Immunomodulatory Activity of Cod Liver Oil

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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
Swiss albino mice were treated with the drug 21 A 50 μl blood
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The phagocytic index (K) was
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via the tail
(0.2 ml of
sulphate
0.2N sulphuric acid. The
turbidity obtained with this solution was expressed as
20 zinc sulphate turbidity (ZST) units.
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 7th and 17th day of
the treatment, the animals were injected subcutaneously
with 0.2 ml of hemorrhagic septicaemic vaccine (HS
vaccine). On the 21st day, the animals were challenged
subcutaneously with 0.2 ml of 25 LD50 dose of
Pasteurella multocida of bovine origin containing
107 cells/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 10th day, neutropenic dose of
CP (200 mg/kg) was injected subcutaneously. This day
was labeled as day ‘zero’. The differential leucocyte
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 0th and 15th 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:

\[
K = \frac{\log \text{OD}1 - \log \text{OD}2}{15}
\]

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 given drugs or vehicle 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 (BaSO4) solution. The standard BaSO4
solution was prepared by adding 3 ml of BaCl2 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.
Rats were pretreated with the drugs for 14 days followed by immunisation with $0.5 \times 10^9$ 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 dilutions with $0.025 \times 10^9$ 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).
**Table 3.** Effect of cold liver oil (CLO) on neutrophil adhesion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neutrophils (%)</th>
<th>Difference (A-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated blood (A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (1 ml/kg, po)</td>
<td>15.66 ± 1.28</td>
<td></td>
</tr>
<tr>
<td>CLO (0.5 g/kg, po)</td>
<td>13.50 ± 1.33</td>
<td>5.33 ± 0.49</td>
</tr>
<tr>
<td>CLO (1 g/kg, po)</td>
<td>12.00 ± 1.53</td>
<td>5.30 ± 1.33</td>
</tr>
</tbody>
</table>

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

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

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 test, 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 anticancer property is known to cause myelosupression resulting in neutropenia [23]. DLC before and after cyclophosphamide administration were determined to study the effect of CLO on haemopoiesis. 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 haemopoietic 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 boost antibody production [24]. The vaccinated animals were challenged with 25 LD50 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 secretory 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.

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