Effect of feeding cuttlefish liver oil on immune function, inflammatory response and platelet aggregation in rats

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The effect of feeding 1% cuttlefish (*Sepia pharaonis*) liver oil for a period of 45 days on immune function, inflammatory response and platelet aggregation was studied in male albino Sprague Dawley rats. In liver oil-treated animals, spleen cell proliferation was significantly higher, especially in the presence of mitogen. The bone marrow cells also showed enhanced proliferation in the treated animals compared to control; however, mitogenic stimulation was not observed. An increase in the number of plaque-forming cells in the spleen and antibody titre in the circulation was also observed in the treated animals, indicating an enhanced humoral immune response. Cuttlefish liver oil feeding also led to a significant decrease in both carrageenan-induced and formalin-induced paw oedema. ADP-induced platelet aggregation was also found to be inhibited in the test animals. The results indicate that feeding a low dose of cuttlefish liver oil can stimulate the immune functions, inhibit inflammatory response and platelet aggregation in rats. The n-3 polyunsaturated fatty acids, especially eicosapentaenoic acid present in the cuttlefish liver oil, may be responsible for the observed beneficial effects.

RECENT research has implicated dietary fish oils in the reduction of eicosanoids formed from n-6 polyunsaturated fatty acids (PUFAs) and amelioration of chronic diseases such as coronary heart disease, atherosclerosis and inflammation. A range of anti-inflammatory and immunomodulatory effects of the n-3 family of PUFAs, particularly those found in fish oils have been identified. It has been ascertained that these fatty acids work in part by antagonizing the production of eicosanoids – prostanoids and leukotrienes), which displace and/or modify the effects of those synthesized from n-6 PUFAs. The beneficial effects of n-3 PUFAs most likely relate to their modification of eicosanoid synthesis and metabolism. The cuttlefish liver, which is discarded during processing, constitutes about 7–12% of body weight and contains high oil content ranging from 6 to 40% and is a rich source of n-3 PUFAs like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). So far, no attempt has been made to exploit this n-3 PUFA-rich resource and study its health beneficial effects as in the case of other marine fish oils. In the present study, the immunomodulatory and anti-inflammatory response in male albino Sprague Dawley rats fed with 1% cuttlefish liver oil in diet, has been evaluated. The effect of feeding cuttlefish liver oil on ADP-induced platelet aggregation was also studied.

Tissue culture media RPMI-1640 and foetal bovine serum (FBS) were purchased from Himedia Laboratories, Mumbai. Phytohaemagglutinin (PHA) was obtained from Genei, Bangalore. ³H-Thymidine was purchased from BRITE, Bhabha Atomic Research Centre, Mumbai. All other reagents were of analytical reagent quality.

Cuttlefish liver was collected from fresh specimens of *Sepia pharaonis*, at the processing plant ‘Bhatsons Aquatic Products’, Alleppey. The liver was cut into small pieces and dried at 60°C in a drier. The dry powder obtained was then extracted with petroleum ether (b.p. 60–80°C) using a Soxhlet extraction apparatus for 8–10 h. The extract was completely evaporated at 40°C in vacuum to obtain the viscous cuttlefish liver oil, which was stored in an amber coloured bottle at −20°C till used for the feeding study.

Male albino Sprague Dawley rats weighing 80–110 g body weight, purchased from College of Veterinary and Animal Sciences, Kerala Agricultural University (KAU), Thrissur were used for the study. The animals were housed in groups of six in polycarbonate cages with a 12:12 light/dark cycle. Sufficient numbers of control groups and test groups were maintained so that at least six animals were available for each assay. The animals in the control group were fed on normal diet purchased from College of Veterinary and Animal Sciences, KAU, Thrissur. The animals of the test group were fed on normal diet +1% cuttlefish liver oil. They were provided with food and water *ad libitum*. The experimental duration was 45 days.

Splenic T-lymphocyte mitogen response was assayed as follows. At the end of the feeding study, the animals were sacrificed, spleen removed aseptically and made into single cell suspension. The cells from both the control and test animals were cultured (10⁶ cells/ml) in RPMI-1640 medium containing 7% FBS (final volume 1 ml) and antibiotics in a humidified atmosphere of 5% CO₂ at 37°C. After 48 h, ³H-thymidine was added (1 µCi/vial) and further incubated for 18 h, at the end of which the DNA was precipitated using 0.8 M perchloric acid and the incorporated radioactivity was counted using a liquid Scintillation Counter (Wallac 1280). Bone marrow cell proliferation assay was carried out following the method of Kumar et al. Total bone marrow cells were collected from control and test animals and made into single cell suspension in RPMI-1640 as described above. The cells (10⁶ cells/ml) were cultured in the presence and absence of mitogen PHA (4 µg/ml). The proliferation of bone marrow cells was determined from the amount of radioactive thymidine incorporated into the DNA.
Modified slide technique of Jern’s plaque assay was adopted for plaque formation cell assay. At the end of the experimental period, the control and test animals were immunized with 1 ml of 5% SRBC intraperitoneally. The spleen was collected from the sacrificed animals on the 9th day following immunization. A single cell suspension of the spleen cells was prepared in HBSS (8 x 10^6 cells/ml). To 0.5 ml of 0.5% agarose prepared in HBSS, 50 µl of 10% SRBC and 50 µl spleen cell suspension were added, mixed well and poured over a glass slide. The slides were allowed to solidify and then incubated with fresh guinea pig serum as a source of complement for 1 h at 37°C. The plaques formed were counted using a colony counter and represented as plaque forming cells (PFCs/million spleen cells).

Circulating antibody titre was determined as follows. Blood was collected from the immunized animals on the 9th day following immunization by cutting the jugular vein. The blood was allowed to clot and serum was separated by centrifugation. Twofold serial dilution of the serum samples was made in physiological saline and mixed (1:1) with 1% SRBC in physiological saline. Agglutination was noted after incubation at room temperature for 3 h.

For determining the antiplatelet aggregating activity, blood from control animals and test animals was collected in anticoagulant solution (2.4% sodium citrate, 1.5% citric acid and 1.8% dextrose). The ratio of blood to anticoagulant solution (2.4% sodium citrate, 1.5% citric acid and 1.8% dextrose) was approximately 5:1. The platelet-rich plasma (PRP) was separated by centrifugation at 1850 rpm for 7 min. PRP was centrifuged at 4500 rpm for 18 min to sediment the platelets. The platelet sediment was dispersed in washing buffer composed of 113 mM NaCl, 4.3 mM KH₂PO₄, 4.3 mM Na₂HPO₄, 24.44 mM NaH₂PO₄ and 5.5 mM dextrose, pH 6.5 and the platelets were collected after centrifugation at 900 g for 10 min. The platelets were then suspended in a buffer composed of 109 mM NaCl, 4.3 mM KH₂PO₄, 16 mM Na₂HPO₄, 8.3 mM NaH₂PO₄ and 5.5 mM dextrose, pH 7.5. The suspension was adjusted to give a final optical density of approximately 0.5 at 600 nm. To 1 ml of platelet suspension, 20 µl 1 mM ADP was added and the OD at 600 nm was measured at 1 min intervals up to 5 min in a spectrophotometer (Jasco V-530, Japan).

Carrageenan-induced paw oedema was used for determining the acute anti-inflammatory activity of the liver oil. In both the control and test groups, inflammation was produced by injecting 0.1 ml of a 1% carrageenan solution in the left hind paw. The paw thickness was measured using vernier calipers before and 3 h after injecting carrageenan.

Chronic anti-inflammatory activity was determined by formalin-induced paw oedema. In both the control and test groups, chronic inflammation was produced by injecting 0.1 ml 2% formalin in the left hind paw of the rats. The paw thickness was measured using vernier calipers before and 6 days after injecting formalin.

Percentage paw oedema was calculated using the formula

\[
\frac{P - P₀}{P₀} \times 100,
\]

where \( P \) is the paw thickness at final time and \( P₀ \) is the paw thickness at the initial time. The mean of the six values was taken as the mean percentage oedema.

The results were expressed as mean ± SD and statistical analysis was done by Student’s t-test.

The results (Table 1) on the effect of feeding 1% cuttlefish liver oil to rats for a period of 45 days showed a two-fold increase in thymidine uptake by spleen cells stimulated with PHA, compared to the control group receiving no cuttlefish liver oil. In the absence of mitogen, there was no significant difference in the thymidine uptake between the control and the test group.

The results (Table 1) also showed that there was a significant increase in the proliferation of bone-marrow cells of rats fed on 1% cuttlefish liver oil compared to the control. Mitogen treatment did not affect the proliferation of these cells, showing the absence of any mature T-cells.

There was a significant increase in the number of plaque-forming cells in rats fed with cuttlefish liver oil (Table 2). There was a 16 times increase in the circulating antibody titre in the serum of test animals (Table 2).

The mean % oedema after 3 h of carrageenan injection was significantly lower in the test animals (Table 3). Formalin-induced paw oedema was also inhibited significantly in the animals fed on cuttlefish liver oil.

Addition of ADP to platelets separated from the blood of control animals showed a decrease in OD at 600 nm indicating aggregation of platelets (Figure 1). In the case of platelets isolated from the test animals, the decrease in OD at 1 min after adding ADP was half that of the control and the OD slowly increased showing that ADP-induced platelet aggregation was inhibited in cuttlefish liver oil-fed animals.

**Table 1. Effect of feeding cuttlefish liver oil on splenic T-lymphocyte mitogen response and bone marrow cell proliferation in rats**

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Spleen cells</th>
<th>Bone-marrow cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>No mitogen</td>
<td>PHA µg/ml</td>
</tr>
<tr>
<td>Control</td>
<td>3279.50 ± 146.68</td>
<td>5303.50 ± 421.88</td>
</tr>
<tr>
<td>Test</td>
<td>3495.87 ± 68.38</td>
<td>11909.16 ± 402.79</td>
</tr>
</tbody>
</table>

*P < 0.001; Values are mean of ± SD of six different estimations.
Several studies have suggested that EPA-rich fish oils boost immune function primarily by mediating the production of eicosanoids. It is also reported that low intake of long chain n-3 fatty acids, i.e. fish oils, enhances certain immune functions, whereas high intakes are inhibitory on a wide range of functions.

In the cuttlefish liver oil-treated animals, spleen cell proliferation was found to be stimulated in the presence of mitogen as seen from the increased \(^3\)H-thymidine incorporation. T-cells respond to plant mitogens like PHA by rapid blastogenesis. The ability of T-cells to get transformed has been shown to bear correlation with in vivo parameters of cell-mediated immunity status of the individual.

Enhanced proliferation of bone marrow cells was also observed in treated animals compared to control. This indicates induction of proliferation of bone marrow stem cells either directly or indirectly, stimulating the release of factors that are involved in the regulation of hemopoiesis. The treated animals also showed an increase in the number of plaque-forming cells in the spleen and antibody titre in the circulation, which are the functions of B-cells.

The fatty acid composition of inflammatory and immune cells is sensitive to change according to the fatty acid composition of the diet. In particular, the proportion of different types of PUFAs in these cells is readily changed and this provides a link between dietary PUFA intake, inflammation and immunity.

Animal studies have shown that dietary fish oils result in altered lymphocyte function and in suppressed production of proinflammatory cytokines by macrophages. Lymphocytes are involved in both the beneficial and detrimental effects of the immune system. Both the level of fat and the type of fatty acid present in the diet can affect lymphocyte functions. The present study reveals that feeding cuttlefish liver oil at a level of 1% in the diet for 45 days, stimulates immune function and inhibits inflammatory response in rats.

The type and content of n-3 PUFAs in cuttlefish liver oil may be responsible for this. The finding is in agreement with the report that low intake of long chain n-3 PUFAs enhances immune function.

In the present study, cuttlefish liver oil exhibited significant anti-inflammatory activity in acute and chronic inflammations in rats. Carrageenan-induced acute inflammation in animals is one of the most suitable test procedures to screen anti-inflammatory agents. The development of carrageenan-induced oedema is biphasic; the first phase is attributed to the release of histamine, 5-HT and kinins and occurs within an hour of injection and is partly due to the trauma of injection, while the second phase is related mainly to prostaglandins, measured around 3 h. Formalin-induced paw oedema is one of the most suitable test procedures to screen chronic anti-inflammatory agents, as it closely resembles human arthritis. The nociceptive effect of formalin is also biphasic, an early neurogenic component followed by a tissue-mediated response. The ability of fish oil to reduce acute and chronic inflammatory response has been well established. The n-3 PUFAs may exert their effects by modulating signal transduction/or gene expression within inflammatory and immune cells. EPA is a substrate for cyclooxygenase and lipoxygenase and gives rise to mediators that often have different biological actions or potencies than those formed from AA. Clinical studies have reported that fish-oil supplementation has beneficial effects in rheumatoid arthritis, inflammatory bowel disease and some asthmatics, supporting the idea that the n-3 PUFAs in fish oil are anti-inflammatory and immunomodulatory.

ADP-induced platelet aggregation was inhibited in the test animals compared to the normal group. This finding is in accordance with the earlier studies with marine fish oils. Under normal circumstances, when linoleic acid (18:2 n-6) is the predominant PUFA in the diet, platelet aggregation and blood clotting are thought to be controlled by the opposing effects of thromboxane A\(_2\) (TXA\(_2\)) produced by platelets and prostacyclin (PGI\(_2\)) synthesized in vessel walls. TXA\(_2\) strongly promotes platelet aggregation and blood clotting, whereas PGI\(_2\) has the opposite effect. The EPA in fish oil can serve as a precursor of TXA\(_3\) and

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**Table 2.** Effect of feeding cuttlefish liver oil on plaque-forming cells and circulating antibody titre

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of PFCs/million spleen cells (mean ± SD)</th>
<th>Circulating antibody titre (mean of six different estimations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>165.166 ± 9.516</td>
<td>4</td>
</tr>
<tr>
<td>Test</td>
<td>381.5 ± 20.334*</td>
<td>64</td>
</tr>
</tbody>
</table>

*P < 0.001.

**Table 3.** Effect of feeding cuttlefish liver oil on carrageenan and formalin-induced paw oedema in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Carrageenan model Mean % oedema after 3 h</th>
<th>Formalin model Mean % oedema after 6 days</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>140.52 ± 2.64</td>
<td>250.23 ± 2.4</td>
<td>0.25</td>
</tr>
<tr>
<td>Test</td>
<td>60.60 ± 1.45*</td>
<td>110.34 ± 1.7*</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*P < 0.001; Values are mean ± SD of six different estimations.

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Figure 1. Effect of feeding cuttlefish liver oil on platelet aggregation in rats. Each point is the mean of six replicates.
PGI$_3$, whereas TXA$_2$ does not induce aggregation of platelets\textsuperscript{23}, whereas PGI$_1$ like PGI$_2$, is a potent anti-aggregating agent\textsuperscript{24}. EPA has a high binding efficiency for platelet cyclooxygenase and thus competes with AA, inhibiting its conversion to TXA$_2$ and PGI$_2$.\textsuperscript{25,26} EPA may also affect platelet aggregation by blocking TXA$_2$ receptors on the cell membrane or by the formation of prostaglandins D$_2$ and E$_3$ as opposed to prostaglandins D$_2$ and E$_3$.\textsuperscript{27,28}

The results of the present study reveal the beneficial effects of feeding low dose of cuttlefish liver oil on immunostimula-
tion, suppression of inflammatory response and inhibition of platelet aggregation in rats. As reported in the case of marine fish oils, the EPA content of cuttlefish liver oil is the cause of the presently observed beneficial immunomodulatory effects. The study also reveals that a low level of n-3 PUFA is sufficient to elicit the above functions.