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E-mail:xbywb@ouc.edu.cn

Assessment and Comparison of *in vitro* Immunoregulatory Activity of Three Astaxanthin Stereoisomers

SUN Weihong[1), 2)], XING Lihong[1)], LIN Hong[2), *], LENG Kailiang[1)], ZHAI Yuxiu[1)], and LIU Xiaofang[1)]

1) *Yellow Sea Fishery Research Institute*, *Chinese Academy of Fishery Sciences*, *Qingdao* 266071, *P. R. China*
2) *College of Food Science and Engineering*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

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Abstract In recent years, the immune-modulatory role of all-*trans* astaxanthin from different pigment sources has been studied. It was reported that all-*trans* astaxanthin might exist as three stereoisomers, and the composition of all-*trans* stereoisomers in natural materials differs from that of synthetic products. However, the different biological effects of various all-*trans* stereoisomers still remain unclear. In the present study, we evaluated the bioactivity of three astaxanthin stereoisomers, (*3S,3'S*)-*trans*-, (*3R,3'R*)-*trans*- and *meso*-*trans*-astaxanthin, in regulating cell-mediated immune response using mice lymphocytes and peritoneal exudates cells (PECs) systems. After the treatment with three astaxanthin stereoisomers ($20\,\mu mol\,L^{-1}$), the lymphocyte proliferation capacity, neutral red phagocytosis of PECs and natural killer (NK) cell cytotoxic activity were comparatively assessed. The results showed that all three astaxanthin stereoisomers significantly promoted lymphocyte proliferation, phagocytic capacity of PECs, and cytotoxic activity of NK cells. Moreover, the (*3S,3'S*)-*trans*-astaxanthin exhibited a much higher response than others.

Key words all-*trans*-astaxanthin; stereoisomer; immunity

1 Introduction

All-*trans*-astaxanthin is an important carotenoid naturally produced by microorganisms, such as green microalgae *Haematococcus pluvialis* (Fan *et al*., 1995) and *Chlorella zofingiensis* (Bar *et al*., 1995) and red yeast *Phaffia rhodozyma* (Bon *et al*., 1997). Given the presence of two chiral carbon atoms at C-3 and C-3', then three stereoisomers exist as all-*trans*-astaxanthin, including a pair of enantiomers (*3R,3'R* and *3S,3'S*) and an optically inactive *meso* form (*3R,3'S*; *3S,3'R*). The stereoisomeric ratio in synthetic astaxanthin is 1:2:1 for the *3R,3'R*/*meso*/*3S,3'S* isomers, while natural astaxanthin is characterized by variable composition of different isomers. The esterified *3S,3'S* enantiomer is the main form found in alga *H. pluvialis* (Wang *et al*., 2008) and wild salmon (Turujman *et al*., 1997), while the predominant optical enantiomer of *P. rhodozyma* astaxanthin is of the *3R,3'R* form (Moretti *et al*., 2006).

Astaxanthin has a feature of coloration, strong antioxidant capacity (Guerra *et al*., 2012; Santos *et al*., 2012), and immunoregulatory effect. A number of researches have demonstrated the immune functions of all-*trans*-astaxanthin *in vitro* and *in vivo*. Astaxanthin can enhance the antibody production ability of mouse spleen cells (Jyonouchi *et al*., 1993), increase cell-mediated and humoral immune responses in cats (Park *et al*., 2011) and dogs (Chew *et al*., 2011), and partially restore the decreased humoral immune responses in old mice (Jyonouchi *et al*., 1994). In addition, dietary intervention with astaxanthin could also enhance immune response, and decrease inflammation of young healthy females (Park *et al*., 2010). In the previous studies, the immune-modulatory role of all-*trans* astaxanthin has been reported but the different biological effects of various all-*trans* stereoisomers still remain unclear.

In this study, the immunoregulatory effect of three all-*trans* astaxanthin was comparatively evaluated *in vitro*.

2 Materials and Methods

2.1 Materials

(*rac./meso*)-Astaxanthin was purchased from Carotenature (Lupsingen, Switzerland). Female SPF KM mice, 6 weeks old, were obtained from Institute for Drug Control in Qingdao (Qingdao, China). K562 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Fetal Bovine Serum and RPMI 1640 were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), tetrahydrofuran (THF), and dimethyl sulfoxide (DMSO) were

* Corresponding author. Tel: 0086-532-82032203
E-mail: linhong@ouc.edu.cn

purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reagent kit for determining lactate dehydrogenase (LDH) was purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). All other reagents at analytical grade were purchased from Beijing Chemicals (Beijing, China).

2.2 Preparation of *3R,3'R* and *3S,3'S trans*-Astaxanthin Stereoisomers

Two enantiomers were prepared using the modified method of Sun *et al*. (2014). Briefly, *(3S,3'S)-trans*-astaxanthin was isolated from *H. pluvialis*. The crude extract was saponified at 4℃ for 15 h, and then 0.02 mol L^{-1} NaOH was added into the reaction mixture at 22℃. After 3 h, the saponified pigment extract was mixed with distilled water and n-hexane at a volume ratio of 1:1:1. *(3R,3'R)-trans*-astaxanthin was directly extracted from *P. rhodozyma*. Subsequently, low-pressure silica-gel column chromatography was used to prepare the high purity (> 75%) enantiomers of all-*trans*-astaxanthin. The supersaturated solution of astaxanthin was crystallized in acetone at 4℃ for 72 h, and it was confirmed by HPLC that the purity of two enantiomers crystalline powder was > 95.0%. The structures of three stereoisomers were elucidated by a combination of HPLC-APCI-MS, ^{1}H NMR, ^{13}C NMR, and HPLC-UV.

2.3 Cell Culture

K562 cell culture was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 μg mL^{-1}) at 37℃ in a humidified atmosphere with 5% CO_2, and the experiment was performed at the exponential phase of growth.

2.4 Cell Viability Assay

K562 cells (5×10^3) were seeded into 96-well plates and incubated in RPMI-1640 medium containing 10% of FBS for 24 h, and then the cells were treated with THF at concentrations of 0, 0.5% and 1%, respectively. After 36 h, the cells were subjected to MTT at a final concentration of 0.5 mg mL^{-1} for an additional 4 h at 37℃, and then the optical density at 570 nm was measured. Each experiment was repeated in triplicate.

2.5 Determination of Lymphocyte Proliferation Capacity

The lymphocyte culture was prepared according to the procedure of Otani and Monnai (1993). An aliquot of astaxanthin stereoisomers (*3R,3'R*, *meso* and *3S,3'S*) (at a final concentration of 10 μmol L^{-1} and 20 μmol L^{-1}, respectively, in THF) was added to the target cells. The same amount of THF, without astaxanthin isomers, was added as the blank control.

2.6 Neutral Red Phagocytosis Assay of Peritoneal Macrophages

Macrophages were obtained from the mice peritoneal exudate using the method of Govindaraj *et al*. (2010).

The astaxanthin stereoisomers (final concentration of 20 μmol L^{-1}) were added to each well with 100 μL cell culture medium, and co-incubated with macrophages for 24 h. Then, the supernatant was discarded and 200 μL 0.075% neutral red was added and incubated for another 30 min. Cells were subsequently washed with PBS three times and incubated with cell lysis buffer (1 mol L^{-1} acetic acid:ethanol =1:1) overnight. Biological evaluation was carried out as previously reported (Cao and Lin, 2004).

2.7 NK Cell Cytotoxic Activity Detection

Modified lactate dehydrogenase (LDH) release assay (Konjević *et al*., 1997; Yaqoob *et al*., 1998; Jurisić *et al*., 1999) was used for the determination of NK cell cytotoxic activity. Target cells (K562 cells) were added to each well at two effector-target (E:T) ratios of 5:1 and 10:1, and the maximal target cell lysis was assessed by incubating K562 cells with lysing reagent, provided in the cytotoxicity kit.

2.8 Statistical Analysis

All the tests were performed in triplicate and the data were expressed as mean ± SEM (indicated by error bars). All the data were analyzed by one-way ANOVA and LSD test for statistical significance ($P<0.05$) using SPSS 19.0 statistical software (IBM Corporation, Somers, NY).

3 Results

3.1 Lymphocyte Proliferation

The lymphocyte proliferation capacity was assayed according to its response to a specific stress. At a concentration of 10 μmol L^{-1}, the three astaxanthin stereoisomers show no significant differences in lymphocyte proliferation. When the experimental concentration was 20 μmol L^{-1}, they all significantly promoted proliferative capacity of mice lymphocytes, and (*3S,3'S*)-*trans*-astaxanthin showed better effect than the other two ($P<0.05$). There was no significant difference for proliferative capacity between the *3R,3'R* and *meso* groups (Fig.1).

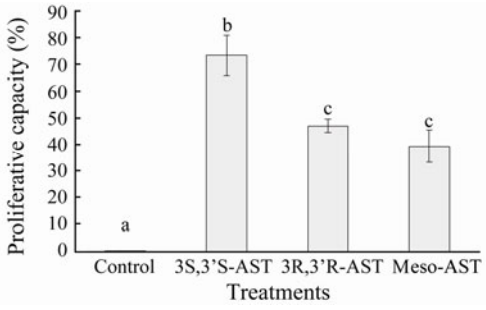

Fig.1 The effect of three astaxanthin stereoisomers on mice spleen lymphocyte proliferation *in vitro*. Different letters indicate significant difference at $P<0.05$.

3.2 Phagocytic Capacity

The phagocytic capacity of PECs was higher ($P<0.05$)

in astaxanthin stereoisomers groups compared to that of the control (Fig.2). Compared to the *3R,3'R* and *meso* stereoisomer groups, the (*3S,3'S*)-*trans*-astaxanthin group promoted a more significant increase in the phagocytosis of PECs ($P<0.05$). However, there was no statistically significance in the phagocytic capacity between the *3R,3'R* treatment and the *meso* groups.

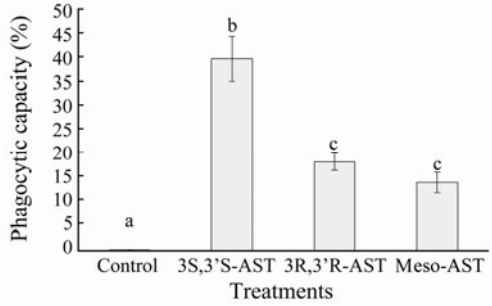

Fig.2 The Effect of three astaxanthin stereoisomers on phagocytic capacity of mice pritoneal macrophages *in vitro*. Different letters indicate significant difference.

3.3 Natural Killer cell Cytotoxic Activity

All of the three astaxanthin stereoisomers groups (E:T ratio, 10:1) showed considerable promotion in NK cell activities ($P<0.05$). What's more, (*3S,3'S*)-*trans*-astaxanthin exhibited the best positive activity. Meanwhile, *3R,3'R* treatment and the *meso* group exhibited almost near moderate effect (20% to 25%). But no significant difference was observed between groups with the E:T ratio of 5:1.

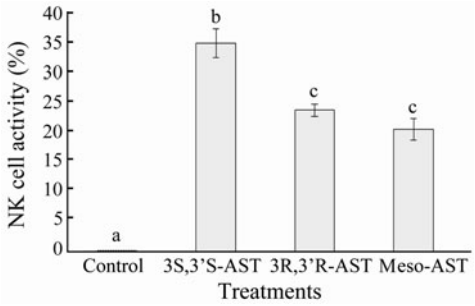

Fig.3 The effect of three astaxanthin stereoisomers on natural killer cell activity *in vitro*. Different letters indicate significant difference at $P<0.05$.

4 Discussion

The biological effects of astaxanthin have been reported previously both *in vitro* and *in vivo* (Ambati *et al*., 2014; Rao *et al*., 2013; Rao *et al*., 2013), while the astaxanthin used in these researches was from different pigment sources. In our study, the effect of three astaxanthin stereoisomers was evaluated in regulating cell-mediated immune response in mice lymphocytes and PECs. The study showed that three astaxanthin stereoisomers significantly promoted the lymphocyte proliferation, phagocytic capacity of PECs, and NK cell cytotoxic activity. In addition, (*3S,3'S*)-*trans*-astaxanthin showed better immunoregulatory effect than that of another two.

Astaxanthin has been approved as a coloring matter for many years in the salmonid fish feeding industry. And astaxanthin from *H. pluvialis* is usually used as a functional food additive and medicinal ingredient. All-*trans* astaxanthin, which predominates in nature, is now commercially available from either natural or synthetic sources. Synthetic astaxanthin is a racemic mixture made of three isomers, while natural astaxanthin also has a variable distribution of different isomers (Moretti *et al*., 2006).

In reference to the biological activities of astaxanthin stereoisomers, the predominant astaxanthin is *3R,3'R* enantiomer in *P. rhodozyma*. It plays an antioxidant role during aging (Schroeder and Johnson, 1993), and can reduce the oxidized oil-induced oxidative stress in rainbow trout by removing oxygen free radical (Nakano *et al*., 1999). Other papers have reported that *P. rhodozyma* increased plasma immunogloblin (Ig) G concentration and splenocyte proliferation stimulated by both concanavalin A and pokeweed mitogen in chicks (Takimoto *et al*., 2007). Astaxanthin in *H. pluvialis* extract all existed in the esterified *3S, 3S'* form, and several studies (Jyonouchi *et al*., 1991; Jyonouchi *et al*., 1993) have demonstrated its immunomodulating effects on mouse lymphocytes *in vitro* by analyzing the mitogen responses of spleen cells. Moreover, astaxanthin also could significantly promote the production of antibody-forming cells of splenocytes. In the previous studies, dietary astaxanthin from synthetic sources efficiently enhanced certain aspects of the non-specific defense mechanism in rainbow trout (Amar *et al*., 2002; Amar *et al*., 2001), which was consistent with those fed *P. rhodozyma* (Amar *et al*., 2004). The biological effects of different astaxanthin stereoisomers remain to be clarified.

In the text, we comparatively evaluated the immunological effect of (*3S,3'S*)-*trans*-astaxanthin, (*3R,3'R*)-*trans*-astaxanthin and *meso*-*trans*-astaxanthin *in vitro*. In lymphocyte proliferation test, (*3S,3'S*)-*trans*-astaxanthin exhibited higher proliferative capacity than that of *3R,3'R* and *meso* stereoisomers. Meanwhile, the colorimetric MTT assay has been introduced to assess proliferative lymphokines, mitogen stimulations and complement-mediated lysis by showing the cellular growth and survival (Mosmann, 1983). Chew *et al.* (1999) have demonstrated that astaxanthin could enhance splenic lymphocyte function in mice. In agreement with Chew's result, we found that three astaxanthin stereoisomers could significantly promote the proliferative capacity of mice lymphocytes, and (*3S,3'S*)-*trans*-astaxanthin showed a significantly better ($P<0.05$) positive activity than *3R,3'R* and *meso* stereoisomers. Macrophages play a central role in immune responses by means of secretion of inflammatory cytokines, phagocytic activity, and antigen presentation (Yokota *et al*., 2000). In neutral red phagocytosis assay of peritoneal macrophages, phagocytic capacity of PECs was assayed by their capacity to proliferate in response to a specific stimulation. In our ongoing research, astaxanthin, especially the *3S,3'S* enantiomer, can sig-

nificantly activate the phagocytic capacity. Several researches have reported that astaxanthin could significantly enhance the phagocytic index and phagocytic ratio in sea urchins (Kawakami *et al*., 1998), improve neutrophil phagocytic and microbicidal capacity (Macedo *et al*., 2010), and recover phagocytic capacity of neutrophils (Guerra and Otton, 2011). NK cells are lymphocytes active in innate responses against viruses, bacteria, and tumors, due to their potent cytotoxic activity and rapid production of cytokines (Moretta *et al*., 2002). In our study, all of the three astaxanthin stereoisomers increased NK cell activity ($P<0.05$), and the (*3S,3'S*)-*trans*-astaxanthin showed a significantly higher response than the other two astaxanthins. Previous studies have demonstrated that astaxanthin from *H. pluvialis* and synthetic sources could increase NK cell cytotoxic activity in application to humans and dogs (Park *et al*., 2010; Chew *et al*., 2011), and also inhibit stress-induced suppression of tumor-fighting natural killer cells in rats (Kurihara *et al*., 2002). Whatever be the case, our research results suggested that the *3S,3'S* enantiomer might have a stronger immunoregaulatory activity than *3R,3'R* and *meso in vitro*.

When synthetic astaxanthin (100 mg kg^{-1} dry diet) is adopted as a source of vitamin A in fish diets short of this vitamin, it does not have any marked effect on innate or specific immunity in its own right and only has little potential as an immunostimulant in rainbow trout (Thompson *et al*., 1995). Astaxanthin stereoisomers deposited in the flesh retained their optical configuration (Bjerkeng *et al*., 1992), and no significant difference is observed between optical isomer composition of astaxanthin in flesh and feed. Storebakken *et al*. (1985) reported that no epimerization occurrs in flesh at the chiral centers at C-3 and C-3' in astaxanthin of Atlantic salmon. We have yet again demonstrated that natural products play a dominant role in the discovery of active agents after the complex envolutionary pre-selection (Cragg *et al*., 2012), since the natural astaxanthin shows better bioactivity than synthetic astaxanthin, and they can be distinguished for their stereo-configuration keeping consistent in the food chain.

In terms of feeding fish with the oral application of astaxanthin, it was reported that *P. rhodozyma* represented a more effective astaxanthin source for pigmentation of Atlantic salmon muscle than synthetic sources (Bjerkeng *et al*., 2007), and the muscle astaxanthin concentration was lower ($P<0.05$) in rainbow trout fed *H. pluvialis* than in those fed synthetic astaxanthin (Choubert *et al*., 2006). Since, in the past, the sights of experts have been dedicated to the role of different sources of astaxanthin in aquaculture, we can suppose that further research should focus on the possible difference in the biological function of the different stereoisomers of astaxanthin.

In a word, our study has demonstrated that (*3S,3'S*)-*trans*-astaxanthin exhibits a better positive immune activity compared with *3R,3'R* and *meso* stereoisomers *in vitro*.

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