# Influence of essential fatty acid deprivation on thrombocyte aggregation in rainbow trout

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## Abstract

Many reports have indicated that differences in blood cells formation and function in fish could be of dietary origin. Essential fatty acids are certainly connected with these cells by virtue of being a source of important compounds like eicosanoids, platelet activating factor in mammals, as well as the cell membrane phospholipids. The thrombocytes from fish fed the commercial diet containing adequate amount of essential fatty acids exhibited greater capacity for aggregation when induced by Collagen Type I (56%), however, this capacity was reduced when fish were fed the essential fatty acids deficient diet (37%). The results obtained in this study demonstrated that EFA exert influence in thrombocytes by affecting their aggregation capacity.

**Key-words:**
Thrombocyte
Essential fatty acids.
Trout.
Aggregation.
Monoclonal antibody.

## Introduction

Lipids are an important component of the diet, serving as a source of essential fatty acids (EFA) which the fish cannot synthesize, but require for both growth and maintenance of healthy tissues [1]. Among macronutrients, lipids in general and EFA in particular exert considerable influence on the health of fish [2]. There are a few studies examining the role of dietary fatty acids on immune response of fish [3,4,5] and a couple of them have examined fatty acid composition, eicosanoids generation and adaptive responses of leucocytes [6,7]. Some of the membrane-related fatty acids are sources of eicosanoids which directly affects the immune cell function. In fish, the n-3 fatty acids play a role in the metabolism of platelet activating factor, a potent lipid involved in normal physiology and in eliciting pathological responses [8].

Factors involved in clotting, coagulation and blood cell formation have been studied for fish health assessment [9]. Many studies have indicated that differences in blood cell formation and its function in fish could be of dietary origin [10,11,12]. It could therefore be considered that thrombocytes of fish may also be similarly influenced by the diet. These cells in fish are considered to be equivalent to platelets in mammals and aggregation has been described as their principal function[13]. The employment of monoclonal antibodies specific to thrombocytes have enabled the isolation of a pure population of these cells, thereby making it possible to examine their aggregatory potential [14].

The present report is perhaps the first ever to have observed changes in the thrombocyte function in fish, related to diet. The focus is on deprivation of dietary essential fatty acids and aggregation capacity of thrombocytes from rainbow trout.

## Materials and Methods

Laboratory reared rainbow trout, *Oncorhynchus mykiss* (Walbaum) maintained on commercial diets up to a size of 85±2g were selected for experimental feeding. Initially, forty fish (each) were introduced to three 100 l polycarbonate tanks and offered either a defatted fish meal diet containing palmitic

acid ester alone as lipid source (deprived groups) or a commercial rainbow trout diet from Nippon Formula Feed Co., Japan (normal group). The fish fed on the commercial diet (one group) were receiving an usual supply of lipids, sourced from the ingredients, which also included fish meal. On the other hand, the fish fed the diet containing completely deffated fish meal (two groups) had access only to palmitic acid and none of the essential fatty acids were available from the diets during the experimental period. Thus, the diets served either as an EFA containing or as an EFA depleted diet and were offered to apparent satiation levels for six months. Sampling was done at this point when the average weight gain for the normal fish was 180g as against 122g for the deficient fish. Thereafter, in order to investigate a possible recovery, one of the groups fed EFA-depleted diets were re-fed on the commercial diet (for 4 months). The water temperature of the flow-through rearing system during the period was 17±2°C.

Fish weighing over 200g were sampled from both the normal as well as of the deficient groups; they were anaesthetized prior to drawing blood to assess the thrombocyte activity. Pooled blood from four fish constituted the sample of a particular group for each of the four trials performed. The livers were removed from fish to confirm the EFA status. They were immediately frozen at -80°C and later fatty acid composition of total lipids was determined by gas chromatography following the AOCS official method Ce 1b-89[15].

Buffy coat of each fish was obtained by centrifugation of the blood samples at 1800´*g*, for 5 min, at 4°C. They were then washed in RPMI-1640 (Nissui Pharmaceutical Co., Japan) for three times and pooled group-wise. Leucocytes were obtained by one-step continuous gradient separation using a Percoll (Pharmacia, Sweden) density of 1.09g/ml. About $3´10^7$ cells, suspended in RPMI supplemented with heat-inactivated fetal bovine serum (FBS; Commonwealth Serum Laboratories, Australia), were pre-incubated with the monoclonal antibody (MAb) TTL-7D11 (anti-trout thrombocytes[14] for 45 min at 4°C. Cells were washed and incubated with a 1:5 dilution of magnetic bead-conjugated goat anti-mouse IgG antibody for 20 min at 4°C (Miltenyi Biotec GmbH, Germany). Leucocytes were washed again and the pellet was re-suspended in degassed RPMI with 10% FBS. Then, they were sorted using a magnetic separation system (Magnetic Cell Separator MACS, Miltenyi Biotec GmbH, Germany). MAb-positive and –negative cells were suspended in degassed RPMI with 10% FBS, washed and suspended again in normal RPMI with 10% FBS.

The aggregation capacity of thrombocytes was investigated by employing collagen Type I (Sigma, USA) prepared at a concentration of 300µl/ml with 0.15M of Tris-buffered saline. Its aggregatory effect on the thrombocytes was verified by using a method modified from Woodward, Smith e Casillas [16]. Ten µl of the collagen solution was mixed with $3´10^6$ cells (suspended in 100 ml of RPMI supplemented with 10%FBS) and carefully rotated for 10 min at 20°C. Control groups were set with 0.15M Tris-buffered saline. After that, aggregated cells were fixed by 1% paraformaldehyde in 0.1M phosphate-buffered saline. The suspensions were observed with a Thoma's hemocytometer where the free cells were counted. When three or more cells were clumped together they were considered to be aggregated. The percentage of aggregated cells for the normal and deficient fish were determined as follows:

$$\text{Aggregated cells (\%)} = \frac{\text{Total cells} - \text{Free cells}}{\text{Total cells}} \times 100$$

where "Total cells" is the initial total number of cells, and "Free cells" is the number of cells that did not aggregate. The same procedures described above were applied later to the EFA-deficiency recovery fish thrombocytes.

## Results, Discussion and Conclusions

On the basis of the fatty acid analysis, the EFA deficiency status of the deprived fish was indicated by the abnormally high levels of eicosatrienoic acid [20:3(n-9)] and monoethylenic fatty acids compared to the normal fish (Table 1). The situation induced through prolonged dietary insult is clearly discernible in the liver, a focal organ in general lipid metabolism. However, it would have been more appropriate if the fatty acid composition of the phospholipids were

Table 1 - Composition[A] of selected fatty acids from total lipid of liver of normal and deficient fish

| Fatty Acid | Diet Group | | |
|---|---|---|---|
| | Normal | Deficient | Recovery |
| Total saturates | 32.47 | 25.62 | 38.18 |
| Total monoenes | 25.03 | 53.63 | 19.63 |
| 18:2(n-9) | Nd[B] | nd | nd |
| 20:2(n-9) | 0.25 | 0.06 | 0.18 |
| 20:3(n-9) | 1.64 | 4.43 | 1.47 |
| Total (n-9) PUFA | 1.89 | 4.49 | 1.65 |
| 18:2(n-6) | 6.01 | 1.10 | 5.04 |
| 18:3(n-6) | 0.21 | 0.07 | 0.09 |
| 20:2(n-6) | nd | nd | nd |
| 20:3(n-6) | 2.36 | 0.76 | 1.24 |
| 20:4(n-6) | 2.58 | 1.22 | 3.08 |
| 22:4(n-6) | 0.70 | 0.19 | 0.80 |
| 22:5(n-6) | 0.57 | 0.71 | 0.18 |
| Total (n-6) PUFA | 12.43 | 4.05 | 10.43 |
| 18:3(n-3) | 0.23 | 0.06 | 0.37 |
| 18:4(n-3) | 0.05 | 0.02 | 0.07 |
| 20:4(n-3) | 0.31 | 0.03 | 0.53 |
| 20:5(n-3) | 1.54 | 0.40 | 3.32 |
| 22:5(n-3) | 1.07 | 0.19 | 1.07 |
| 22:6(n-3) | 22.11 | 4.91 | 21.73 |
| Total (n-3) PUFA | 25.31 | 5.61 | 27.09 |
| Total PUFA | 39.63 | 14.15 | 39.17 |
| 20:3(n-9)/ 22:6(n-3)[C] | 0.07 | 0.92 | 0.07 |

[A] area %; mean values from pooled samples for 4 fish.
[B] nd = not detected.
[C] this ratio above 0.40 indicates EFA deficiency in rainbow trout[20].

examined. Nevertheless, it has been demonstrated that the dietary fatty acids clearly are reflected in the phospholipid of fish leucocytes [17,18]. Therefore, the thrombocytes isolated in our study may have had altered their phospholipid fatty acid composition in response to the dietary provisions.

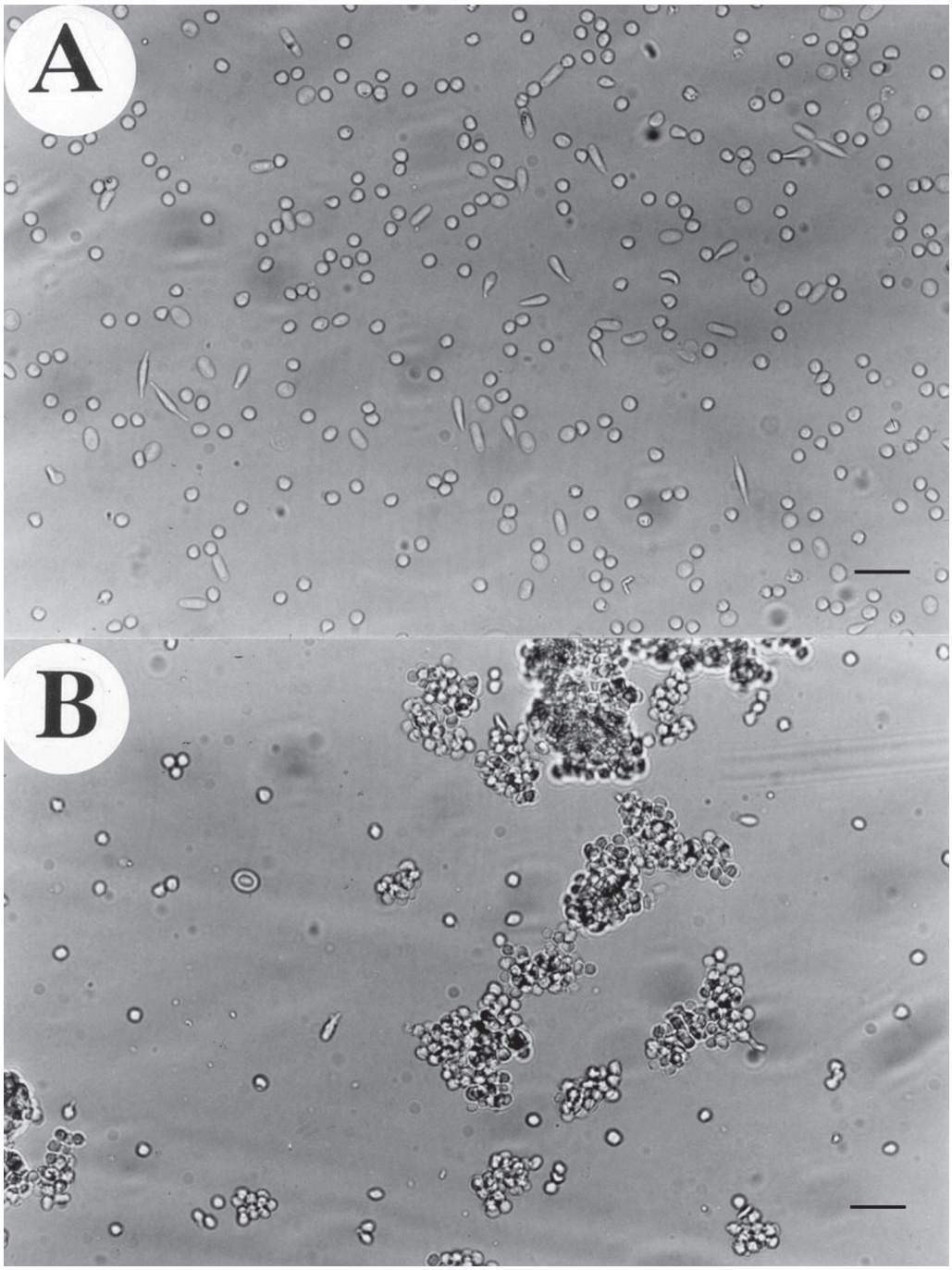


Figure 1 - Effect of 0.15M Tris-buffer saline (A) and collagen Type I (B) on rainbow trout thrombocytes isolated by magnetic cell separation using the MAb TTL-7D11. Bar, 45mm

In the four trials performed, the thrombocytes from the fish fed the commercial diet containing adequate amount of essential fatty acids exhibited greater capacity for aggregation when induced by collagen (Figure 1). The mean values obtained were 55.7±3.0% for the normal fish as against 37.0±4.0% for the deficient fish. The aggregation percentage values presented by thrombocytes from EFA-deficiency recovered fish was very similar to the normal fish (55.8±9.8%). The differences were statistically significant ($p< 0.025$) when tested using unpaired *t*-test.

Platelets play a central role in haemostasis of homeotherms by virtue of their adhesive and aggregatory behaviour. A change in proportions of fatty acids types in cell membranes may alter the fluidity of the membranes and affect its functions including the activities of membrane-bound enzymes, receptor expression, etc. [19,20,21] thereby influencing platelet activation. The agents that control the functional ability of platelets include platelet-activating factor and eicosanoids, but it has been indicated that the former did not have a significant pro-aggregatory activity for trout thrombocytes[7]. Rainbow trout thrombocytes have been found to produce eicosanoids derived from arachidonic acid like 12-hydroxyeicosate-traenoic acid (12-HETE), as well as the corresponding product from eicosapenta-enoic acid[22]. In vitro studies with exogenous Lipoxin (Lx) $A_4$ and Leukotriene (LT) $B_4$ demonstrated a certain degree of aggrega-tion of trout thrombocytes [7]. All these point to the pertinent role of fatty acids in thrombocyte functioning.

The aggregatory activity noted for the normal fish in this study must be the natural haemostasis operating in the animal. In contrast, the thrombocytes from fish deficient in dietary essential fatty acids were weaker in their aggregatory response. However, this response seems to recover after the deficient fish had been re-fed the commercial diet. The deprivation-induced changes at the cellular level may have impaired the function of thrombocytes from these fish, the reasons for which have not been elucidated by this study. It would be interesting to observe the shifts, in the aggregatory ability and to relate it to the phospholipid fatty acids composition of the cells on the basis of dietary sources.

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## Influência da privação dos ácidos graxos essenciais na agregação de trombócitos em trutas arco-íris

## Resumo

Indicações sobre alterações na formação das células sanguíneas e em suas funções nos peixes têm sido relatadas em diversos trabalhos. Os ácidos graxos essenciais (EFA) certamente estão ligados a essas células devido ao fato de constituírem fonte de componentes importantes, como os eicosanoides, fatores de ativação de plaquetas nos mamíferos, bem como de fosfolipídios de membrana. Trombócitos oriundos de peixes alimentados com uma dieta comercial contendo quantidades adequadas de EFA mostraram uma grande capacidade de agregação quando induzidos por colágeno do tipo I (56%), contudo, essa capacidade encontrou-se reduzida quando os peixes foram alimentados com uma dieta deficiente em EFA (37%). Os resultados obtidos nesse estudo demonstraram que os ácidos graxos essenciais exercem influência nos trombócitos afetando sua capacidade de agregação.

**Palavras-chave**:
Trombócito.
Ácidos graxos essenciais.
Truta.
Agregação.
Anticorpo monoclonal.

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