Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2312

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **4 (3):** 104-111 (2016)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

Analysis of Fish Oil as Potential Oxidative Stress Inhibitor in C57BL/6 Mice

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Received: 10.06.2016 | Revised: 19.06.2016 | Accepted: 24.06.2016

ABSTRACT

The aim of present study was to evaluate the effect of dietary supplementation of fish oil (FSO) on the oxidative stress of liver tissue in c57bl/6 mice. The thiobarbituric acid reactive substances (TBARS), xanthine oxidase (XO), superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-PX) were studied in mice after oral gavage of 4, 8 or 16 mg/ kg body weight (bw.) FSO (rich in omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid) for 4 weeks and the levels were compared to untreated control mice. The mice in the treated group had significantly higher SOD activity ($P<0.01$), GSH ($P<0.01$), catalase ($P<0.01$) and decreased TBARS levels ($P< 0.01$) with respect to control mice whereas XO activities were not observed to be significantly different between the groups. FSO supplementation also caused a significant ($p < 0.05$) decrease in inflammatory cytokines (TNF-α, and IL-1β) and increase in the anti-inflammatory ones (IL-10) at a dosage of 16 mg/kg bw.

Thus, it can be concluded that dietary supplementation with FSO may enhance resistance to oxidative stress and validate the notion that FSO is an effective dietary supplement for management of various oxidative stress induced diseases.

Key words: *Catalase, fish oil, Glutathione reductase, polyunsaturated fatty acids, reactive oxygen species, Superoxide dismutase.*

INTRODUCTION

A disturbance altering the equilibrium of pro-oxidant/antioxidant systems in intact cells is termed as Oxidative stress[1]. It has been implicated in a myriad of human disorders with dietary constituents being pointed out as its causative agents or protective agents in the antioxidant defense mechanism[2]. Epidemiological and experimental studies suggest that that n-3 polyunsaturated fatty acids (PUFAs) in the fish oil (FSO) are beneficial with regard to combating the cardiovascular diseases[3,4]. Research is needed to clarify and validate the mechanisms by which n-3 PUFAs effect the physiological functions. Atherosclerosis, insulin resistance, SLE are some of the multi-factorial diseases which can be modulated through dietary intervention and altering the cellular oxidative stress status. It has been reported that oxidative modification of LDL is a key factor in development of atherosclerosis[5].

Cite this article: Bashir, S., Sharma, Y. and Khan, F., Analysis of Fish Oil as Potential Oxidative Stress Inhibitor in C57BL/6 Mice, *Int. J. Pure App. Biosci.* **4(3):** 104-111 (2016). doi: http://dx.doi.org/10.18782/2320-7051.2312

Dietary PUFAs may potentially affect the susceptibility of oxidative modification of LDL by incorporation into the lipoproteins. Eicosapentaenoic acid [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)] are the major n-3 PUFAs found in fish oil (FSO).

The production of reactive oxygen species (ROS) is a normal during cellular functioning. These include superoxide anions ($O^{\cdot-}_2$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). However, there needs to be a continuous check on their production in order to keep their levels at minimum as they are highly reactive leading to the oxidation of DNA, proteins or lipids. The major antioxidant enzymes that are involved in the regulation of the cellular levels of ROS are superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). Increased levels of ROS are the key risk factors in pathogenesis of a number of diseases. Thus the antioxidant system of the body needs to be a balanced one. Increase in production of reactive oxygen species and body's inability to detoxify it as well as the intermediates produced is termed as oxidative stress. However, cells have several protective mechanisms against oxidative stress which succeed in preventing cell damage to the extent that these protective mechanisms are effective. Many constituents of diet are important sources of protective agents that vary from anti-oxidant vitamins and minerals to food additives that might boost the action of natural anti-oxidants.

Immunomodulators can be used as a highly effective strategy for combating infectious diseases by enhancing host defenses either preventatively or therapeutically. Also immunomodulators exert their effect by boosting host immune response rather than targeting pathogen directly. Their role thus can be exploited to develop new types of neutraceuticals to ensure an appropriate immune response. The effectiveness of many drugs currently in use is due in part to neutraceuticals, nutrients that help enhance and appropriately skew the immune response. Thus essential oils may be helpful to combat oxidative stress induced diseases and could be the effective tools in the development of new neutraceuticals in future.

In the present study the effect of dietary intervention of omega-3 fatty acid rich fish oil on the indices of antioxidant status of liver in mice was analyzed. Plasma thiobarbituric acid-reactive substances (TBARS), as well as activities of xanthine oxidase (XO), superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH) of mice after supplementation of fish oil rich in omega-3 fatty acids were compared with untreated mice.

MATERIALS AND METHODS

Animals and diet

Male *c57bl/6* mice (20-25g) were used for the study. Central animal house facility, Jamia Hamdard was used for breeding and maintenance of the mice after approval by the University's animal ethics committee. Mice were divided into three groups, 5 mice each (receiving 4, 8 or 16 mg/kg body weight FSO orally) and the control group given standard rodent diet. FSO was given through oral administration for 4 weeks.

Tissue preparation

The tissue preparation was carried out as described earlier [6]. A 10 % tissue lysate (liver homogenate) was prepared after its aseptic removal. It was washed in ice-cold phosphate buffer solution. Approximately 2g of liver tissue was weighed, minced and homogenized in 0.1M phosphate buffer (pH 7.4) containing 1.15% KCl using a homogenizer.

Determination of TBARS levels

The plasma TBARS level was determined based on the reaction with thiobarbituric acid (TBA) at 90–100^{0}C[7]. In this test, malondialdehyde (MDA) or MDA-like substances and TBA react to form a pink colored product which absorbs maximally at 532 nm. The reaction was carried out at a pH of 2–3 at 90^{0}C for 15 min. The sample and ice cold 10% (w/v) trichloroacetic acid were mixed at a ratio of 1:2 to precipitate the protein. The precipitate was separated by centrifugation, and supernatant was mixed with an equal volume of 0.67% (w/v) TBA in boiling water for 10 min. The absorbance was measured at 532 nm and the results were expressed as µmol/mg protein.

SOD activity determination

SOD activity was measured by the inhibition of pyrogallol auto oxidation as per protocol

described by Marklund & Marklund[8]. SOD converts superoxide radical to hydrogen peroxide that does not interfere with the auto-oxidation process. Tris buffer (50 mM Tris and 1 mM EDTA, pH 8.5) was used. Pyrogallol solution was prepared by dissolving 20 mM pyrogallol in distilled water. Tissue sample (preparation described above) from each group were taken. In each test sample 2.8 ml tris buffer, 0.1 ml pyrogallol and 0.1 ml tissue sample were taken. After an induction period of 90 seconds absorbance was recorded first in control as well as test samples every 30 seconds upto 3.0 minutes at 420 nm.

The induction period was allowed to achieve a steady state for authorization of pyrogallol. Rate of change of absorbance per minute in the control as well as test sample were noted to calculate the SOD activity.

SOD activity was calculated using the following formula-

$$\text{SOD} = \frac{\text{O.D.} \times \text{volume of assay} \times 1000000000 \times \text{vol. of PMS taken}}{\text{(M.E.C.) } 4.02 \times 1000 \times \text{path length} \times 1000 \times \text{vol. of enzyme} \times \text{mg protein}}$$

Determination of effect on GSH

GSH (EC 1.6.4.2) activity was assessed by the method described earlier[9]. The reaction in the tube, containing NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, started by the addition of H_2O_2 and the change in absorbance was monitored by a spectrophotometer at 340 nm. Activity was expressed as µmol/mg protein.

Effect of FSO on Catalase content

The assay mixture of 3.0 ml consisting of 1.99 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml of Hydrogen peroxide (0.019 M) and 10 µl PMS (10% w/v) was prepared. Decrease in absorbance due to the disappearance of hydrogen peroxide was recorded at intervals of 30 seconds up to 3 minutes at 230 nm spectrophotometrically. Catalase activity was calculated as nmol of hydrogen peroxide consumed/min/mg protein using a molar extinction coefficient value 0.081 × 1000 /M/cm.

XO activity determination

XO (EC 1.2.3.2) activity was measured according to Prajda and Weber's method[10] after the formation of uric acid from xanthine at the absorbance of 293 nm, the calibration curve using 10–50 mU/ml concentrations of standard XO solutions was constructed. One unit of activity represents 1 mmol of uric acid formed per minute at 37^0C (pH 7.5) and is represented as units U/ml.

Cytokine analysis

Serum was separated from the blood collected from the retro-orbital plexus of the mice. Cytokines (TNF-α, IL-1β and IL-10) were detected and their levels monitored using ELISA according to their respective kits (e-bioscience, USA).

Statistical Analysis

Data were statistically analysed using Student's t-test with GraphPad InStat software (San Diego, CA) to determine significant differences in the data of various groups. p values less than 0.05 were considered significant. The values are expressed as mean ±SEM

RESULTS

Effect on Malondialdehyde content

Effect of FSO on Malondialdehyde content in mouse liver homogenate is shown in Figure-1. After the oral administration of FSO, the content of malondialdehyde was evaluated. It was found to be significantly increased at dose levels of 8 and 16 mg/kg body weight as compared to control group. The findings suggest that oral administration of FSO significantly enhances the MDA levels in a dose dependant manner.

Effect on the reduced glutathione

Effect of FSO on the content of reduced glutathione in mouse liver homogenate is that the level of GSH was increased at all dosage levels. The reduced glutathione level was

observed to be significantly ($p < 0.05$) high in 16 mg/kg bw. dosage. The results are shown in figure 2.

Effect of FSO on Superoxide dismutase

Effect of FSO on the content of Superoxide dismutase in mouse liver homogenate is depicted in Figure 3. The level of SOD was enhanced at all dosage levels. Significant increase ($P<0.05$) in Superoxide dismutase level was observed at all the FSO 4, 8 or 16 mg/kg bodyweight dose concentration however, a maximum increase in Superoxide dismutase (1185.89±44.61) was observed in the mice treated with a dose of 16 mg/kg bw.

Effect of FSO on XO content

XO activity (Figure 4) was measured in the liver tissue spectrophotometrically after the formation of uric acid from xanthine through the change in absorbance at 293 nm. 10–50 mU/ml concentrations of standard XO. XO levels were not observed to be significantly different from the control.

Effect of FSO on Catalase content

Catalase content was increased at all dose concentration upon oral administration of FSO for 4 weeks. Significant increase ($p < 0.05$ and $p < 0.01$ resp.) in the levels of catalase were observed at 4 and 16 mg/kg bw. dose concentration. Maximum increase in catalase levels (1.125 ± 0.063) were observed in the category of mice treated with dose of 8 mg/kg body weight.

Cytokine analysis

Cytokine analysis is an important parameter for inflammation and immune disorders. FSO supplementation caused a significant ($p < 0.05$) decrease in inflammatory cytokines (TNF-α, and IL-1β) and increase in the anti-inflammatory ones (IL-10) at a dosage of 16 mg/kg bw. (figure 6).

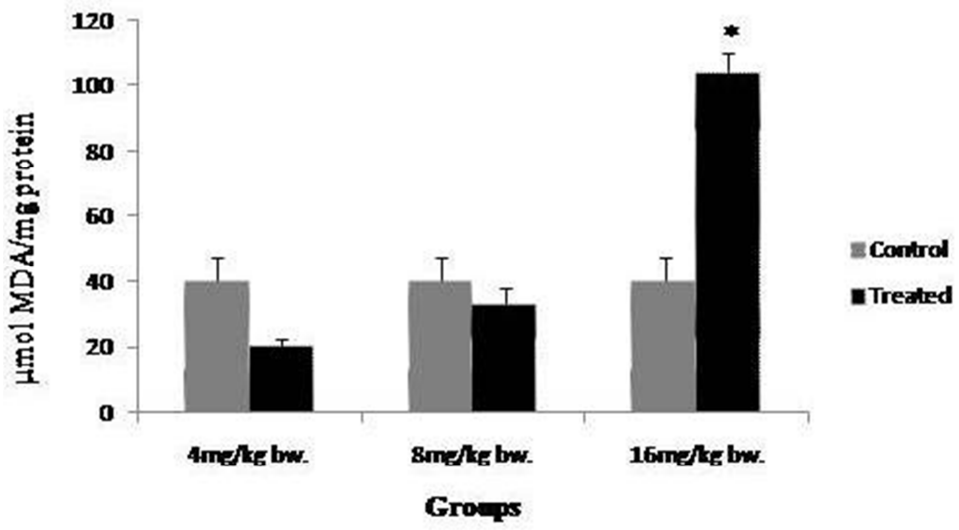

Fig. 1: Effect of FSO on Malondialdehyde (MDA) content in liver tissue in C57bl/6 mice given 4, 8 or 16 mg/kg bw. FSO. Data are plotted as mean ± SEM, number of animals (n) = 3.

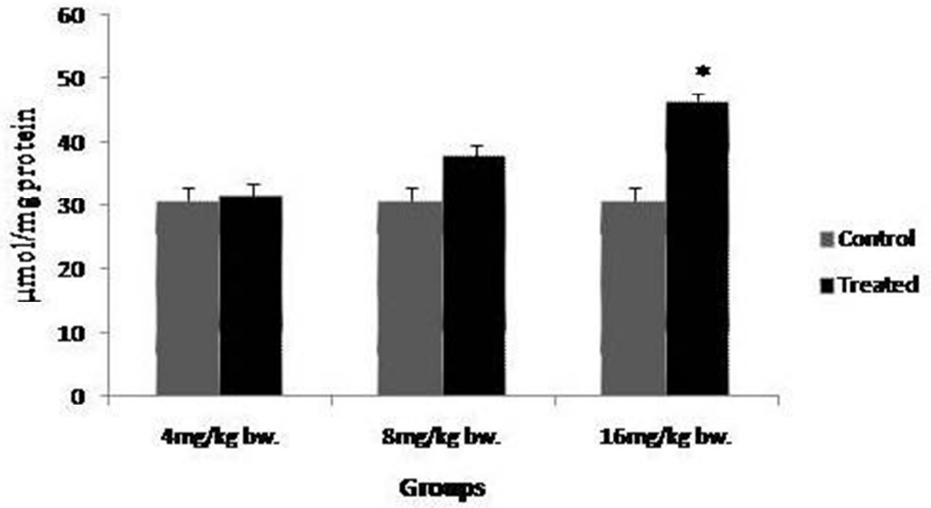

Fig. 2: Effect of FSO on GSH content in liver tissue in C57bl/6 mice given 4, 8 or 16 mg/kg bw. FSO. Data are plotted as mean ± SEM, number of animals (n) = 3.

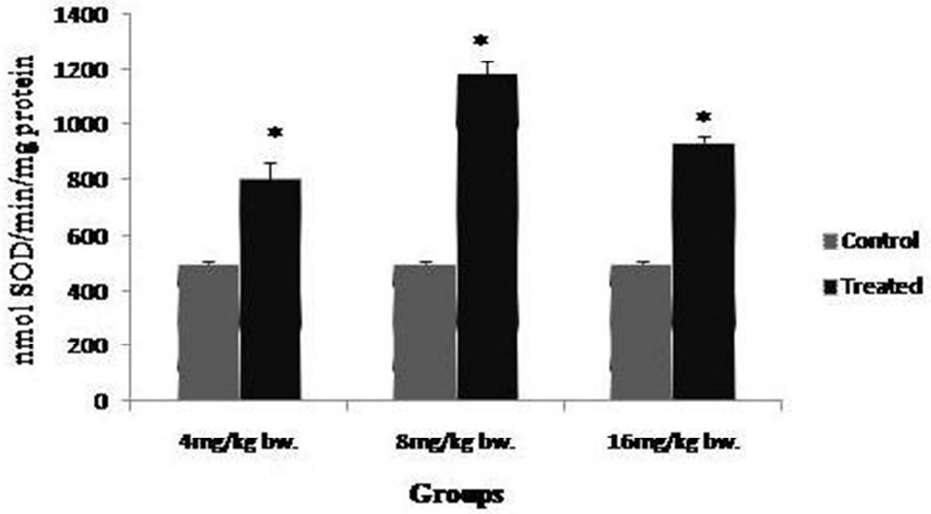

Fig. 3: Effect of FSO on SOD content in liver tissue in C57bl/6 mice given 4, 8 or 16 mg/kg bw. FSO. Data are plotted as mean ± SEM, number of animals (n) = 3.

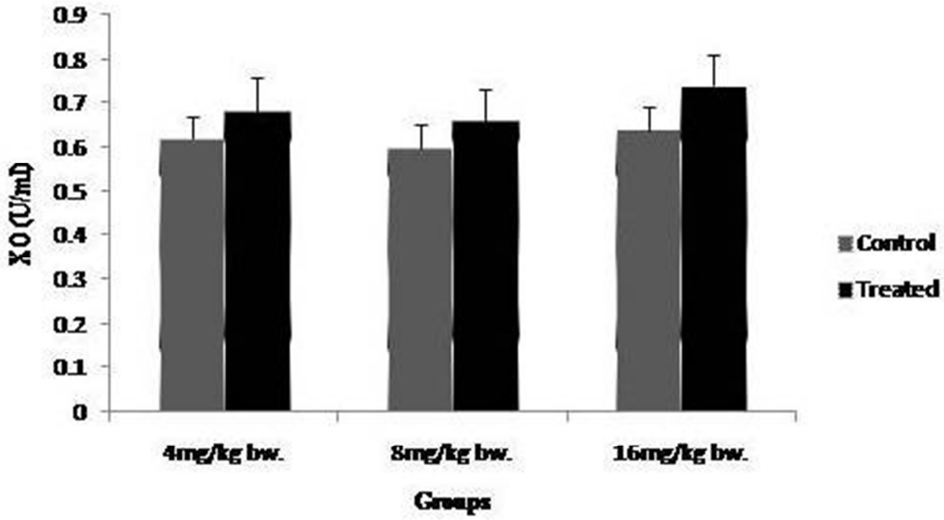

Fig. 4: Effect of FSO on XO content in liver tissue in C57bl/6 mice given 4, 8 or 16 mg/kg bw. FSO. Data are plotted as mean ± SEM, number of animals (n) = 3.

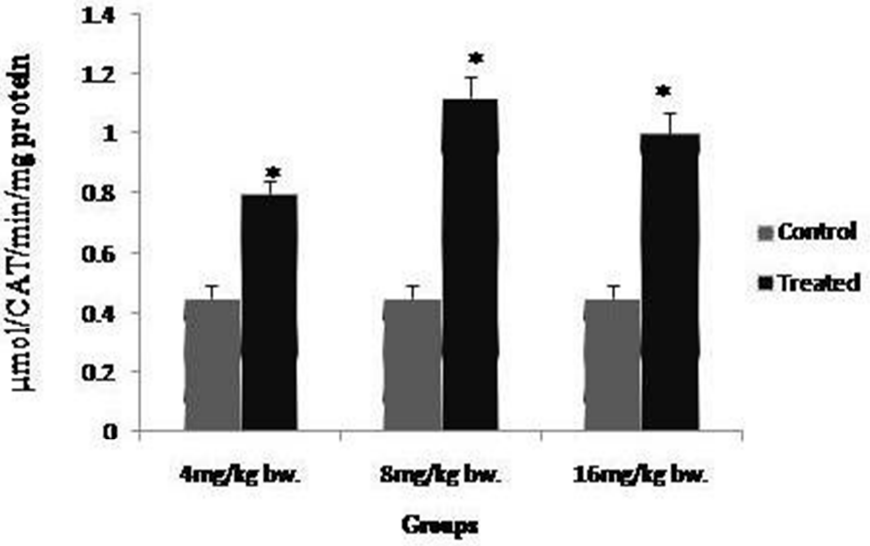

Fig. 5: Effect of FSO on CAT content in liver tissue in C57bl/6 mice given 4, 8 or 16 mg/kg bw. FSO. Data are plotted as mean ± SEM, number of animals (n) = 3.

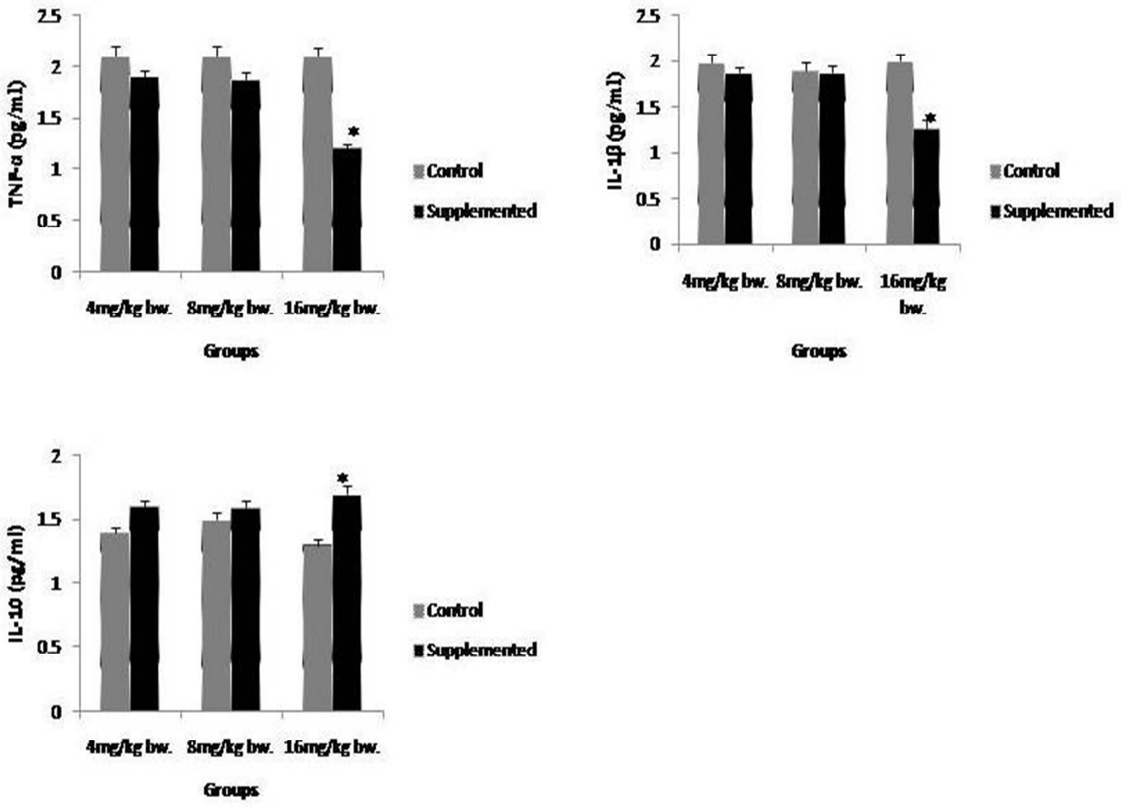

Fig. 6: Effect of FSO on serum cytokine levels in C57bl/6 mice given 4, 8 or 16 mg/kg bw. FSO. Data are plotted as mean ± SEM, number of animals (n) = 3.

DISCUSSION

In the present study, Antioxidant activities of FSO have been evaluated. Oxidative stress affects many cellular functions by various mechanisms such as the alteration in gene expression through the activation of transcription factors, or by the induction of permeability transition in mitochondria with fatal consequences. Increased oxidative stress has been reported in a variety of hepatic

dysfunctions like cirrhosis and hepatocellular carcinoma. The induction of oxidative stress is an important feature in the destruction of parenchyma and the activation of stellate cells that lead to liver dysfunctions[11,12]. Oxidative stress may be activated by the hydroxyl radical scavengers. In fact, a decrease in the antioxidant levels may indicate an increase in free radical level and thereby an increase in cellular damage.

Increase in lipid peroxidation has been found in numerous oxidative stressed cells and tissues[13,14] which is accompanied by a decrease in the antioxidant status[15]. The gradual progression of cell injury has been related to a significant increase in lipid peroxidation (LPO) and reduction in reduced glutathione in serum and liver[12]. Glutathione detoxifies the toxic metabolites of drugs, regulates gene expression, apoptosis and trans-membrane transport of organic solutes, and are essential in maintaining the reduced status of the cell/tissue[16]. Oral administration of FSO was observed to replenish the level of glutathione in a dose dependant manner.

Antioxidants scavenge free radicals and prevent oxidative damage in cells. The major enzymes that have been reported to protect cells from damage due to free radicals (are superoxide dismutase (SOD) and catalase (CAT)[17,18] have reported that the level of SOD decrease in oxidative stressed cells. Decrease in the level of SOD, CAT and reduced glutathione (GSH) and an increase in lipid peroxidation has also been reported[12]. These enzymes forage superoxide and peroxide radical species.

Oral administration of FSO significantly affects the level of malondialdehyde (MDA) content which is the key marker of lipid peroxidation. Increase level of reduced glutathione in liver of CS treated mice suggests that FSO might have the potential to maintain the reduced environment and help to regulate the oxidative stress. Increased levels of SOD and CAT enzymes in FSO treated mice also indicate that it might help in neutralizing the reactive oxygen species and convert them into the water and oxygen molecule. FSO also rearranges the cytokines towards anti-inflammatory state.

In conclusion, the dietary supplementation with FSO enhances resistance of mice to free radical induced stress. Thus the results validate the notion that omega-3 fatty acid rich is a helpful for the proper management of oxidant/antioxidant stress related diseases. Further, human case studies are required in subjects with disease states to assess the underlying antioxidant protective action of fish oil and related oils.

Acknowledgements

The authors acknowledge funding of work by Jawahar Lal Nehru fellowship for doctoral studies.

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