decreased MDA and increased both superoxide dismutase (SOD) activity and GSH level; thus, n-3 fatty acids increase total antioxidant capacity (TAC) level and prevent the enhancement of oxidative stress [41].

The reduction of the ROS (superoxide and hydrogen peroxide) production by supplementation of n-3 fatty acids may be attributed to the stimulation of neutrophils [42].

Attenuating of NE, SE and DA levels in treated groups in this study may be attributed to the effect of omega-3 fatty acids in improving brain functions, amendment of neuronal membrane fluidity, receptors' affinity and nerve growth factor [43].

Omega-3 fatty acids act by two ways, directly by replacing arachidonic acid (omega-6) and eicosanoid substrate and indirectly by inhibiting its metabolism via alteration of inflammatory genes expression through transcription factor activation [42,44]. In agreement, cytokines (IL-1 α and TNF- α) levels were decreased in treated groups compared to LPS group in our study.

Calder [45] explained the reduction of IL-1 α and TNF- α by n3-PUFAS administration; he indicated that fish oil decreased IL-1 production by monocytes and decreased serum TNF- α concentration in addition to the inhibition of the expression of IL-1 α and TNF- α messenger RNA.

N-3 fatty acids showed their anti-inflammatory action by inhibiting NF κ B activity that has a crucial role in inflammatory signaling pathways and plays a key role in regulating the immune response to infection so it controls several cytokines. Other mechanism included that n-3FA attenuates the conversion of arachidonic acid (AA) to pro-inflammatory eicosanoids, inhibits the formation of pro-inflammatory cytokines and promotes levels of anti-inflammatory decosanoids [46].

In the present study, the brain cerebrum of treated group showed positive result of COX-1 that appears as a brown color compared to a negative result of COX-1 which appear as absence of brown color in LPS group. On the other hand the brain cerebrum of treated groups showed negative result of COX-2 that appears as absence of brown color compared to a positive result of COX-2 which appears as a brown color in LPS group, these results were in agreement with Lee *et al.* [47] who found that EPA and DHA can suppress COX-2 activity. One of the main anti-inflammatory mechanisms is that EPA and DHA act as a competitive inhibitor for proinflammatory arachidonic acid (AA) on cyclooxygenase (COX), which produces proinflammatory eicosanoids. When EPA and DHA are subjected to oxygenation by COX the less inflammatory prostaglandin E₃ (PGE₃) is generated instead of highly pro-

inflammatory prostaglandin E2 (PGE2) which produced by AA [48]. Also Liu et al. [49] found that omega-3 intervention suppressed the LPS-induced up regulation of COX-2. The beneficial effects of omega-3 may be associated with the suppression of TLR4 signaling.

In conclusion, the current results suggest that flaxseed and fish oils effectively attenuated the elevation of oxidative stress during brain injury due to their antioxidant and anti-inflammatory effects. There was no statistically significance difference between the effect of fish and flaxseed oils in improvement of brain injury and oxidative stress.

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