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A comparative study: *In vitro* effects of EPA and DHA on immune functions of head-kidney macrophages isolated from large yellow croaker (*Larmichthys crocea*)

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ABSTRACT

Comparative effects of different concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on immune responses of head-kidney macrophages isolated from large yellow croaker were studied *in vitro*. After exposing to serum-free medium for 1 day, cultured cells were incubated in medium supplemented with graded levels of EPA or DHA (0, 5, 25, 100, 200 and 1000 μM, respectively) in the form of fatty acid bovine serum albumin (FA-BSA) complex for 12 h, 24 h and 36 h, respectively. Control samples were incubated in the absence of EPA or DHA (2% bovine serum albumin, BSA). Following stimulation, cell viability, lipid peroxidation, secretary phopholipase A_2 ($sPLA_2$) and prostaglandin E_2 (PGE_2) production as well as some immune parameters including phagocytosis, respiratory burst activity and interleukin 1β (IL-1β) production were determined. Results showed that EPA and DHA affected cell viability in dose-dependent and time-dependent manners. In particular, cell viability was significantly decreased after 24 h and 36 h incubation with 1000 μM EPA or DHA ($P < 0.05$). Higher levels of EPA (200 and 1000 μM) caused a significant increase in the production of malondialdehyde (MDA) ($P < 0.05$), while DHA did not significantly affect the MDA production. EPA significantly increased the intracellular superoxide anion synthesis which, on the contrary, was significantly reduced by DHA. Phagocytosis percentage (PP) values were significantly higher in treatments with 5 μM DHA ($P < 0.05$), but significantly decreased by 200 and 1000 μM EPA and DHA compared to the control group ($P < 0.05$). Decreased PGE_2 production was produced by cells treated with relatively low doses of EPA or DHA. When high levels of stimulants (1000 μM EPA or DHA) were used, PGE_2 levels were elevated and reached a significant level ($P < 0.05$). Both EPA and DHA significantly inhibited the production of $sPLA_2$, where DHA exerted the more potent inhibitory effects than EPA. No pronounced effect was observed on IL-1β production among all the treatments, and IL-1β level in cell culture supernatant was fairly low (only approximately 6 pg/ml). Those findings suggested that EPA and DHA could influence the immunity and physiological conditions of macrophages from head kidney of large yellow croaker *in vitro*.

1. Introduction

Currently, the increasing demand for fish oil together with the stagnant worldwide supplies of marine oils and meals have created the necessity to use alternative oils in marine fish diets. Those alternative oils are usually rich in C18 polyunsaturated fatty acids (PUFAs) but lacking n-3 long chain highly unsaturated fatty acids (HUFAs). The inclusion of oil lacking n-3 HUFAs, in particular EPA and DHA, in fish diet has influenced fish health and resistance to stress and diseases [1,2]. In addition, precautious consideration of drug use in disease treatment and urgent needs for healthy aquatic products have brought about the requirement to reduce basic risk of disease outbreak in aquaculture practice through nutritional manipulation and fishery management [3,4]. Therefore, a considerable amount of research effort took place to identify the important roles of n-3 HUFAs in affecting growth and in regulating immune functions of marine fish. Several studies have shown that dietary EPA and DHA can significantly affect fish specific and nonspecific immunity [5–7], fish capacity to fight parasite infection [8] as well as the expression of immune-related genes [9–11]. Nonetheless, due to some limitations including competitive interactions of HUFAs in the biochemical and physiological processes *in vivo*, potential interferences with rearing

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conditions, temperature, stress *etc.* [12], these fatty acids are generally taken into account together when it comes to investigate the requirements and immune effects of HUFAs [13]. Moreover, fish immune responses tend to show a high degree of variation, and existing results were often in conflict [14]. For those reasons, dietary trials to explore immune regulatory mechanisms of individual fatty acid were scarce in fish.

In vitro experiments have been reported to reflect the *in vivo* situation of experimental animals and results obtained *in vitro* has shown similarities to those obtained *in vivo* according to a large number of published references [15–17]. Thus considerable *in vitro* studies have been designed to identify the mechanisms of how fish immune responses were regulated by immuno-stimulants including LPS, glucan, vitamin, *etc.* [18–21]. However, so far, no *in vitro* study exploring the relationship between EPA/DHA and fish immunity has been reported. To the contrary, a majority of studies have been conducted on human, mammals and poultry, and several mechanisms have been hypothesized to elucidate the immunological roles played by EPA and DHA, which was related: alterations in the physical properties of the membrane, regulation of immunity-related gene expression through effects on receptor activity, intracellular signaling processes or on transcription factor activation, modulation in the synthesis of bioactive lipid mediators [22,23].

The proposed immune responses and related mechanisms mentioned above provide the premise to explore the mechanism of how EPA or DHA modulate fish immunity. In a previous *in vitro* study with large yellow croaker, an aquaculture species with great commercial value, ARA were found to influence cell viability, lipid peroxidation and some immune responses of isolated head-kidney macrophages, which to some extent provided a clearer understanding of the immune regulatory effects of ARA [24]. In the present study, using the same "*in vitro*" model, effects of EPA and DHA on immune responses and physiological conditions of head-kidney macrophage derived from large yellow croaker were investigated and compared.

2. Material and methods

2.1. Experimental fish

Large adult yellow croaker weighing between 1000 and 1600 g were obtained from a commercial fish farm in Ningbo, China. The fish were held in floating sea cages with water temperature ranging from 24 to 28 °C under natural photoperiods. The fish were fed low-value fish twice a day. Only healthy fish, as indicated by their activity and exterior appearance, were picked out for experiments. Each time one fish were sampled and assays were conducted in duplicate.

2.2. Culture media and fatty acid preparation

Cell suspension, adherence and priming steps were performed with L-15 medium (Invitrogen, USA) consisting of 0.1 g l^{-1} $NaHCO_3$, 2.38 g l^{-1} HEPES (Amresco, USA), 0.33 g l^{-1} glucose (Sigma, USA), 50 mg/l heparin (Sigma–Aldrich, USA) and 0.1% fetal bovine serum (FBS, Gibco, USA). Medium pH was adjusted to 7.4. All the solutions contained 200 IU ml^{-1} penicillin/streptomycin (P/S, Amresco, USA) and 0.05% butylated hydroxytoluene (BHT) as antioxidant.

EPA (99% purity, fish oil source, Matreya, Pennsylvania, USA) or DHA (99% purity, algae source, Matreya, Pennsylvania, USA) were supplemented to cell cultures in the form of bovine serum albumin (BSA, fatty acid free, Wako, Osaka, Japan) complex according to the methods described previously [25,26]. Fatty acids were added to 2% BSA solution prepared beforehand by dissolving BSA in cell assay medium, sonicated for 5 min under a steady stream of nitrogen at room temperature to protect from oxidative damage. The solutions were sterilized by filtration through 0.22 μm filters, then diluted with 2% filter-sterilized BSA solution to give a 1 mM FA-BSA complex. The FA-BSA complex was kept in brown vials, gassed with N_2 before sealing and stored at −20 °C. Prior to use, assay FA-BSA complex was diluted to 5, 25, 100, 200, 1000 μM with 2% BSA solution supplement with 0.01% BHT and 200 IU ml^{-1} P/S.

2.3. Isolation and primary culture of head kidney macrophages

Handling procedures with experimental fish were performed according to Guidelines for the Use of Fishes in Research [27]. Fish was immersed in near-freezing water for half an hour to mitigate pain or stress before being euthanized by a lethal blow to head without anesthesia, and then head kidney was removed aseptically. Macrophages were isolated and maintained according to modified procedure described previously [28,29]. In brief, tissue was dissected and pushed carefully through a 100 μm nylon mesh. The resultant cell suspension was loaded onto 31%/45% Percoll (Pharmacia, USA) density gradients prepared beforehand followed by centrifugation (400× *g* at 4 °C for 30 min). The macrophage-rich cellular band lying at the gradient interface was collected with care, and Percoll was removed from cell pellet by repeated centrifugation in L-15 medium. Subsequently, macrophage was suspended in cell culture medium, and cell viability was determined quickly by the trypan blue (Sigma, USA) exclusion test. Cell number was adjusted to 5.0×10^6 cells ml^{-1} and seeded in 96-well cell culture plates (Nunc, Denmark) or 48-well cell culture plates (Nunc, Denmark) at aliquots of 200 μl $well^{-1}$ or 400 μl $well^{-1}$, respectively. After overnight incubation in serum-free medium at 26 °C to allow adherence, old medium was aspirated out and non-adherent cells were removed by two washes with medium. Cell population profiles of the remaining cell monolayers were analyzed by Wright's-Giemsa staining (NJJCBIO assay kits, China). Only cells with macrophage purity exceeding 95% were used in the experiments.

Physiological assays.

2.4. Cell viability

Cell viability was assessed using quantified spectrophotometrical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich, USA) assay [30]. EPA and DHA of different concentrations (5 μM–1000 μM) were incubated with cells. Control cells were given 2% BSA. After incubation for 6, 24 and 36 h, cell media was aspirated out and collected for further use, aliquots of 100 μl MTT working solutions (5 mg/ml) were added to the wells and incubated for 4 h followed by fully dissolving formazan crystals in DMSO. Absorbance was measured in 96 microplate readers at 570 nm. The decrease of absorbance was considered as a loss of cell viability.

2.5. Lipid peroxidation assays

To determine whether lipid peroxidation was associated with cytotoxicity induced by HUFAs, malondialdehyde (MDA) as one of the metabolites derived from lipid peroxidation was measured. Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay for MDA in line with the manufactory's protocol (NJJCBIO assay kits, China). A number of metabolites derived from lipid peroxidation, including MDA, react with thiobarbituric acid to produce a pink-colored material that can be readily monitored by spectrophotometry to give an overall indication of the level of lipid peroxidation. Briefly, at the end of 36 h incubation with fatty acids, cell media was collected and centrifuged to remove cell debris. Supernatant was incubated with 20% trichloroacetic acid and 0.67% thibarbituric acid

at 95 °C for 40 min. Absorbance were read at 532 nm with distilled H_2O as blanks, results were converted to nmol/ml of MDA using a standard sample of 10 nmol/ml Malonaldehyde diethyl acetal. Cells treated without fatty acids were taken as control.

2.6. Immunological assays

2.6.1. Respiratory burst activity

Respiratory burst activity was determined by the reduction of nitroblue tetrazolium (NBT, Amresco, USA) to formazan as described previously with little modification [31,32]. After 24 h-incubation with fatty acids, medium was collected and centrifuged at 3500 rpm for 20 min, cell-free supernatant were transferred to −80 °C for Elisa analysis. Cells were incubated with 1 mg/ml NBT solution for 30 min with 1 μg/ml phorbol myristate acetate (PMA, Applichem, German) as a trigger for superoxide anion production. Cells were then washed twice by pre-warmed PBS and fixed in absolute methanol for 10 min, washed once with 70% methanol to remove extracelluar formazan, air dried and then intracellular fomazan was dissolved in 120 μl 2 M KOH and 140 μl DMSO (Amresco, USA). Absorbance was measured by a microplate reader at 630 nm using KOH/DMSO as a blank. The mean number of cells, obtained from at least three wells was determined by counting nuclei under microscope after removal of medium and addition of 100 μl of lysis buffer containing 0.1 M citric acid (Sigma, USA), 1% Tween 20 (Sigma, USA) and 0.05% crystal violet (Sigma, USA). Results were adjusted to absorbance per 10^5 cells and expressed as means of at least four replicates ± S.E.M.

2.6.2. Phagocytosis

Phagocytic activity was determined by a modified method of Pulsford et al. [33]. In the present study, opsonized yeasts were used as engulfed material. For opsonization, yeasts were sterilized in boiling water. After several washes with germ-free PBS, yeasts were incubated overnight with FBS at room temperature [15]. After 36 h incubation EPA or DHA, cells were mixed with opsonized yeasts to obtain an approximate 10:1 ratio of yeasts to cells. Phagocytosis proceeded at 26 °C for 1 h, then stopped by washing with ice-cold PBS. Phagocytosis was evaluated by observation under light inverted microscopy [34]. At least one hundred cells were counted and phagocytosis was expressed as Phagocytosis Percentage (PP) which was calculated:

Phagocytosis Percentage (PP) = (Number of cells ingesting yeast/Number of adherent cells observed) × (Number of yeast ingested/Number of adherent cells observed) [35].

2.7. ELISA analysis

Production of secretary phopholipase A_2 ($sPLA_2$), prostaglandin E_2 (PGE_2) and interleukin 1β (IL-1β) were assayed in the head kidney macrophages culture medium according to manufacture's protocols (Invitrogen, Maryland, USA). Quantitative determination for PGE_2 is based on competitive binding ELISA reaction while $sPLA_2$ and IL-1β assays on solid sandwich ELISA reaction. The intensity of color is inversely proportional to the concentration of PGE_2 and directly proportional to that of $sPLA_2$ or IL-1β present in the sample.

2.8. Statistical analysis

The normality of data and homogeneity of variance were analyzed by Kolmogorov–Smirnov and Levene test, respectively. All data were subject to one-way ANOVA analysis in SPSS 13.0 for Windows. When equal variances were assumed, differences between the means were tested by Tukey's multiple range test, whereas when equal variances were not assumed, then Tamhanes's T2 test was applied. Differences were considered statistically significant at probability (P) values <0.05. Results were shown as means ± S.E.M. (standard errors of the mean).

3. Results

3.1. Cell viability

Cell viability decreased significantly in dose-dependent and time-dependent manners. No significant difference on cell viability was observed with all treatments after 12 h incubation. After 24 h incubation, cell treated with 1000 μM EPA or DHA presented a significant decrease in viability compared to control cells ($P < 0.05$). After 36 h incubation, cell viability was much lower than that of cells after 12 h and 24 h at each level, and cell viability decreased with increasing fatty acid levels. In particular, viability of cells treated by 1000 μM EPA or DHA was significantly lower than control cells ($P < 0.05$). DHA appeared to have stronger capacity to induce cell viability loss compared to EPA (Fig. 1).

3.2. Lipid peroxidation

Distinct effects of EPA and DHA on lipid peroxide production by cells were observed after 36 h incubation. MDA production was not

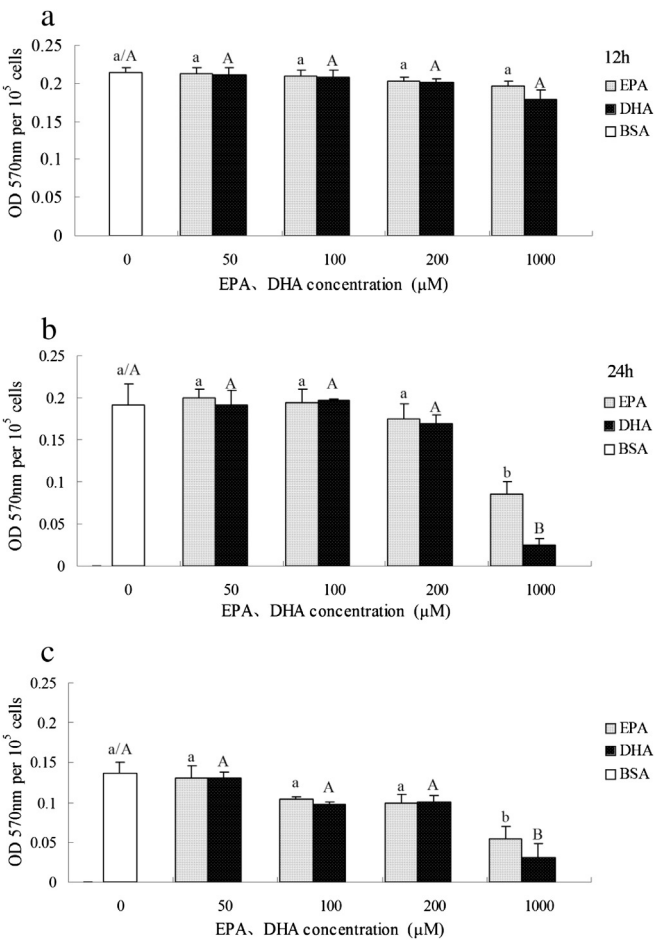

Fig. 1. Viability of large yellow croaker (LYC) head kidney macrophage after incubation with EPA and DHA for 12 h (a), 24 h (b), 36 h (c). Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

significantly influenced by incubation with DHA, even when used over 200 μM. While higher levels of EPA (200 and 1000 μM) significantly enhanced the MDA production of macrophages compared to the control group ($P < 0.05$) (Fig. 2).

3.3. Respiratory burst activity

Respiratory burst activity affected by EPA and DHA was apparently different even at the lowest level (5 μM). Stimulation of macrophages with PMA after 24 h incubation with EPA resulted in significant increase of superoxide anion production. In contrast, DHA exhibited inhibitory effects on the production of superoxide anion (Fig. 3).

3.4. Phagocytosis

Macrophage phagocytosis was enhanced according to fatty acid dose up to 25 μM, this enhancing response was abolished when EPA or DHA was supplied at relatively higher levels (Fig. 4). Higher levels of EPA or DHA (200 and 1000 μM) caused significant decrease in macrophage phagocytic activity compared to control, and the inhibitory effect of DHA was more potent than that of EPA (Fig. 4).

3.5. PGE_2 production

PGE_2 production by macrophages in culture supernatant was measured and compared. When used at 5 and 25 μM, EPA can reduce the PGE_2 production to a significant extent, while high doses of EPA showed significantly elevating effects. Similar effect was observed with DHA, but no statistically significant effects were detected. Interestingly, significant enhancement of PGE_2 level was observed in cells treated with 1000 μM EPA or DHA, wherein EPA resulted in 800.47 ± 18.08 pg/ml PGE_2 which was much higher than 223.41 ± 12.34 pg/ml PGE_2 induced by DHA (Fig. 5).

3.6. $sPLA_2$ analysis

As expected, the production of $sPLA_2$ was significantly lower in treatments of both EPA and DHA than control cells. The inhibitory effects induced by DHA on the $sPLA_2$ production of cells were more pronounced as compared to EPA (Fig. 6).

3.7. IL-1β analysis

In this set of experiment, no distinct effects were observed in the release of IL-1β by macrophages in large yellow croaker. Moreover, the IL-1β concentration determined in the culture supernatant was fairly low, reaching merely an approximate amount of 6 pg/ml (Fig. 7).

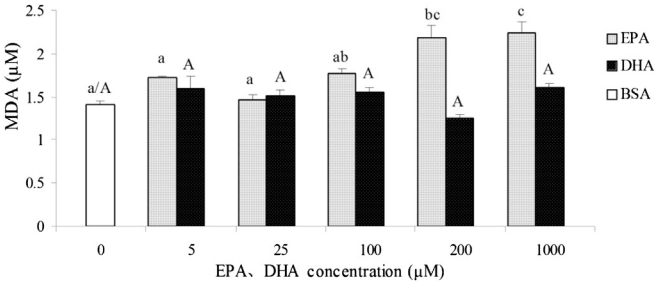

Fig. 2. MDA production of LYC head kidney macrophage after incubation with EPA and DHA for 36 h. Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

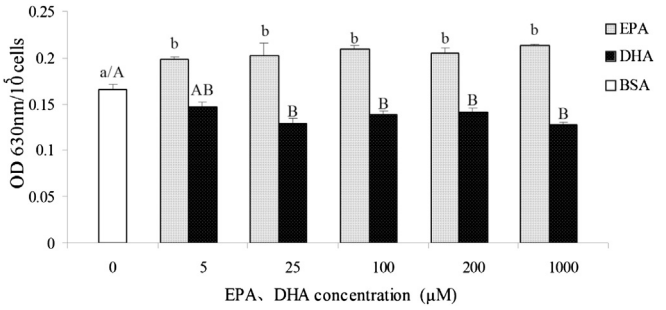

Fig. 3. Respiratory burst activity of LYC head kidney macrophage after incubation with EPA and DHA for 24 h. Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

4. Discussion

With incubation time extending and levels of fatty acids increasing (0–1000 μM), cell viability decreased markedly. In addition, DHA possessing 22 carbon atoms and 6 double bonds appeared to cause more significant cell viability loss than EPA with 20 carbon atoms and 5 double bonds. Concomitant evidence obtained with trypan blue exclusion test also indicated higher degree of cell membrane integrity loss induced by DHA (cell survival rate, 43.2% for 24 h treatment with 1000 μM DHA) compared to EPA (cell survival rate, 61.9% for 24 h treatment with 1000 μM EPA). The present finding agreed well with studies on human cell [36] which showed that EPA presented a lower cytotoxicity at the same concentrations and incubation time compared to DHA. It has been well established that exogenous fatty acid could be incorporate into cell membrane [17,23], and that the organization level of biological membrane is dependent on acyl chain length and unsaturation degree as well as fatty acid level [37]. As suggested by Eldho et al. [38], DHA has been shown to have a stronger membrane disordering effect than EPA using NMR and X-ray diffraction techniques. However, according to the study on a macrophage cell line J774 [36], wherein the cytotoxicity was assessed by loss of membrane integrity and DNA fragmentation, toxicity of fatty acids on J774 cells was not related to carbon-chain length and number of double bonds, but to the ability of fatty acids to incorporate into triacylglycerol (TAG). Cnop et al. [39] also noted that toxicity of fatty acids was attributed to the inability of the cells to incorporate them into neutral lipid droplets. In this study, we observed that DHA was more effective than EPA in the capacity to reduce cell size, enhance granularity and induce cytoplasmic lipid droplets which could be clearly seen through light microscopic analysis (Fig. 8). Probably,

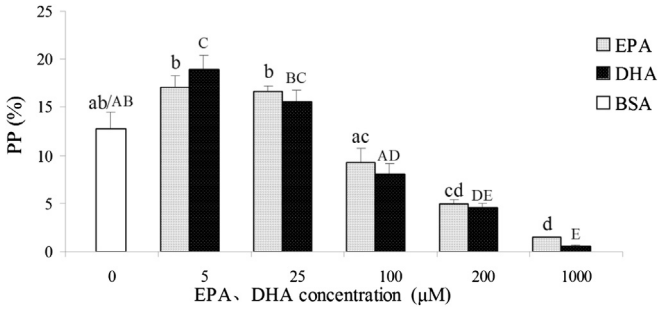

Fig. 4. Phagocytic percentage (PP) of LYC head kidney macrophage after incubation with EPA and DHA for 36 h. Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

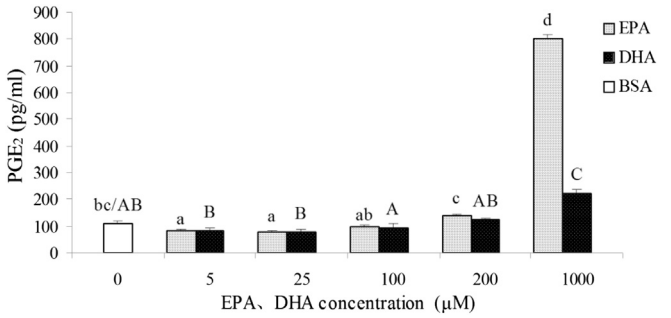

Fig. 5. PGE_2 production of LYC head kidney macrophage after incubation with EPA and DHA for 24 h. Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

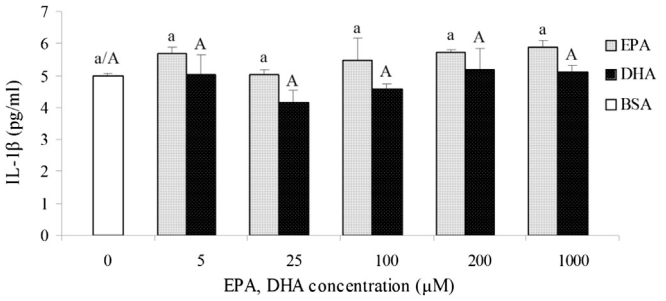

Fig. 7. IL-1β production of LYC head kidney macrophage after incubation with EPA and DHA for 24 h. Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

compared to EPA, the weaker ability of DHA to be incorporated into TAG contributed to the more pronounced toxic effects observed in this work [36].

In this study, distinct differences on respiratory burst activity of fish macrophage exhibited by EPA and DHA could be clearly detected. EPA up-regulated the production of superoxide anion whilst DHA showed inhibitory effect, which was partly in agreement with previous finding in goat neutrophils [40], although no statistically significant effects was produced by EPA. Studies on human retinal cells [41] and murine macrophages [42] also showed that DHA could decrease the oxidative stress and the over-production of ROS. However, the physiological effects of EPA and DHA as modulators of ROS production were contradictory in light of the existing publications. Some authors reported that both EPA and DHA produced an increased respiratory burst activity (measured by chemiluminescence) on cord blood neutrophils [43]. To the contrary, Chen et al. [44] found that EPA and DHA significantly suppressed the PMA-stimulated superoxide generation by human neutrophils. The difference in literature reports may perhaps be attributed to the dual roles that EPA and DHA exert on ROS production through mitochondrial respiratory chain and NADPH oxidase [45]. To date, little knowledge is readily available to address the impacts of HUFA on the ROS production of *in vitro* fish model. Therefore many points are still very elusive and remain to be fully established.

In general, when fatty acid especially HUFAs are incorporated into the membrane lipid raft, cells are prone to undergo the process of lipid peroxidation [46]. This is particularly damaging to cell membranes and has hazardous effects on cell viability [47]. In this work, using TBA assaying system [46], significantly higher degree of lipid peroxidation was observed on EPA-treated cells, while no

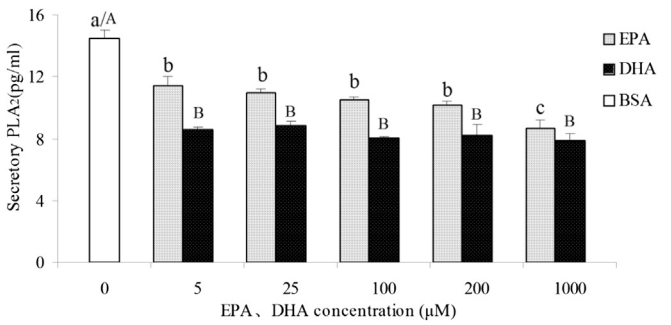

Fig. 6. $sPLA_2$ production of LYC head kidney macrophage after incubation with EPA and DHA for 24 h. Data were expressed as mean ± S.E.M. ($n = 2$ fish per treatment). Groups labeled with same letters are not significantly different as compared to control treatment, $P > 0.05$.

significant effect on lipid peroxidation was detected with DHA. Results of the present study matched to some extent those by Falconer et al. [48], Pupe et al. [49] and Muralidhar et al. [50] who also confirmed the enhancing effects of EPA on lipid peroxide production and by Begin et al. [51] who found in human breast cancer cells that DHA was ineffective in raising lipid peroxide. Since lipid peroxidation occurs when ROS attack PUFA at their double bonds, setting off a chain reaction of hydrogen abstraction and lipid radical formation [52], the significant differences exerted by EPA and DHA were probably due to their corresponding effects on ROS production and the amount of HUFAs accessible to cells.

Macrophage phagocytic activity appears at the early stage of fish innate immune response capacity and has been used as an indicative immunologic parameter to evaluate immune function [53,54]. *In vitro* studies have clearly demonstrated that modifications in fatty acid composition of phagocyte membrane are associated with altered phagocytic capacity [55,56]. This may be related in part to altered expression of receptors involved in phagocytosis, but also to the physical nature of membrane [57]. In this study, low doses (5 and 25 μM) of EPA and DHA were found to enhance phagocytic activities, which was reinforced by the observation in goat neutrophils where cultured cells with EPA and DHA up-regulated the phagocytosis [40]. When fatty acids levels went higher, the enhancing effects on fish cells were abolished but that stimulatory effects on goat cells were still retained. The reasons are not easily discerned, may be due to the difference in the types of engulfing material and incubation duration employed. *In vivo* study also indicated that dietary intake of EPA and DHA could enhance the phagocytic activities of human immune cells [57]. However, it is necessary to note that high levels of EPA and DHA exhibited markedly inhibitory effects, which, as indicated by the present result, could be due to the fact that EPA and DHA induce macrophage death at high concentrations. Therefore, it is important to emphasize that both of them increased the phagocytic activities of macrophages at non-toxic concentrations [36].

PGE_2 is produced from phospholipids by a cascade of enzymatic reactions involving cyclooxygenase (COX), phospholipase A_2 (PLA_2), and prostaglandin E synthase (PGES) [58]. In the present study certain levels (5, 25 and 100 μM) of EPA and DHA decreased the production of PGE_2, although to an insignificant extent. This result was comparable to published surveys both *in vitro* [59,60] and *in vivo* [61,62]. It has been well documented that increased amount of EPA and DHA incorporated into cell membrane act as competitive substrate to ARA for COX and lipoxygenase (LOX) enzymes, giving rise to less biologically active eicosanoid mediators like PGE_3 or resolvins [13,63]. Another potential mechanism for the reduced PGE_2 production in 5, 25 and 100 μM fatty acids treatments might be partly related with the inhibitory effects of EPA and DHA on the

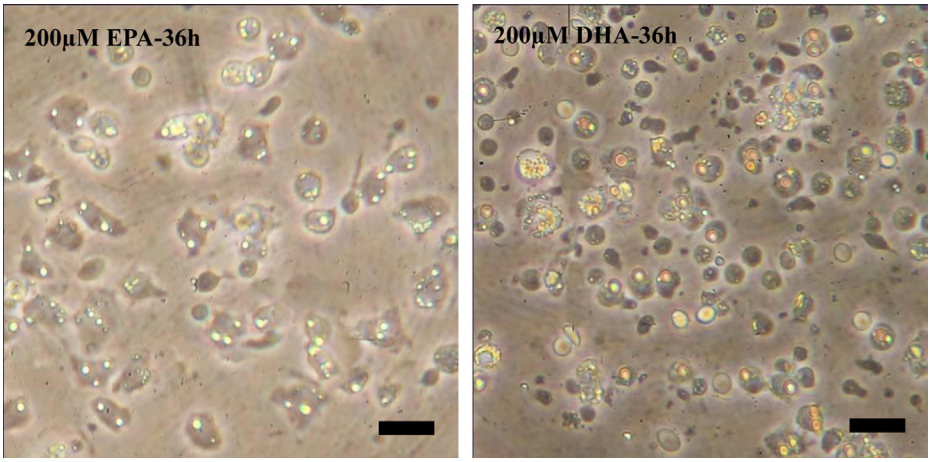

Fig. 8. Light microscopic analysis of large yellow croaker head-kidney macrophages after 36 h incubation with EPA and DHA. Cytoplasmic lipid droplets induced by fatty acid could be clearly seen. Bar = 10 μm.

activity of $sPLA_2$ [64]. Interestingly, the manner of PGE_2 production did not correlate well with the production of $sPLA_2$, since DHA seemed to be more potent than EPA in inhibiting the $sPLA_2$ production, however, their respective PGE_2 productions did not differ at each level. It has been reported that PGE_2 production is dependent on the enzymatic activities of two principle phospholipase A_2s, cytoplasmic phospholipase A_2 ($cPLA_2$) and $sPLA_2$, which can catalyze the hydrolysis of phospholipids at the sn-2 position, a reaction that produce the precursor, arachidonic acid. The metabolic pathway of PGE_2 formation is variable. In some cases, the process of PGE_2 production is mediated by $cPLA_2$, in other cases, PGE_2 was produced through $sPLA_2$-mediated way [65,66]. Probably, in our case, $sPLA_2$-mediated pathway is not the dominant metabolic pathway of PGE_2 formation. Similarly, our earlier work with ARA also suggested, it was likely that $sPLA_2$ was probably not the only rate-limiting enzyme in the process of ARA liberation and production of PGE_2 [24]. As for the sudden increase of PGE_2 production determined in medium supplemented with 1000 μM EPA or DHA, one possible explanation was that because high levels of EPA or DHA induced the formation of cytoplasmic lipid bodies where an alternative pathway for PGE_2 production occurred. Through a series of receptor-driven signaling pathways rather than the general COX and LOX enzymes pathways, lipid bodies and $cPLA_{2\alpha}$ facilitate ARA mobilization and synthesis of PGE_2 [67]. Further research is needed to reveal whether or not this is the case.

Fish macrophage has been reported to be able to produce IL-1β which plays essential role in the immunity-regulating process and inflammatory response in fish [68]. Therefore, the activity of IL-1β could be the indication of inflammatory events in fish. Herein we choose IL-1β as the indicator to represent the cytokine production, because IL-1β shows a high degree of homology (75%–78%) between different species through sequence comparison analysis, which is the precondition for the obtainment of reliable Elisa analysis data on the basis of commercial Elisa assay kit specifically developed for human use [69]. It is well recognized that n-3 HUFAs for instance, EPA and DHA were consigned to drive immune responses into anti-inflammatory direction [70]. Considerable studies have clearly described that EPA and DHA down-regulated the expression levels of genes linked with inflammation in mammal and human macrophages, which resulted in a consequently reduced IL-1β production, and this inhibitory effect of DHA was reported to be more potent as compared to EPA [30,71,72]. However, little relative information was available on fish. To the best knowledge of the author, it is the first time the effect of EPA and DHA on inflammatory cytokine production of fish macrophages is investigated. In the present study, although IL-1β production by DHA-treated macrophages was lower than that produced by EPA-treated cells, no significant difference on the production of IL-1β among treatments was observed. According to earlier studies, the anti-inflammatory effects exhibited by EPA and DHA were dependent on the activation of macrophages which could be induced by an appropriate type and dose of stimulants [71,73]. Therefore, the unclear anti-inflammatory effects of EPA or DHA were probably due to a lack of stimuli priming process, which was also evidenced by the overall low levels of IL-1β in control group.

In conclusion, primary fish macrophage as a model system was employed to identify the effects of EPA and DHA on immune responses *in vitro*. The present study shows that EPA and DHA presented pronounced effects on fish macrophage cell viability, lipid peroxidation, phagocytosis, respiratory burst as well as production of eicosanoid and cytokines, which in turn modulate the immune responses of fish macrophage comprehensively. In addition, although the work was restricted to *in vitro* investigations, those data would be useful to explain the controversial findings obtained from *in vivo* studies. Furthermore, to elucidate the clear mechanisms of actions of EPA and DHA in modulating immune responses in fish, it is of great necessity to examine the data and proposed mechanisms obtained from *in vitro* effects through dietary studies.

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