IJPT
1735-2657/12/111-20-25
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 11: 20-25, 2012

ORIGINAL ARTICLE

Immunomodulatory Activity of Cod Liver Oil

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Received June 27, 2011; Revised October 4, 2011; Accepted November 18, 2011

This paper is available online at *http://ijpt.iums.ac.ir*

ABSTRACT

The immunomodulatory activity of Cod liver oil (CLO) was evaluated by mice lethality test, carbon clearance assay, neutrophil adhesion test, cyclophosphamide-induced neutropenia, indirect haemagglutination test and effect on serum immunoglobulin levels. CLO was administered orally at a dose of 0.5 g/kg or 1 g/kg body weight. In mice lethality test, low dose of CLO (0.5 g/kg, po) produced 40% decrease in the mortality ratio compared to the control suggesting an increase in specific immunity against an invading pathogen. The low dose of CLO (0.5 g/kg, po) also showed significant increase in phagocytic index in carbon clearance assay, increased serum immunoglobulin levels (ZST values) and also showed an increase in antibody titer value in indirect haemagglutination test. The high dose of CLO (1 g/kg, po) was effective only in preventing mortality by 33.3% in mice lethality test. However, both the doses of CLO did not show any significant effect on neutrophil adhesion and failed to prevent cyclophosphamide induced neutropenia. It was concluded that CLO in low dose increases humoral immunity.

Keywords: *Carbon Clearance, Cod liver oil, Cyclophosphamide, Humoral immunity Phagocytic index*

The rise in immunological disorders due to environmental and other dietary habits is a major hurdle faced by medical fraternity. These disorders include multiple sclerosis, congestive heart failure, rheumatoid arthritis and several other inflammatory disorders. Agents that alter the immune system either by stimulating or depressing it are of great significance in treating these disorders and are known as immunomodulators**.** Since dietary habits are one of the major causes for these disorders, considerable work has been done to study the effect of various dietary sources on the immune system [1].

Cod liver oil (CLO) obtained from the cod fish consists of polyunsaturated fatty acids (PUFA) that includes C_{20} fatty acids (17%), palmitoleic acid (7%) and C_{22} fatty acids (11%), vitamin A not less than 850 USP units/g and vitamin D not less than 85 USP units/g [2,3]. Since CLO contains vitamin-D (Vit-D), it is widely used in the treatment of rickets and osteomalacia as dietary supplement [4].

Vit-D has immune regulating effects and is reported to be an immunosuppressant [5-7]. Further, Omega 3 fatty acid present in CLO causes depression of humoral responses and phagocytic functions [8]. On the contrary, vitamin-A (Vit-A) has immune stimulant properties. In large clinical trails, administration of Vit-A to children with measles resulted in major reduction of morbidity and mortality [9]. Vit-A is also shown to possess significant immunomodulatory effect in mice [10].

Since CLO contains Vit-A, Vit-D and omega 3 fatty acid, the present study was carried out to evaluate its effect on the immune system in experimental animals.

MATERIALS AND METHODS

Experimental animals

Albino Wistar rats weighing between 200-250 g and Swiss albino mice weighing between 25-35 g were used. Institutional Animal Ethics Committee approved the experimental protocol; animals were maintained under standard conditions in an animal house approved

by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Chemicals

CLO (Seacod batch no: Ao3B41 Sevenseas. Universal Medicare Mumbai, India), Leishmann's stain (Merck, Mumbai, India), WBC diluting fluid, zinc sulfate, barium chloride (Nice Chemicals, Mumbai, India), cyclophosphamide (Endoxan Injection- German Remedies, Mumbai, India), Indian ink (Bril, Bangalore, India), gluteraldehyde (Merck, Mumbai, India), percoll (Amersham Biosciences, Uppsala, Sweden), hemorrhagic septicaemic vaccine (Industrial Veterinary Research Institute, Bangalore, India).

Determination of purity of CLO

The qualitative and quantitative analysis of CLO was carried out using methods described in the pharmacopoeias. The saponification value, acid value and refractive index test were carried out as per the British pharmacopoeia [11]. Assay of Vit-A was done following the United States pharmacopoeia method [2] and Vit-D assay was carried out using the HPLC procedure mentioned in the Indian pharmacopoeia [12].

Preparation of drug solution [13]

The CLO was formulated into an emulsion using dry gum method. Three parts of the oil was mixed with two parts of water and one part of the gum acacia and triturated in a mortar to form a primary emulsion. This primary emulsion was diluted with water to required concentration.

Treatment

The animals were divided into three groups of six animals each. The first group served as control (vehicle 1 ml/kg, po), and animals of other two groups received CLO orally at a dose of 0.5 g/kg and 1 g/kg body weight respectively. The dose of CLO was selected based on earlier reports [14]. Mice lethality test had four groups, out of which two groups served as controls, one as negative and the other positive control. The remaining groups received the treatments as mentioned above.

Mice lethality test [15]

Male Swiss albino mice were treated with the drugs or vehicle orally for 21 days. On the 7^{th} and 17^{th} day of the treatment, the animals were injected subcutaneously with 0.2 ml of hemorrhagic septicaemic vaccine (HS vaccine). On the 21^{st} day, the animals were challenged subcutaneously with 0.2 ml of 25 LD_{50} dose of *Pasteurella multocida* of bovine origin containing 10^7cells per ml. The animals were observed for a period of 72 hr and the mortality ratio was determined using the formula.

Mortality ratio = Number of animals dead ÷ Total number of animals

Cyclophosphamide (CP)-induced neutropenia [16]

Male Swiss albino mice were divided into four groups and were treated with the drugs or the vehicle orally for 10 days. On the 10^{th} day, neutropenic dose of CP (200 mg/kg) was injected subcutaneously. This day was labeled as day 'zero'. The differential leukocyte count (DLC) were performed prior to and on day 3 after injection of cyclophosphamide. The neutrophil count (% of the total leucocytes) in treated group was compared with the values of the control group.

Neutrophil adhesion test [17]

Male albino rats were given drugs or vehicle for 14 days. On the day 14, blood samples were withdrawn and analyzed for DLC. After the initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 10 min at 37°C. The incubated blood samples were analyzed again for DLC.

Carbon clearance test [18]

Male albino mice were divided into four groups of six animals each. The animals were treated with the drugs or vehicle orally for 5 days. After 48 h of the last dose, mice were injected 0.1 ml of Indian ink via the tail vein. Blood samples were withdrawn from the retro orbital plexus at 0^{th} min and 15^{th} min. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index (K) was calculated using the following equation:

$K= (Log_e OD1-Log_e OD2)/15$

Where OD1 and OD2 are the optical densities at 0 min and 15 min, respectively.

Effect on serum immunoglobulins levels [19]

Female albino rats were treated with the drugs orally for 21 days. Six hours after the last dose of drug, blood was collected from the retro-orbital plexus under ether anesthesia and the serum was separated and used for estimation of immunoglobulin levels. For each serum sample to be measured, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution (408 mg/ml) were prepared. To each of these tubes, 0.1 ml of serum was added from a pipette. The tubes were inverted to enable complete mixing of the reagents and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample - blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of $BaCl_2$ solution (1.15% w/v) to 97 ml of 0.2N sulphuric acid. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units.

Table 1. Effect of cold liver oil (CLO) on mice lethality test

Treatment	Number of dead mice in group of six			Mortality Ratio
	Day-1	Day-2	Day-3	
Vehicle (1 ml/kg, po)	2	2	2	100%
Positive control (Vehicle + vaccination)	1	1	3	83.3%
CLO (0.5 g/kg, po) + vaccination	Nil	1	1	33.3%
CLO (1 g/kg, po) + vaccination	Nil	1	2	50%

Table 2. Effect of cold liver oil (CLO)on cyclophosphamide (CP)-induced neutropenia

Treatment	Neutrophil count (%)		Difference (A-B)	% Reduction of neutrophils
	Before CP Treatment (A)	After CP Treatment (B)		
Vehicle (1 ml/kg, po)	13.83 ± 0.401	7.00 ± 0.810	6.83 ± 0.870	49.38%
CLO (0.5 g/kg, po)	15.16 ± 0.703	9.16 ± 1.195	6.00 ± 1.340	39.5%
CLO (1 g/kg, po)	13.50 ± 1.839	9.83 ± 0.874	6.33 ± 1.085	49.40%

All values are mean ± SEM, n = 5-6.

Indirect haemagglutination test [20]

Rats were pretreated with the drugs for 14 days followed by immunisation with $0.5x10^9$ sheep red blood cells (SRBCs) per rat by ip route, including control rats. The day of immunization was referred as day 0. The drug treatment was continued for 14 more days and blood samples were collected from each rat next day for determination of haemagglutinating antibody (HA) titre. The titre value was determined by titrating serum dilu tions with $0.025x10^9$ SRBCs using microtiter plates. The microtiter plates were incubated at room temperature for 2 hr and examined visually for agglutination. The highest number of dilution of serum showing haemagglutination has been expressed as HA titre.

Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test. The values were expressed as mean ± SEM and $p < 0.05$ was considered significant.

RESULTS

Analysis of CLO

The saponification value of CLO was 185.13 and the acid value was found to be 1.12. The refractive index was 1.475. The sample contained 2133 USP units of Vit-A and 114 USP units of Vit-D per g. All the above values were within the limits prescribed by the United States pharmacopoeia and British pharmacopoeia.

Mice lethality test

The low dose of CLO (0.5 g/kg, po) was effective in reducing the mortality. The mortality ratio in the high dose (1 g/kg, po) group was more compared to that of low dose-treated animals (Table 1).

Cyclophosphamide (CP)-induced neutropenia

Both low dose (0.5 g/kg, po) and high dose (1 g/kg, po) of CLO did not show any significant effect on neutrophil count before CP treatment and after CP treatment. Similarly no effect on difference in the neutrophil count was observed when compared to control group (Table 2).

Neutrophil adhesion test

None of the treatments showed any significant effect on neutrophil adhesion when compared to control (Table 3).

Carbon clearance assay

The low dose of CLO (0.5 g/kg, po) produced a significant increase in the phagocytic activity of the macrophages as indicated by a significant increase in phagocytic index ($p < 0.01$). The high dose of the CLO (1 g/kg, po) was ineffective (Table 4).

Effect on serum immunoglobulins

The low dose of CLO (0.5 g/kg p.o.) produced a significant increase in ZST values when compared to the control group indicating an increase in serum immunoglobulin levels (Table 4).

direct action on human peripheral mononuclear cells,

Table 3. Effect of cold liver oil (CLO) on neutrophil adhesion

Treatment	Neutrophils (%)		Difference (A-B)
	Untreated blood (A)	Nylon-fiber-treated blood (B)	
Vehicle (1 ml/kg, po)	15.66 ± 1.28	10.33 ± 1.05	5.33 ± 0.49
CLO(0.5 g/kg, po)	13.50 ± 1.33	7.66 ± 0.98	6.50 ± 0.34
CLO(1 g/kg, po)	12.00 ± 1.53	9.66 ± 1.84	5.30 ± 1.33

All values are mean ± SEM, n = 5-6

Table 4. Effect of cold liver oil (CLO) on phagocytic index in carbon clearance assay, serum immunoglobulin levels and haemagglutinating antibody titre in indirect haemagglutination test

Treatment	Phagocytic Index	Serum immunoglobulin	HA titre levels (ZST Units)
Vehicle (1 ml/kg, po)	0.03343 ± 0.00245	21.12 ± 0.419	21.91 ± 8.94
CLO (0.5 g/kg, po)	0.07840 ± 0.01605**	23.06 ± 0.290**	120.00 ± 17.80*
CLO (1 g/kg, po)	0.03968 ± 0.00506	22.23 ± 0.487	53.33 ± 8.43

All values are mean ± SEM, n = 6. *$p < 0.05$, **$p < 0.01$ when compared to control group

Indirect haemagglutination test

The antibody titer value in CLO (0.5 g/kg p.o.) treated group were significantly more when compared to that of control (Table 4).

DISCUSSION

In the present study, CLO showed immunostimulant activity only in lower dose (0.5 g/kg, po). CLO (0.5 g/kg, po) prevented the mortality of mice challenged with *Pasteurella multocida* in mice lethality text, increased phagocytic index in carbon clearance test, in creased serum immunoglobulin levels and also increased the antibody titer in indirect agglutination test. CLO at both low and high dose did not show any significant effect on cyclophosphamide induced neutropenia and neutrophil adhesion.

Fish oils are widely used as dietary supplements. The effect of some of the fish oils on the immune system has been reported. Fish oils in general are known to possess immunosuppressant effect due to the presence of omega 3 fatty acids. The omega 3 fatty acids present in the fish oils are recommended for the treatment of autoimmune diseases like rheumatoid arthritis [1,8]. Although many fish oils are reported to possess immunosuppressant activity, oil obtained from liver of fish such as shark liver oil is an immunostimulant and also possess haemopoetic properties. The shark liver oil also contains omega 3 fatty acid along with other alkylglycerols. The immunostimulant activity is attributed to the presence of alkylglycerols [21].

Cod liver oil is a fish oil that is rich in omega 3 fatty acid and unlike other fish oils also contain natural Vit-D and Vit-A. As mentioned earlier, considerable work has been done to study the effect of Vit-D on the immune system. Vit-D produces anti-proliferative effect by decreases interleukin (IL)-2 production and is also reported to inhibit cell-associated IL-1 activity in monocytes. It is also reported to suppress antibody production by B-cells and proliferation of T-cells [5-7]. Vit-A on the contrary is reported to possess immunostimulant property. Administration of Vit-A reduces morbidity and mortality in children suffering from measles [9]. Vit-A deficiency is associated with increased risk of vertical transmission of HIV-1 and of disease progression and mortality among HIV infected adults [22].

The effect of cod liver oil was evaluated using different animal model to determine its effect on various immunological responses. The carbon clearance assay was used to evaluate the effect on reticulo endothelial cell mediated phagocytosis [18]. Colloidal carbon acts as a foreign particulate that triggers macrophage phagocytosis and it is preferred for carbon clearance assay because of its small particle size and even distribution through out the suspension. When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. Rate of clearance of (carbon particles) ink from blood is known as phagocytic index. The low dose of CLO produced an increase in phagocytic index suggesting its effect on reticuloendothelial system.

Cyclophosphamide, nitrogen mustard with anti-cancer property is known to cause myleosupression resulting in neutropenia [23]. DLC before and after cyclophosphamide administration were determined to study the effect of CLO on haemopoesis. There was no significant change in reduction of neutrophils after treatment with both doses of CLO indicating that CLO does not have any effect on the haemopoeitic system in immune suppressed states.

Cell adherence property of neutrophils is one of the earliest responses of both immunological and physical injury [17]. In neutrophil adhesion test, cell adherence property of neutrophils was assessed in blood sample

from different groups, by treating with nylon fibers to which the neutrophils adhere. Both doses of CLO failed to show any significant effect on neutrophil adhesion.

In mice lethality test, all the treated groups except negative control were sensitized with HS vaccine to boast antibody production [24]. The vaccinated animals were challenged with 25 LD_{50} dose of *Pasteurella multocida* bacterial culture and observed for period of 72 hr for mortality. Low dose of CLO (0.5 g/kg, po) showed good protection but the survival period was less; while the high dose provided protection was less compared to the low dose of CLO.

Estimation of immunoglobulin level is a direct measure of humoral immunity. Immunoglobulin levels were determined using zinc sulphate turbidity test. This method is dependent on the correlation between the concentration of specific immunoglobulin present in the serum and the intensity of the zinc sulphate reaction developed [19]. The ZST units in low dose treated (0.5g/kg, po) animals were significantly more compared to that of control animals proving their effect on humoral immunity.

The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation to antibody secreting plasma cells [18]. In indirect haemagglutination test, SRBCs that act as specific antigens were injected subcutaneously. The SRBCs trigger production of specific antibodies that are estimated in serum samples of different treated groups. The mean antibody titer values of CLO (0.5 g/kg, po) treated was significantly more compared to control further confirming their effect on humoral immunity.

The results of the present study suggest that CLO can be used as dietary supplement in the treatment of immunodeficiency states and CLO should not be given to patients suffering from autoimmune disorders. The immunostimulant activity of CLO may be due to the presence of Vit-A.

ACKNOWLEDGEMENTS

The authors are thankful to Prof Suresh Nagpal, Chairman, Krupanidhi Educational Trust and Prof Sunil Dhaminigi, Secretary, Krupanidhi Educational Trust for providing facilities to carry out the work. The authors are also thankful to Prof Amit Kumar Das, Principal, Krupanidhi College of Pharmacy for his suggestions during the work.

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