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In vitro effects of arachidonic acid on immune functions of head kidney macrophages isolated from large yellow croaker *(Larmichthys crocea)*

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ABSTRACT

The experiments were conducted to determine the effects of arachidonic acid (ARA) on macrophages isolated from large yellow croaker head kidney. After exposing to serum-free medium for 1 day, cultured cells were incubated in medium supplemented with graded levels of ARA (0, 5, 25, 100, 200 and 1000 μM) in the form of fatty acid bovine serum albumin (FA-BSA) complex for 12 h, 24 h and 36 h, respectively. Cells treated with 0 μM ARA (2% bovine serum albumin, BSA) were included as control at each incubation time. Following stimulation, cell viability, lipid peroxidation, secretory phopholipase A_2 ($sPLA_2$) production and some immune parameters including phagocytosis, respiratory burst activity, prostaglandin E_2 (PGE_2) and Interleukin 1β (IL-1β) production were examined. Results showed that cell viability decreased with prolonged exposure and increasing ARA concentration. Especially after 36 h, cell viability was significantly decreased by incubation with higher levels of ARA (200 and 1000 μM) ($P<0.01$). In the meanwhile, higher levels of ARA (200 and 1000 μM) caused a significant increase in the production of malondialdehyde (MDA) ($P<0.05$, $P<0.01$). Phagocytosis percentage (PP) values were significantly higher in treatments with 25 and 100 μM ARA ($P<0.05$), but significantly lower in treatments with 1000 μM ARA compared to the control group ($P<0.01$). Enhanced intracellular superoxide anion (O_2^-) synthesis was found in cells treated with each level of ARA with an exception of 1000 μM ARA ($P>0.05$). The significantly promoted production of $sPLA_2$ seemed to be irrelevant to increased ARA concentration, while PGE_2 production was significantly increased in an ARA dose dependent way ($P<0.01$). No pronounced effect on IL-1β production was observed between the control and ARA group, and levels of IL-1β in cell culture supernatant were fairly low (only approximately 6 pg/ml). These findings suggested that ARA could influence the immunity and physiological conditions of macrophages from head kidney of large yellow croaker *in vitro*.

1. Introduction

Arachidonic acid (ARA) is an essential fatty acid (EFA) for marine fish since they lack the ability to biosynthesize ARA but still require an optimal amount of ARA to maintain good growth and normal physiological activities. Over the recent years, considerable work has been extensively conducted due to the fact that ARA plays an important role in modulating fish immunity. One possible mechanism is that ARA fulfills a variety of roles within immune cells like macrophages (Ellis, 1999; Enane et al., 1993). Fish macrophages are the prime component in innate immune systems by performing immune-related functions such as phagocytosis, production of reactive oxygen, nitric oxide and secretion of immune modulating bioactive materials (Russo et al., 2009; Secombes and Fletcher, 1992). ARA can affect physical stability of cell membrane, signaling pathways and membrane-associated enzymes activities by modifying fatty acid profiles of cell membrane, which subsequently alter immune functions of macrophages (Calder, 2008; Waagb, 2006). In addition, ARA acts as the most important precursor for eicosanoids production, through the action of cyclooxygenase and lipoxygenase, giving rise to metabolites including prostaglandins (PGs), leukotrienes (LTs) and tromboxanes (TXs) (Ganga et al., 2005; Rowley et al., 1995). Furthermore, via modulation of eicosanoids synthesis, ARA probably interferes with cytokine release (Baker, 1990). The eicosanoids and cytokines possessing different immunity-modulating potencies might also expect to influence the immunity of fish macrophages.

Considerable research concerning relationships between ARA and fish immunity have been carried out *in vivo*, which have shown that dietary ARA was closely associated with fish health and resistance to stress and diseases (Bell and Sargent, 2003; Ganga et al., 2005; Montero et al., 2003; Xu et al., 2010). However, due to the existence of high degree of variation in immune response of fish (Haugland et al., 2005; Purcell et al., 2004) and biochemical competition between ARA and other types of EFA *in vivo* (Sargent et al., 1999; Tocher, 2010), the mechanism by which ARA affects fish immunity is still not fully understood.

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In vivo modes could be simplified by *in vitro* experiments which could be free from interferences such as complex cell interactions, stresses from environments and feeding conditions. Therefore relevant studies could be undertaken under highly controlled conditions (Castillo et al., 2009; Ryckaert et al., 2010; Seierstad et al., 2009). To date, there have been a large number of reports on the application of *in vitro* cell model to investigate the modulation of fish immunity by immunostimulants such as LPS, glucan, vitamin, etc. (Calduch-Giner et al., 1997; MacKenzie et al., 2006; Paulsen et al., 2001; Sarmento et al., 2004; Sealey and Gatlin, 2002). But few attempts were made to study the mechanism of how ARA regulates fish immunity on the basis of *in vitro* model, and the unique role of individual ARA fulfilled in fish immune responses remains unclear.

In this study, an *in vitro* model was developed to investigate the specific effects of ARA on the physiological functions and immune responses of macrophages isolated from head kidney of large yellow croaker which is an economically important species for aquaculture in South China (Ai et al., 2006; Mai et al., 2006). Isolated cells were incubated with graded levels of ARA for certain duration of time. Following stimulation, immune functions as well as possible related physiological indicators of fish head kidney macrophages were assessed.

2. Materials and methods

2.1. Experimental fish

Large yellow croaker (1000–1600 g) was obtained from a commercial fish farm in Ningbo, China and kept in floating sea cages at a water temperature range of 20–22 °C with natural photoperiods. Fish was fed low-value fish twice a day. Every time one healthy fish without physical trauma, as indicated by activity and exterior appearance, was selected for experimental use. All the experiments were reproducible.

2.2. Cell culture and ARA-BSA preparation

After the fish was sacrificed by a lethal blow to the head, head kidney was removed aseptically, and placed in ice-cold L-15 medium (Invitrogen, USA) containing 2% fetal bovine serum (FBS, Gibco, USA) and 200 IU ml^{-1} penicillin/streptomycin (P/S, Amresco, USA). Isolation of head kidney macrophages was conducted according to the method described previously with little modification (Braun-Nesje et al., 1982; Sorensen et al., 1997). Briefly, head kidney was washed three times in medium, dissected and pushed carefully through a 100 μm nylon mesh. Then the resultant cell suspension was loaded onto 31%/45% Percoll (Pharmacia, USA) density gradients which have been proven to be the optimal gradients for macrophage isolation beforehand followed by centrifugation (400×*g* at 4 °C for 30 min). Thereafter, cell banding at the interface was collected and washed with care, then resuspended in medium. Cells were counted in a haemocytometer (Boeco, Hamburg, Germany) and viability was determined quickly by trypan blue (0.4% 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 25 °C to allow adherence of macrophages, non-adherent cells were removed by two washes with medium. Cell population profile of the remaining cell monolayer was analyzed by Wright's-Giemsa staining (NJJCBIO assay kits, China). Only macrophage purity exceeded 90% would be used in the experiment.

Typically 0.01 mmol ARA (99% purity, fungal-source, Matreya, Pennsylvania, USA) was dissolved in absolute ethanol as stock solutions of 10 mM. Bovine serum albumin (BSA, fatty acid free, Osaka, Japan) was dissolved in cell assay medium to give a final 2% concentration. ARA-BSA complex solution was prepared beforehand and stored in aliquots at −20 °C. Briefly, 1 ml ARA stock solution was placed in a reaction vial, solvent evaporated under a soft stream of nitrogen and followed by adding 10 ml 2% BSA solution, sonicated for about 5 min, sterilized by pushing through 0.22 μm filter to give a 1 mM ARA-BSA complex (Håversen et al., 2009; Tocher et al., 1996). Prior to use, assay ARA-BSA complex was diluted to 0, 5, 25, 100, 200 and 1000 μM with 2% BSA solution supplemented with 0.01% butylated hydroxytoluene (BHT) as antioxidant and 200 IU ml^{-1} P/S.

2.3. Physiological parameter

2.3.1. Cell viability

After two washes to remove non-adherent cells, graded levels of ARA (0, 50, 100, 200 and 1000 μM) were added into the cell culture plates for 12 h, 24 h and 36 h. At the termination of incubation, cell viability was assessed using quantified spectro-photometrical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) assay (Weldon et al., 2007). Aliquots of 100 μl MTT working solutions were added to cell plate wells and incubated for 4 h followed by fully dissolving formazan crystals in DMSO, and absorbance was measured in 96 microplate readers at 570 nm. The decrease of absorbance was considered as signs of cell viability loss.

2.3.2. Lipid peroxidation

To determine whether lipid peroxidation was involved in the process of cytotoxicity induced by ARA, malondialdehyde (MDA) as one of the metabolites derived from lipid peroxidation was measured using the thiobarbituric acid (TBA) assay kit (Nanjing Jiancheng Bioengineering Institute, China). MDA reacts with thiobarbituric acid to produce a pink-colored material that can readily be monitored by spectro-photometry to give an overall indication of the level of lipid peroxidation (Gavino et al., 1981). At the end of 36 h incubation with ARA, cell-free supernatant was collected, then incubated with 20% trichloroacetic acid and 0.67% thibarbituric acid at 95 °C for 40 min. Absorbance was 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.

2.4. Immune parameter

2.4.1. Phagocytosis activity

Before use, yeasts were opsonized by FBS and washed several times by centrifugation. For opsonization, yeasts were disinfected in spoiled water for 30 min, washed 3 times with germ-free PBS by centrifugation. Subsequently, yeasts were incubated with FBS at room temperature for overnight (Russo et al., 2009, Zhang et al., 1992). Cells were incubated with ARA for 36 h, then opsonized yeasts were added to wells obtaining an approximate 10:1 ratio of yeasts to cells. After 1 h incubation, detached cells and extracellular yeasts were washed off with PBS, then phagocytosis was subjected to light inverted microscope (Nikon TS-100, Japan) for observation (Jensch-Junior et al., 2006). At least one hundred cells were examined and phagocytic capacity was expressed as Phagocytosis Percentage (PP) which was calculated as follows:

Phagocytosis Percentage (PP) = (Number of cells ingesting yeasts/Number of adherent cells observed)×(Number of yeasts ingested/Number of adherent cells observed) (Ai et al., 2007).

2.4.2. Respiratory burst activity

The respiratory burst activity was determined by the reduction of nitroblue tetrazolium (NBT, Amresco, USA) as described previously by Choi et al. (2006) with minor modification. After 24 h incubation with ARA, medium was aspirated out and centrifuged at 3500 rpm for 20 min. Then cell-free supernatant were collected and stored at −80 °C for enzyme linked immunosorbent assay (Elisa) analysis.

Cell monolayers were incubated with 1 mg/ml NBT solution for 30 min containing 1 μg/ml phorbol myristate acetate (PMA, Applichem, Germany) as a trigger for O_2^- production. Cells were then washed twice by pre-warmed PBS and fixed in absolute methanol for 10 min, washed once with 70% methanol, air dried and intracellular fomazan was dissolved in 120 μl 2 M KOH and 140 μl DMSO (Amresco, USA). The mean number of cells per well was determined by counting nuclei 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). Absorbance was measured by a microplate reader at 630 nm using KOH/DMSO as blank. Results were adjusted to absorbance per 10^5 cells.

2.4.3. ELISA analysis

$sPLA_2$, PGE_2 and IL-1β production were determined in head kidney macrophages culture medium according to manufacturer's protocol (Invitrogen, Maryland, USA). Quantitative determination of PGE_2 is based on the competitive binding between PGE_2 and a PGE_2-alkaline phosphatase tracer for a limited amount of PGE_2-specific monoclonal antibody. IL-1β and $sPLA_2$ assay kits are developed on the basis of solid phase sandwich ELISA reaction. Particular antigen ($sPLA_2$ or IL-1β) simultaneously bound to the immobilized antibody coated onto the solid phase. Following a wash to remove excess conjugate and unbound sample, substrate solution was added to the wells. The color development was stopped and absorbance was read at 405 nm. The intensity of the color was inversely proportional to concentration of PGE_2 and directly proportional to that of $sPLA_2$ or IL-1β present in the sample.

2.5. Statistical analysis

Data were subjected to analysis of variance in SPSS 13.0 for Windows. Differences between the means were tested by Tukey's multiple range test, and were considered significant when probability (P) values <0.05 were obtained. Results were expressed as means ± S.E.M (standard errors of the mean).

3. Results

3.1. Physiological parameter

3.1.1. Cell viability

Cell viability was not significantly affected by media ARA levels after 12 h and 24 h incubation. After 36 h incubation, cell viability was lower than that of cells after 12 h and 24 h at each level of ARA, and cell viability decreased with increasing ARA levels, particularly the viability of cells treated with 200 μM and 1000 μM ARA was significantly lower than the control cells (P<0.01) (Fig. 1).

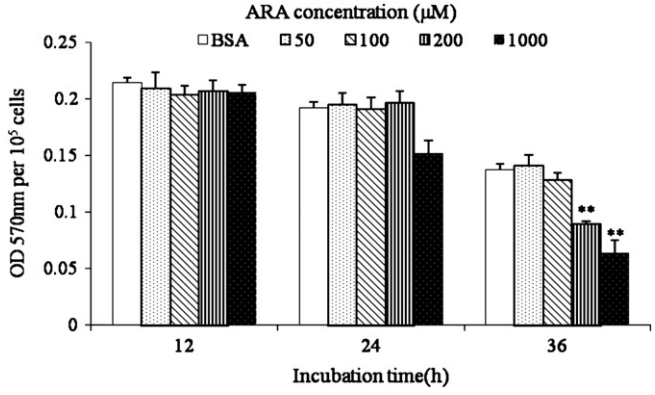

Fig. 1. Effects of ARA on viability of large yellow croaker (LYC) head kidney macrophages after being incubated for 12 h, 24 h, 36 h. Data were expressed as mean ± S.E.M. (n = 4 replicate wells per treatment). * P<0·05 and ** P<0·01 as compared to the control treatment.

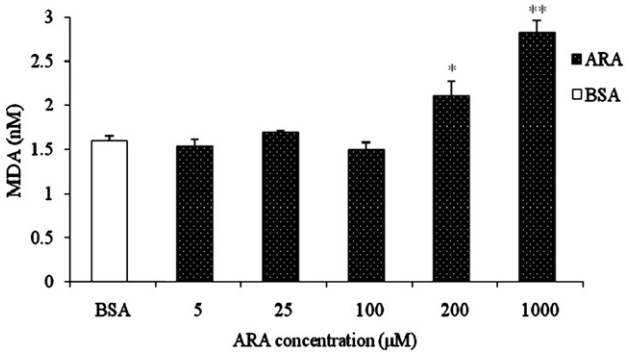

Fig. 2. Effects of ARA on MDA production of LYC head kidney macrophage after being incubated for 36 h. Data were expressed as mean ± S.E.M. (n = 4 replicate wells per treatment). *P<0·05 and **P<0·01 as compared to the control treatment.

3.1.2. Lipid peroxidation

After 36 h incubation, the MDA production of cells treated with relatively low levels of ARA (5, 25 and 100 μM) was not significantly different from the control group. However, higher levels of ARA (200 and 1000 μM) significantly enhanced the MDA production of macrophages compared to the control group (*P<0.05,**P<0.01) (Fig. 2).

3.2. Immune parameter

3.2.1. Phagocytosis

With the increase of ARA levels, phagocytosis activity increased and reached a peak point when ARA was supplemented at a level of 25 μM into the medium, and thereafter declined. Cells treated with moderate levels of ARA (25 and 100 μM) showed significantly higher phagocytic activity than the control group (P<0.01). On the other hand, high level of ARA (1000 μM) caused a significant decrease in the phagocytic activity compared to the control group (P<0.01). No significant difference was observed between the control and treatments with 5 μM and 200 μM ARA (Fig. 3).

3.2.2. Respiratory burst activity

Production of O_2^- increased with the increase of ARA levels from 0 to 200 μM, and decreased at 1000 μM ARA. No significant difference in the O_2^- production from large yellow croaker head kidney macrophages was observed between the control and treatments with ARA (Fig. 4).

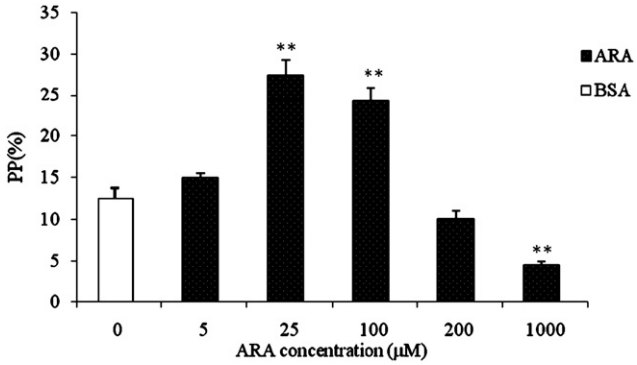

Fig. 3. Effects of ARA on phagocytosis percentage (PP) of LYC head kidney macrophage after being incubated for 36 h. Data were expressed as mean ± S.E.M. (n = 4 replicate wells per treatment). * P<0·05 and ** P<0·01 as compared to the control treatment.

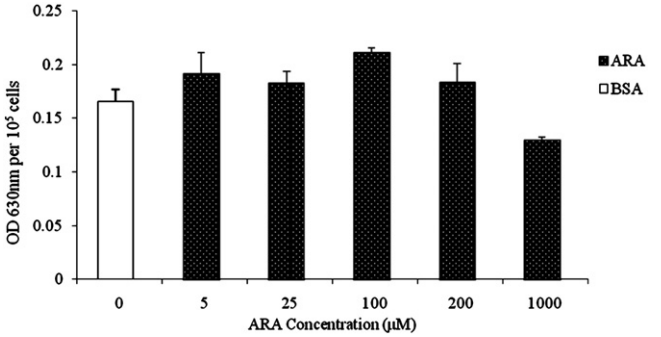

Fig. 4. Effects of ARA on respiratory burst activity of LYC head kidney macrophage after being incubated for 24 h. Data were expressed as mean ± S.E.M. ($n = 4$ replicate wells per treatment).

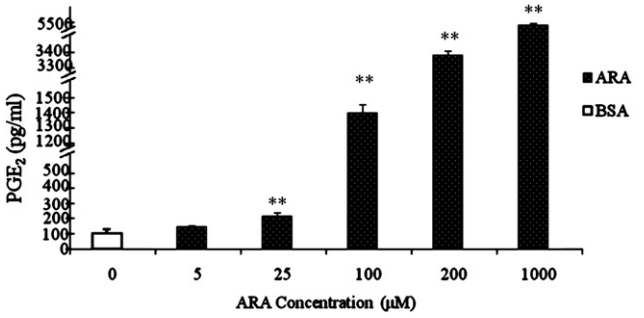

Fig. 6. PGE_2 concentration in the supernatant of LYC head kidney macrophages culture after 24 h incubation with ARA. Data were expressed as mean ± S.E.M. ($n = 3$ replicate wells per treatment). *$P<0.05$ and **$P<0.01$ as compared to the control treatment.

3.2.3. $sPLA_2$ activity

As expected, ARA supplemented in the media significantly elevated the production of $sPLA_2$ compared to the control cells. The $sPLA_2$ productions by cells treated with all tested ARA levels were approximately 20.0 pg/ml, which were significantly higher than the control group (14.5 pg/ml) ($P<0.01$) (Fig. 5).

3.2.4. PGE_2 production

The results herein showed that PGE_2 concentration was largely increased with the increment of ARA level, particularly at the higher levels of ARA, where PGE_2 productions by cells were 10 to 50 fold the concentration of the control group (104.16 ± 23.05 pg/ml), reaching 1532.62 ± 26.6 pg/ml at 100 μM ARA, 3298.8 ± 11.2 pg/ml at 200 μM ARA, 5153.7 ± 40.5 pg/ml at 1000 μM ARA (means ± S.E.M.) ($P<0.01$), respectively (Fig. 6).

3.2.5. IL-1β activity

There were no distinct effects on the production of IL-1β in large yellow croaker macrophages between the control group and treatments with all tested ARA levels. Additionally, IL-1β concentrations determined in cell culture supernatant were relatively low (only approximately 6 pg/ml) (Fig. 7).

4. Discussion

4.1. Cell viability

In the present study, cell viability expressed by absorbance of MTT assay decreased markedly in a time and ARA concentration-dependent fashion. Similar results have been described in several cell types (Cury-Boaventura et al., 2006; Otton and Curi, 2005). With regard to the cell viability loss, previous studies indicated that exogenous fatty acid could incorporate into cell membrane and bring about modification of cell fatty acid profile and physical property of biological membrane (Calder, 2008; Tocher and Dick, 1990). In particular, high levels of fatty acids have been reported to adversely induce cell DNA fragmentation, loss of membrane integrity and disorder of membrane permeability, and eventual cell death (Gorjão et al., 2009; Ji et al., 2005).

The involvement of lipid peroxidation in the induction of cell death by ARA has been observed in some *in vitro* studies, including human monocyte-derived macrophages (Muralidhar et al., 2004) and HepG2 cells (Chen et al., 1997). When fatty acids especially HUFAs incorporate into the membrane lipid draft, cells are subjected to the risk of lipid peroxide, and it has been clearly indicated that the fatty acids with three or more double carbon bonds are more susceptible to lipid peroxidation. In the present study, cell performance observed in the present study appeared to correlate well with the degree of lipid peroxidation (measured as MDA production) induced by ARA with four carbon-carbon bonds per molecule. However, Martins de Lima et al. (2006) indicated that fatty acid-induced viability loss of J447 cells was independent of the double carbon bonds in the molecules. Probably, difference in cell types, experimental methodology and culture condition could help to explain the discrepancies.

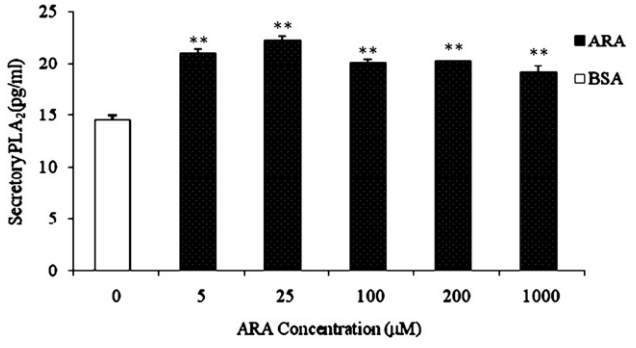

Fig. 5. $sPLA_2$ production in the supernatant of LYC head kidney macrophages culture following 24 h incubation with ARA. Data were expressed as mean ± S.E.M. ($n = 3$ replicate wells per treatment). * $P<0.05$ and ** $P<0.01$ as compared to the control treatment.

4.2. Phagocytosis and respiratory burst activity

The present study showed that optimal dose of ARA could enhance phagocytic capacity of fish macrophages. The result was in agreement with *in vitro* studies on human monocytes (Lennartz and Brown, 1991). One possible mechanism lies in the modification of membrane fatty acid profile. Several studies demonstrated that altered phagocytic capacity could relate in part to altered expression of receptors involved in phagocytosis, but more related to physical nature of membrane (Calder, 2008; Calder et al., 1990; Gorjao et al., 2006). Exogenous ARA incorporated into cell membrane contributed to promoted abilities of membrane fluidity and expansion, which consequently enhanced their phagocytic capacity. Another proposed interpretation suggested that release of ARA from membrane by the action of phospholipase A_2 (PLA_2) has been demonstrated to be essential in Fc-receptor mediated phagocytosis (Lennartz and Brown, 1991). Optimal dose of ARA seems to be necessary for membrane pseudopodia extension and development, underlying alien particles to be phagocytosed (Karimi and Lennartz, 1995).

As an indicative immune parameter in fish non-specific immune system, oxidative responses play an important role in killing and clearance of microorganisms (Novoa et al., 1996; Skarmeta et al., 1995). Previous literature surveys revealed that ARA could enhance the ability of *in vitro* human neutrophils to produce O_2^- (Hardy et al., 1994; Hii and Ferrante, 2007). Also, Calder (2001) noted that the

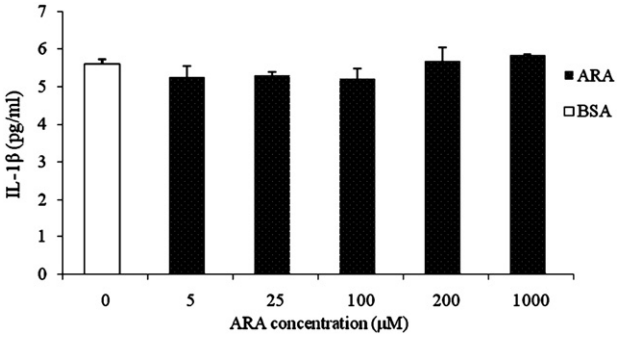

Fig. 7. IL-1β concentration in the supernatant of LYC head kidney macrophages after 24 h incubation with ARA. Data were expressed as mean ± S.E.M. ($n = 3$ replicate wells per treatment).

leukotriene B_4 (LTB_4) derived from ARA enhanced the respiratory burst activity of vertebrate leucocytes. In addition, results on juvenile sea bass also seemed to correlate well with those previous findings (Xu et al., 2010). In this study, although optimal ARA concentration could slightly increase the respiratory burst activity of macrophages, no significant difference compared to the control was detected, which was partly comparable to the results obtained on an *ex vivo* plasma incubation model (Seierstad et al., 2009). The difference may be due to the type of the cells, priming time and the experimental protocols used. In conclusion, the works related to the effects of ARA on fish immune systems is still limited, and further research concerning the modulation mechanism is needed.

Besides the positive immune enhancing effects, PP value and O_2^- production were found to be inhibited by 1000 μM ARA, which was comparable to previous work by de Lima et al. (2006), who reported that optimal dose of ARA could stimulate NO production of murine macrophage while high levels of ARA could exert toxic effects on cells and suppress NO production. Those findings were indicative that ARA could promote immune functions of fish macrophages on condition that concentrations of fatty acids tested match the physiologically acceptable range. In the meanwhile, the inhibitory effects exerted by ARA were mainly due to their cytotoxicity.

4.3. Eicosanoid production

Results of this study showed that exogenous supplementation of ARA could significantly elevate the PGE_2 production of fish macrophages, and similar results have been found on bone cells (Coetzee et al., 2005) and mouse peritoneal cells (Kakutani et al., 2010). Generally, increased amount of ARA incorporated into cell membrane increased the availability of ARA as substrate for eicosanoids production, resulting in the enhanced production of ARA-derived eicosanoid mediators like PGE_2 (Bell and Sargent, 2003; Sargent et al., 1999). Additionally, the release of ARA from phospholipids by phopholipase A_2 (PLA_2) is a rate-limiting step for PGE_2 production (Boyanovsky and Webb, 2009; Natarajan and Nadler, 2004). However, the present study indicated that the increase of $sPLA_2$ production induced by ARA did not show the same concentration-dependent fashion as that of PGE_2. Thus, $sPLA_2$ probably not the only rate-limiting enzyme in the process of ARA liberation and production of PGE_2. Finally, it is important to note an alternative pathway for PGE_2 production which took place in cytoplasmic lipid bodies. Lipid bodies and $cPLA_2$-α facilitate ARA mobilization and synthesis of PGE_2 through a series of receptor-driven signaling pathways rather than the general cyclooxygenase and lipooxygenase pathways (Moreira et al., 2009). Although no relevant reports on fish cells is available, the cytoplasmic lipid droplets (Fig. 8) along with concomitant increased PGE_2 levels observed in present study is an area worth more research for the understanding of enhanced PGE_2 production stimulated by ARA.

4.4. Cytokine production

Fish macrophage has been reported to show potent capability of IL-1β expression (Pelegrín et al., 2001). Although it is well recognized that ARA exert pronounced pro-inflammatory effects (Calder, 2006), so far, little information regarding *in vitro* effects of ARA on inflammatory cytokine production of fish macrophages is available. The present work showed that ARA had no significant effects on the production of IL-1β. Presumably lack of immune stimuli like LPS priming process was accountable for the phenomenon (Coetzee et al., 2005; MacKenzie et al., 2003; Weldon et al., 2007). The pro-inflammatory effects by ARA were dependent upon the LPS dose and these effects may be missed by using less appropriate concentrations of stimuli or not using at all (Mullen et al., 2009).

4.5. In vivo/in vitro correlation

As the same case with oral intake of certain fatty acid-rich diets, *in vitro* culture of these cells in medium enriched with specific fatty acids could also modify fatty acid composition of immune cells (Calder, 1999; Ghioni et al., 1997; Tocher et al., 1995). Moreover, considerable studies have described the effects of dietary ARA on some indicative parameters of fish immunity including phagocytosis activity (Montero et al., 2003), respiratory burst activity (Bell et al., 1996; Montero et al., 2003; Xu et al., 2010) and eicosanoid production (Bell et al., 1996; Ganga et al., 2005), which also confirmed the findings obtained from the present study. All the evidences suggested that *in*

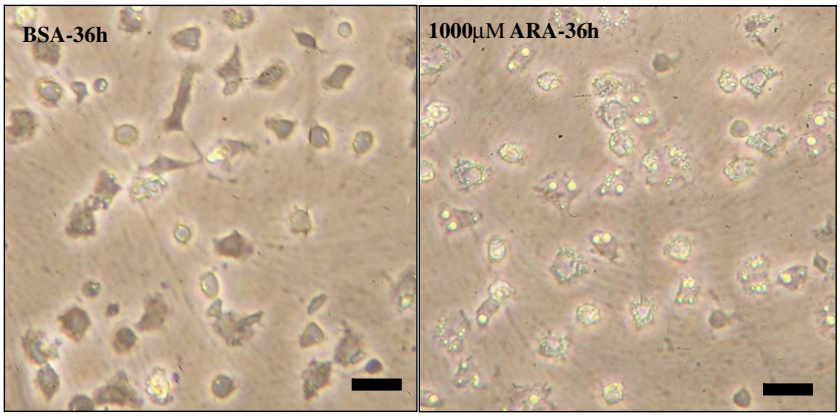

Fig. 8. Light microscopic analysis of LYC head kidney macrophages after 36 h incubation with ARA. Cytoplasmic lipid droplets induced by 1000 μM ARA could be clearly seen. Bar = 10 μm.

vitro models could reflect, at least in part, the *in vivo* situation of fish. However, *in vitro* models could not fully substitute all-animal models after all, and results from many *in-vitro* assays were often in controversy. Therefore in order to validate *in vitro* assays it is necessary to establish *in vitro*/*in vivo* correlations by comparing the information derived from *in vitro* assays and the results from *in vivo* studies (Villena, 2003).

5. Conclusion

In summary, primary macrophage as a model system could be used to identify the effects of ARA on immune responses of fish macrophages *in vitro*. The present study demonstrated that ARA exerted pronounced effects on cell viability, lipid peroxidation as well as immune responses of macrophage in large yellow croaker. To elucidate the complex mechanism involved in the immune-modulatory effects, further studies dealing with some possible regulatory pathways proposed in this study are necessary.

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