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A study on neuroprotective effect of cod liver oil in bilateral common carotid artery occlusion induced global cerebral ischemia in rats

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Abstract
Cerebral is chemia is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain and it leads to various pathological modalities such as mitochondrial damage, neuronal cell death and also associated with oxidative stress and DNA fragmentation. The present study reports the neuroprotective activity of Cod liver oil in cerebral ischemic rats. Cerebral ischemia was induced in rats by the bilateral common carotid artery occlusion (BCCAO) and Cod liver oil was evaluated at four different time points i.e., 30mins before cerebral ischemia (day 1), followed by 24, 48 and 72 hours post first dose. Biochemical parameters *viz*., lipid peroxidase (LPO), acetyl cholinesterase (AChE), reduced glutathione (GSH) and total protein levels were estimated. The different doses of Cod liver oil have significantly improved the altered levels of LPO, AChE, GSH and total protein levels in treatment groups which prior undergone occlusion procedure for 10mins in rats. Histopathological observation supports the prevention in architecture of the brain due to the treatment with Cod liver oil against occlusion induced cerebral ischemia in rats. The results obtained from the study suggested that the neuroprotective effect of Cod liver oil was mediated through the antioxidant, free radical scavenging activity and also return of biochemical marker level near to normal values.

Keywords: Acetyl cholinesterase, carotid artery occlusion

Introduction
The brain is particularly vulnerable to oxidative stress because of its high metabolic rate and low antioxidant defences [1]. Neuroprotection within the nervous system protects neurons from apoptosis or degeneration, for example following a brain injury or as a result of chronic neurodegenerative diseases. Cerebrovascular diseases include some of the most common devastating disorders such as ischemic stroke, hemorrhagic stroke, cerebrovascular anomalies, etc. They cause two lakhs deaths each year and are of major cause of disability [2]. Stroke has been ranked third most common cause of death worldwide and it is the leading cause of adult disability.

Neurodegeneration is the term for the progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases including Parkinson's, Alzheimer's and Huntington's occur as a result of neurodegenerative processes [3]. Cerebral ischemia is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain and it leads to various pathophysiological modalities such as reactive oxygen species (ROS), calcium overload, mitochondrial damage, neuronal cell death and also associated with oxidative stress and DNA fragmentation [3]. The most common form of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. This pathway controls the activation of caspase-9 by regulating the release of cytochrome c from the mitochondrial intermembrane space [4]. Over production of ROS is a central feature of all neurodegenerative disorders. Reperfusion after cerebral ischemia further add to the complications of stoke by releasing various mediators such as pro-inflammatory cytokines and free radical generation. This increases the oxidative stress to the brain and ultimately leading to neuronal cell death [5]. India shows a crude stroke prevalence rate of about 203 per 100,000 populations above 20 years of age, amounting to a total of about 1 million cases. The male to-female ratio was estimated to be 1:7 Most studies carried out in India show that about 10% to 15% of strokes occur in the population below 40 years, which is a higher proportion compared with other countries. However, it was

estimated that stroke represented 1.2 % of the total deaths in the country, when all ages were included. The proportion of stroke death increased with age, and in the oldest group (>70 years of age) stroke contributed to 2.4% of all deaths [6]. According to the World Health Organization, 15 million people suffer stroke worldwide each year, 5 million die and another 5 million are permanently disabled. The concept of neuroprotection is derived from the studies of ischemic brain injury. It has been well documented that abrupt deprivation of oxygen and glucose to neuronal tissues elicits a series of pathological cascades, leading to spread of neuronal death of the numerous pathways identified, excessive activation of glutamate receptors, accumulation of intracellular calcium cations, abnormal recruitment of inflammatory cells, excessive production of free radicals and initiation of pathological apoptosis are believed to play critical roles in ischemic damage, especially in the penumbral zone. Thus, it is logical to suggest that if one is able to interrupt the propagation of these cascades, at least part of the brain tissue can be protected [7]. Brain ischemia or cerebral ischemia is a condition which leads to alterations in brain metabolism, reduction in metabolic rates and energy crisis where the brain cannot perform aerobic metabolism due to lack of oxygen supply [8]. Though a large number of therapeutic agents like thrombolytics, NMDA receptor antagonists, calcium channel blockers, antioxidants, sodium-channel and potassium channel openers, glutamate antagonists, magnesium sulphate, glycine antagonists, GABAergic compounds (such as clomethiazole), growth factors (such as basic fibroblast growth factor), free radical scavengers (such as tirilazad), and anti-inflammatory compounds (such as enlimomab) have been used, there remains a large gap between the benefits by these agents and properties that an ideal drug for stroke should offer [9].

Cod liver oil is an abundant source of nutrients difficult to obtain elsewhere, such as vitamin A, arachidonic acid, DHA, and the B, D vitamins. It has an effective and stable antioxidant configuration. It is promoted as dietary supplement used to boost the immune system, fights off infections and heal wounds and to treat cancer. Because of their immune-boosting effects they are also claimed to help against arthritis. Most of the scientific studies with Cod liver oil have focused on its possible benefits against cancer and infections [10]. Cod liver oil has powerful antioxidant and cytoprotective effects. Cod liver oil has been used both as a therapeutic and preventive agent. Some of the other fish oils are found to produce significant cytoprotective properties. Cod-liver oil has been used effectively in Rheumatism [11], Inflammatory bowel disease [12], Atherosclerosis [4].

Material and Method

Chemicals

Cod liver Oil was purchased from Seven Sea Ltd, Hull, UK. Acetyl thicholine, 2-thiobarbituric acid, Ellman's Reagent (5,5'-dithiobis (2-nitrobenzoic acid), Sodium lauryl sulphate A.R (Sodium dodecyl sulphate), EDTA (Ethylene diamine tetra acetic acid) A.R, Tris Hydrochloride A.R, 2,3,5 triphenyl tetrazolium chloride (TTC) (tetrazolium salt), Pyrogallol A.R. 98.5%, Sodium phosphate monobasic monohydrate A.R and Sodium phosphate dibasic dehydrate A.R were obtained from Himedia Laboratories, Mumbai, India.

Instruments

Electronic balance from Adventurer, OHAUS, USA, U.V. Spectrophotometer Shimadzu Corporation, Japan, Auto analyzer- Rapid Diagnostics, Delhi, pH meter- Micropro Labmate, India, Homogenizer and Centrifuge -Remi Motors Pvt. Ltd. India.

Animals

Albino wistar rats of either sex weighing 250-300g were used. The animals were purchased from Sri Venkateshwara Enterprises, Bangalore, India. They were maintained in the animal house of Soniya Education Trust's College of Pharmacy, Dharwad, India for experimental purpose. The animals were maintained under controlled conditions of temperature (23±2°C), humidity (50±5%) and 12-h light-dark cycles. They were having free access to standard pellets as basal diet and water ad libitum. The animals were housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. Animals were acclimatized in laboratory conditions for a minimum period of 5 days prior to experimental initiation to minimize if any of non-specific stress. The experiment conducted was approved by the Institutional Animal Ethical Committee (IAEC) of Soniya Education Trust's College of Pharmacy, Dharwad, India (REG.No.112/1999/CPCSEA) according to prescribed guidelines of CPCSEA, Government of India.

Experimental Design

Albino Wistar rats of either sex (weighing 250-300g) were divided into five groups of six animals in each group (n=6). Group I Control (distilled water 1ml/p.o.), Group II Sham with (only surgery), Group III is cerebral ischemic group (induced by BCCAO), Group IV and V were treated with Cod liver oil (500 and 1000 mg/kg,b.w respectively). Dosing with Cod liver oil was done at four different time points i.e., 30 mins before cerebral ischemia (day 1), 24 hours after first dose (day 2), 48 hours after 1st dose (day 3) and 72 hours after 1st dose (day 4). After 72 hours all the animals were subjected to different parameters.

Induction of cerebral ischemia by bilateral common carotid artery occlusion (BCCAO)

Rats were anaesthetized with chloral hydrate (400 mg/Kg, ip). A midline incision was made in the region between neck and sternum and the trachea was exposed. Both the right and left common carotid arteries were located lateral to sternocleidomastoid, freed from surrounding tissues and the vagus nerve was separated. Cerebral ischemia was induced by clamping both the arteries with the help of aneurysm clips (Figure:1). After 10mins of cerebral ischemia; the clips were removed from the arteries to allow the reflow of the blood through the carotid arteries. The incision was sutured back in layers with the surgical suture. While performing the surgical procedure; the body temperature was maintained at 37ºC by heated IR lamp. All the surgical instruments used in the procedure were sterilized prior to use [13].

Preparation of post-mitochondrial supernatant

Following decapitation, the brain was removed and washed in cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized as 10%

(w/v) in cold phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 1000 rpm for 10mins at 4ºC and post-mitochondrial supernatant (PMS) was kept on ice until assayed [14].

Biochemical Estimation

Estimation of Total Protein

The peptide bonds of protein react with copper II ions in alkaline solution to form blue-violet complex, (biuret reaction). The protein was estimated using kit of Erba as per Biuret methods, one ml of this working reagent was pipette out in each of the test tubes labeled as blank, standard, test. 20 µl of distilled water, 20 µl of standard and 20 µl of sample homogenate was pipetted out and added to test tubes labelled as blank, standard and test respectively. Test tubes were incubated for 10 mins at 37 ºC. Reading was obtained by measuring the absorbance at 546 nm (520-560nm) in the auto analyser of standard and each test against reagent blank [15].

Estimation of GSH

GSH is a major non-protein thiol and endogenous antioxidant that counters balance free radical mediated damage. It is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reaction. The different solution was added and incubated at room temperature for 15 mins. Absorbance was read at 412 nm using a UV spectroscopy [16].

Estimation of LPO

Oxidative stress is associated with peroxidation of cellular lipids, which is determined by the measurement of thiobarbituric acid reacting substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS results in increased oxidative free radicals, which attacks the polyunsaturated fatty acids in cell membranes and cause lipid peroxidation. The malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS. 15 ml centrifuge tube for sample was labelled accordingly. 100 µl of the sample was pipette out into the respective test tubes. To that 200 µl of 8.1 % SDS solution added and swirled followed by the addition of 1.5 ml of 20 % acetic acid and 1.5 ml of 0.8 % aqueous solution of TBA was added to each tube. Final volume was made up to 4 ml with biological grade water. The tube were heated at 95-100ºC in boiling water bath for one hour. After one hour tubes were cooled immediately under running tap water for 10mins and centrifuged at 4000 rpm for 10mins. The supernatant was collected and optical density was read at 532 nm using UV-Visible Spectrophotometer [17].

Estimation of acetyl cholinesterase enzyme activity of whole brain

On 5thday, exactly 60mins after the scopolamine treatment the rats were decapitated and the whole brain was taken out quickly, suspended in phosphate buffer and weighed accurately. The whole brain was homogenized in a tissue homogenizer. 400mg of brain was homogenized in 0.1M Phosphate buffer pH 8 (10% w/v), the homogenized tissue was centrifuged to 10,000 rpm for 10mins. An aliquot 0.4 ml of the supernatant was added to a cuvette containing 2.6ml phosphate buffer (0.1M, pH 8) and 100µl of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air and the absorbance was measured at 412 nm. When the absorbance reach a stable value, 20µl of substrate i.e., acetylthiocholine was added and change in absorbance was recorded for a period of 10mins at intervals of 2 mins. Change in the absorbance per minute was thus determined. AchE activity is calculated using the formula [18].

$$R = 5.74 \times 10\text{-}4 \times A/CO$$

Where,

R = Rate in moles of substrate hydrolyzed /minute /gm of brain tissue

A = Change in absorbance / minute.

CO = Original concentration of the tissue (mg/ml)

Measurement of infarction area

The infarction area was measured by 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining method. After ischemia and reperfusion animals were sacrificed, and brains were removed within 2-3 mins. Two coronal slices were made at 5 and 7 mm from the frontal pole, and brain slices were immersed in 2% solution of TTC stain in normal saline at 37º C for 30 mins. After which sections were fixed in 10% phosphate buffered formalin for photograph. Then the cerebral infarction area was observed and compared between various treatment group and negative control group [14].

Histopathological examination

The animals were deeply anaesthetized with pentobarbitone. Following decapitation, the brains were taken out and fixed in 10% formalin. Multiple, paraffin-embedded, coronal sections (5-Am thick) were taken from each brain (spanning through striatum to caudal hippocampus). Serial sections (spaced apart by 250 Am, 15 in total for each brain) were selected for histopathological analysis of neuronal damage. After staining with hematoxylin and eosin, the slides were examined using light microscopy by an observer blinded to experimental groups. The brain was subjected to histopathology and observed for infarct cells. The samples were submitted to Jeevan Lab Pvt Ltd. (Belgaum, India) for histopathological examination [19].

Statistical analysis

Results were expressed as mean ± S.E.M. (Graph Pad Prism software, San Diego, CA, version 5.01). Differences among data were determined using one-way ANOVA followed by Dunnet's Multiple Comparision Test (Graph Pad Prism software, San Diego, CA, version 5.01).

Result

Effect of Cod liver oil (500 and 1000 mg/kg) on biochemical parameters

Total protein activity (g/dL)

The total protein activity showed significant decrease ($P<0.001$) in total protein level of positive control group (Cerebral ischemia induced group) compared to vehicle group and sham control group. Cod liver oil treated group showed significant ($P<0.001$) increase in total protein level compared to positive control group (Cerebral ischemia induced group). The results are summarized in Table1.

Lipid Peroxidase activity (µmol/g)

The Lipid peroxidas assay showed significant (P<0.001) increase in LPO activity of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham group. Cod liver oil treated (500 and 1000 mg/kg p.o.) group showed significant (P<0.01) reduction in LPO activity compared to the positive control group. The results are summarized in Table 1.

Reduced Glutathione activity (µmol/g)

The reduced glutathione assay showed significant decrease (P<0.001) in GSH level of positive control group (Cerebral ischemia induced group) compared to vehicle control and sham control group. Cod liver oil treated group showed significant (P<0.001) increase in GSH level compared to positive control group (Cerebral ischemia induced group). The results are summarized in Table 1.

Acetyl cholinesterase (AChE) activity (µmols/min/mg)

The Acetyl cholinesterase assay showed significant increase in AChE level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Cod liver oil treated group showed significant (P<0.001) decrease in AChE level compared to positive control group (Cerebral ischemia induced group). The results are summarized in Table 1.

Effect of Cod liver oil on cerebral infarction

Bilateral carotid artery occlusion induced cerebral ischemia in rats resulted in cerebral tissue damage resulting in large infarction area, as indicated by lesser TTC staining when compared to control. Ischemia followed by reperfusion lead to further increase in infarction area, as indicated by further less TTC staining when compared to control.

Pretreatment with Cod liver oil prevented tissue damage and hence infarction induced by ischemia and reperfusion as shown by increase in TTC staining, when compared to cerebral ischemia (Ci) group. Hence, pretreatment with Cod liver oil showed marked reduction infarction area near to normal as indicated by further increase in TTC staining when compared to cerebral ischemia (Ci) group [Figure 2 (A-E)]

Histopathology of brain

No change was found in histoarchitecture of brain in the control group. The brain sections of bilateral carotid artery occlusion induced cerebral ischemia in rats showed marked cerebral edema, moderate cerebral congestion and mild neutrophilic Infiltration. Ischemia followed by reperfusion resulted in moderate cerebral edema, marked cerebral congestion, moderate neuronal vacuolization and mild neutrophilic infiltration. Pretreatment with Cod liver oil reduced these alterations as indicated by mild cerebral edema, cerebral congestion and mild neutrophilic infiltration. Pre-treatment with Cod liver oil further prevented the pathological changes as shown by mild cerebral edema, cerebral congestion and focal neutrophilic infiltration. [Figure 3 (a-e)]

Fig 1: Bilateral Carotid Artery Occlusion by using Atraumatic clamps.

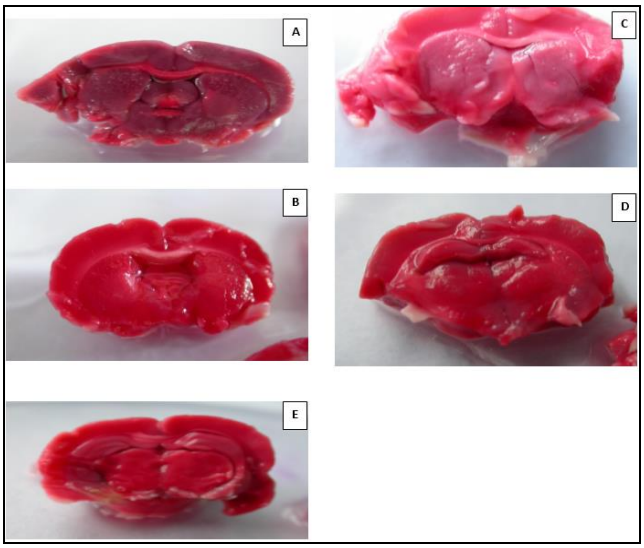

Fig 2: T.S. of Rat Brain

Effect of Cod liver oil on cerebral infarction (Transverse Section of rat brain for each Group I to V denoted as A-E respectively).

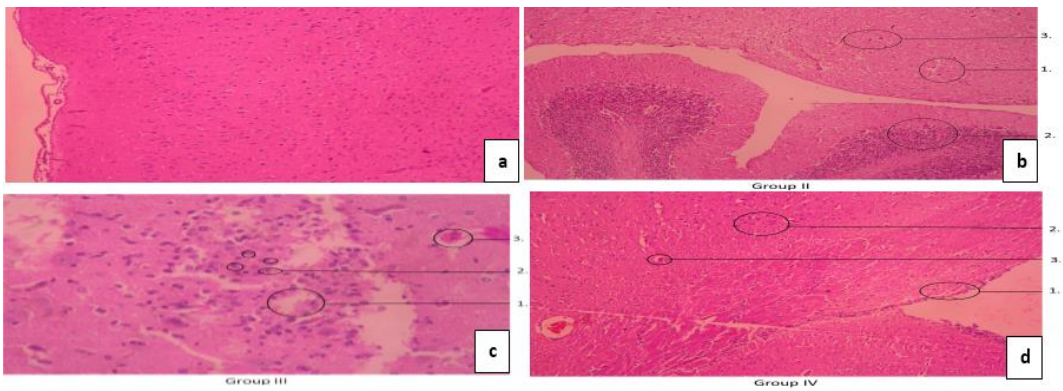

Fig 3: Histopathology of Brain

Histopathology of brain on effect of cod liver oil. (a) No congestion, edema, neutrophilic infiltration (b) Marked cerebral odema, neutrophill infiltration & cerebral congestion, hence overall damage was marked. (c) Marked cerebral odema, neutrophill infiltration & cerebral congestion, hence overall damage was marked. (d) Moderate cerebral odema, neutrophill infiltration & cerebral congestion. Hence overall damage was Moderate. (e) Moderate cerebral odema, neutrophill infiltration & also Moderate Cerebral congestion. Hence overall damage was Moderate.

Table 1: Effect of Cod liver oil on LPO, Total protein, GSH and AChE levels in BCCAO induced global cerebral ischemia in rats

S. No	Groups	LPO (µmols/g)	GSH (µmols/g)	Total Protein(g/dL)	AChE (µmols/min/mg)
1	Vehicle	0.28±0.008	3.31±0.127	1.42±0.040	6.59±0.14
2	Sham Only Surgery	0.35±0.011	3.21±0.107	1.50±0.032	8.36±0.13
3	Cerebral Ischemia (Ci)	0.48±0.011$^{###}$	0.58±0.043$^{###}$	1.04±0.040$^{###}$	9.03±0.081$^{###}$
4	Cod Liver oil (500mg/kg) + Ci	0.34±0.008**	1.73±0.107***	1.54±0.043***	7.21±0.081***
5	Cod Liver oil (1000mg/kg) +Ci	0.31±0.005**	2.59±0.129***	1.56±0.073***	7.52±0.055***

Each value represents Mean ± S.E.M ($n=6$), ***p<0.001, **p<0.01, compared to (Cerebral ischemia) group and ###p<0.001, compared to (Sham only Surgery) group. One way ANOVA followed by Tukey's multiple comparison tests. Significant differences between (Sham only Surgery) group and (Cerebral ischemia) group denoted with #, Significant differences between (Cerebral ischemia) group and Treatment groups denoted with *.

Abbreviation

AChE	Acetyl Cholinesterase
ANOVA	Analysis of variance
BCCAO	Bilateral Common Carotid Artery Occlusion
b.w	Body Weight
Ci	Cerebral Ischemia
EDTA	Ethylene diamine tetra acetic acid
GSH	Reduced Glutathione
IAEC	Institutional Animal Ethical Committee
CPCSEA	Committee for Purpose of Control and Supervision of Experiments on Animals
LPO	Lipid Peroxidase,
p.o.	Per Oral
ROS	Reactive oxygen species
S.E.M	Standard Error of Mean
TTC	2, 3, 5-triphenyl tetrazolium chloride
TBARS	Thiobarbituric acid reacting substance

Discussion

In the present study Cod liver oil was evaluated for neuroprotective activity in bilateral common carotid artery occlusion induced global cerebral ischemia model. Assessment of neuroprotective activity was done by measuring various biochemical parameters such as LPO, GSH, total protein, acetyl cholinesterase. It was reported in literature that, when global cerebral ischemia is produced by carotid artery occlusion, a makeable change is observed in histopathology of brain tissue. Marked destruction of different lobes and cells of brain could be seen [19]. The ischemic parts were confirmed by staining different regions of brain with hematoxylin and eosin stains. The most common form of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. This pathway controls the activation of caspase-9 by regulating the release of cytochrome-c from the mitochondrial intermembrane space [4]. Over production of ROS is a central feature of all neurodegenerative disorders. Reperfusion after cerebral ischemia further adds to the complications of stoke by releasing various mediators such as proinflammatory cytokines and free radical generation [4]. This increases the oxidative stress to the brain and ultimately leading to neuronal cell death [5]. This study has shown following major findings cerebral ischemia is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain and it leads to various pathophysiological modalities such as reactive oxygen species (ROS), calcium overload, mitochondrial damage, neuronal cell death and also associated with oxidative stress and DNA fragmentation [3]. The reduced glutathione assay showed significant decrease (P<0.001) in GSH level of positive control group (cerebral ischemia induced group) compared to vehicle group and sham control group. Cod liver oil treated group showed significant (P<0.001) increase in GSH level compared to positive control group (Cerebral ischemia induced group). The Acetyl cholinesterase assay showed significant increase in AChE level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Cod liver oil treated group showed significant (P<0.001) decrease in AChE level compared to positive control group (Cerebral ischemia induced group). The lipid peroxidase (LPO) assay showed significant (P<0.001) increase in LPO activity of positive control group (cerebral ischemia induced group) compared to vehicle control group and sham group. Cod liver oil treated (500 and 1000 mg/kg p.o.) group showed significant (P<0.001) reduction in LPO activity compared to the positive control group. The total protein activity showed significant decrease (P<0.001) in total protein level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Cod liver oil treated group showed significant (P<0.001) increase in total protein level compared to positive control group. During ischemia, free radicals may be produced to such an extent that endogenous antioxidant systems are overwhelmed. Free radicals are demonstrated to promote lipid peroxidation. In the present study there was a significant increase in acetyl cholinesterase, lipid peroxidation levels and significant decrease in reduced glutathione, as well as total protein levels. Treatment with Cod liver oil at a doses of 500 and 1000 mg/Kg showed in decreased acetyl cholinesterase, lipid peroxidation levels and increase in reduced glutathione as well as total protein levels. Histopathological observation supports the prevention in architecture of the brain due to the treatment with Cod liver oil against occlusion induced cerebral ischemia in rat. Improvement in all antioxidant enzyme levels to significant values suggest antioxidant and free radical scavenger activity of Cod liver oil against cerebral ischemia.

Conclusion

Bilateral common carotid artery occlusion significantly produced cerebral ischemia and caused neuronal damage at 10 mins time point. Pretreatment with Cod liver oil (dose of 500 mg/kg and 1000 mg/kg b.w oral) showed significant neuroprotective effect. Various biochemical parameters measures such as lipid peroxidase (LPO), reduced glutathione (GSH), acetyl cholinesterase (AChE), and total brain protein showed a significant improvement in the levels compared to that of bilateral common carotid artery occlusion induced cerebral ischemia group. Hence, present findings indicate the possible exploitation of Cod liver oil for neuroprotective activity.

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