Does squalene alter the antioxidant potential of astaxanthin and fucoxanthinol? In vitro evidence in RAW 264.7 cells, a murine macrophage

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Abstract Astaxanthin (Ax) and fucoxanthin/fucoxanthinol (FuOH) are marine xanthophylls exhibiting anti-oxidant effects. Squalene (SQ) is a triterpenoid and is a precursor of sterols. This study aimed to determine if SQ can improve the effect of Ax/FuOH on lipid peroxidation. RAW 264.7 cells were treated with different concentrations of Ax, FuOH and SQ and corresponding rate of cell survival was noted. In addition, combination groups - Ax + SQ and FuOH + SQ- were also run. Cells treated with Ax, FuOH, SQ, Ax + SQ and FuOH + SQ were stimulated with lipopolysaccharide and lipid hydroperoxides were estimated. Results showed that 5 μ M Ax, 2 μ M FuOH and 10 μ M SQ supported cell survival. In presence of SQ, cell viability improved for higher concentrations of FuOH (5, 10 μ M). Lipid hydroperoxides were suppressed by Ax, FuOH, Ax + SQ and FuOH + SQ and were

significantly lower in Ax + SQ, indicating the synergistic effect of Ax and SQ. To conclude, combination of Ax with SQ enhances its ability to suppress lipid peroxidation while with FuOH, SQ attenuates the toxic effect at higher doses. Moreover, this is the first time that the combined effect of SQ and carotenoids has been studied and reported.

Keywords Astaxanthin · Fucoxanthinol · Lipid peroxidation · RAW264.7 · Squalene

Abbreviations

Ax	Astaxanthin
FuOH	Fucoxanthinol
DMSO	Dimethyl sulfoxide
DPPP	diphenyl-1-pyrenylphosphine
SQ	Squalene

Research Highlights

- This is the first report indicating the combined effect of squalene and marine xanthophylls
- Results indicate the potential of combining squalene with marine xanthophylls that would enhance the activity of the latter
- Squalene enhanced tolerance of cells to higher concentration of fucoxanthinol and synergistically decreased lipid peroxidation in cells with astaxanthin
- The results have significance in utilizing the combination of astaxanthin and squalene in cosmeceuticals, food and feed formulations

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Introduction

Lipids, present ubiquitously in biological systems, are prone to peroxidation by free radicals that are continuously being generated as a result of various processes. Under normal conditions, endogenous antioxidants as well as other antioxidants neutralize these free radicals to maintain a healthy equilibrium and prevent damages to the internal system and health. However, under certain circumstances such as disease conditions, this precarious balance is disrupted, resulting in increased oxidative stress and damage to the physiological system (Pham-Nuy et al. 2008). Oxidative stress and lipid peroxidation are often a cornerstone of several diseases and disorders, including chronic and degenerative diseases such as diabetes, cancer, neurological and cardiovascular diseases among others, as well as conditions such as malnutrition and

deficiency disorders. Astaxanthin (Ax) and fucoxanthin are xanthophylls of marine origin that are known to exhibit potent antioxidant activity. For instance, Lee et al. (2003) and Ohgami et al. (2003) have reported the antioxidant and anti-inflammatory property of Ax in lipopolysaccharide (LPS) stimulated RAW 264.7 cells. Reports of the antioxidant effect of Ax in other cell types are also available (Nakajima et al. 2008, Kim et al. 2009, Li et al. 2013). Saw et al. (2013) have moreover reported the antioxidant effect of Ax alone and in combination with EPA and DHA. Shiratori et al. (2005); Heo et al. (2008) and Maeda et al. (2015) amongst others have reported the antioxidant and anti-inflammatory effects of fucoxanthin and its metabolites in cell line studies. Squalene (SQ), is a triterpenoid whose major source is shark liver oil. Moreno (2003) attempted to study the antioxidant effect of SQ and other components of olive oil in RAW 264.7 macrophage system but did not find any significant results for SQ. While attempts have been made to ascertain the antioxidant effect of SQ and Ax (separately) in combination with n-3 long chain fatty acids (Dhandapani et al. 2007, Saw et al. 2013), no study demonstrating the combined effect of carotenoids and SQ is available. Against this background, we studied the effect of Ax and Fucoxanthinol (FuOH) on lipid peroxidation, in combination with SQ, in RAW 264.7 cells that were stimulated with LPS to induce oxidative stress. FuOH was selected as it is a metabolically active metabolite of fucoxanthin in vivo.

Materials & methods

RAW 264.7 cells (DSP Biomedicals, Osaka, Japan) were cultured in RPMI-1640 with 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, NY, USA) and 10 % foetal bovine serum (Thermo Trace, Melbourne, Australia) at 37 °C, in a humidified atmosphere of 95 % air and 5 % CO₂. Astaxanthin (Ax), fucoxanthinol (FuOH) (Sigma, MO, USA), and squalene (SQ) were dissolved in dimethyl sulphoxide (DMSO) and added to the culture medium. The final concentration of DMSO in the culture medium was less than 0.5 %. RAW 264.7 cells were pre-incubated for 24 h under the conditions mentioned and then stimulated with 0.1 and 1 µg/ml LPS from *E. coli* (Sigma, MO, USA) for 6, 12 and 24 h. Cell viability was measured and expressed as % of the control by measuring the density of colour produced with the WST-1 dye at 450 nm. Cells (5×10^4) were incubated with media containing different concentrations of Ax (5–10 µM), FuOH (2–10 µM) and SQ (5–50 µM) separately for 24 h. Control group (containing only DMSO) was run alongside. Cell viability was measured with WST-1 dye at 450 nm. After ascertaining the highest concentration of Ax, FuOH and SQ that supported maximum cell survival, the effect of Ax + SQ and FuOH + SQ on cells was determined using WST-1 dye. Based on the results obtained from the measurement of cell viability, RAW 264.7 cells were

incubated for 48 h with Ax (5 µM), FuOH (2 µM), SQ (10 µM), Ax (5 µM) + SQ (10 µM) and FuOH (2 µM) + SQ (10 µM). Thereafter, cells were exposed to 0.1 µg/ml LPS and incubated for 6 h. The cells were harvested and the lipids extracted by the method of Folch et al. (1957). Lipid hydroperoxides react with non-fluorescent diphenyl-1-pyrenylphosphine (DPPP) to give fluorescent DPPP oxide, which was measured using reverse phase HPLC. Briefly, the lipid extract was weighed and dissolved in chloroform: methanol (2:1, v/v) (containing 10 mg butyl hydroxy toluene per ml chloroform). To the sample solution (100 µl), 50 µl of DPPP solution (1 mg/10 ml chloroform) was added and left to stand in a water bath for 60 min at 60 °C. Thereafter, the solution was cooled on ice and 3 ml of 2-propanol was added to it, diluted with mobile phase and injected to the HPLC system. The HPLC system used was Hitachi L-2350 HPLC system (Hitachi, Tokyo, Japan) and consisted of a pump (L-2130), an auto-sampler (L-2200) and a fluorescence detector (L-2485). The DPPP oxide was measured at 40 °C with a Develosil-ODS column (UG-5, Nomura Chemicals, Aichi, Japan), protected by a ODS guard column (10 × 4.0 mm i.d.). The mobile phase consisted of a mixture of butanol and methanol (10:90, v/v), and the flow rate was set at 1.0 ml/min. The fluorescence detector was set at Ex. 352 nm and Em. 380 nm. The DPPP standard curve was used to calculate the lipid hydroperoxide concentration in the samples and expressed as nmol/g lipid. SQ, DMSO, WST-1, DPPP and other reagents/solvents were from M/s Wako Pure Chemicals (Osaka, Japan). Statistical analysis was performed on Microsoft Excel. Mean separation was achieved by Students T-test ($p < 0.01$).

Results & discussion

Ax, FuOH and SQ are lipid based molecules of marine origin and are established nutraceuticals. To study the effect of SQ

Fig. 1 Effect of lipopolysaccharide (LPS) at different concentrations over a period of 6 to 24 h on cell viability. *LPS = µg/ml media. Columns sharing a common alphabet are not significantly different ($p > 0.01$) ($n = 8$)

Fig. 2 Effect of different concentrations of astaxanthin (Ax), fucoxanthinol (FuOH), squalene (SQ) and combination of Ax/FuOH with SQ on cell viability. Columns in a group sharing a common alphabet are not significantly different ($p > 0.01$) ($n = 8$)

combined with Ax or FuOH, LPS stimulated RAW 264.7 cells were harvested and lipid hydroperoxides were estimated. Based on the previous reports (Ohgami et al. 2003, Konishi et al. 2008), LPS was chosen as the oxidative stress stimulant. RAW 264.7 cells were stimulated with 0.1 μg/ml and 1 μg/ml LPS for 6, 12 and 24 h. Cell viability (% compared to control) was 91, 98 and 125 with 0.1 μg/ml LPS at 6, 12 and 24 h respectively, while it was 102, 104 and 110 with 1 μg/ml LPS respectively (Fig. 1). This indicates that LPS concentrations of 0.1 and 1 μg/ml had an effect on the cells upto 6 and 12 h respectively and this was more pronounced in the former, resulting in its selection for studying the effect of Ax, FuOH and SQ in the cells. Stimulation of RAW 264.7 cells using a similar concentration of LPS has been previously reported (Konishi et al. 2008).

RAW 264.7 cells incubated with media containing 5 and 10 μM of Ax showed a cell viability of ~92 % (Fig. 2). Nakajima et al. (2008) have used similar concentrations of Ax (0.1–10 μM) to study its effect on oxidative stress in RGC-5 cells. Ohgami et al. (2003) have reported the anti-inflammatory and antioxidant effects of Ax at concentrations of 12.5 μM and higher in RAW 264.7 cells. FuOH, at concentrations of 5 and 10 μM reduced the cell viability to 5 and 17 % of control ($p < 0.01$) respectively. Whereas, at a

concentration of 2 μM of FuOH, the cell viability was 90 % (of control). Das et al. (2010) have reported similar result in RAW 264.7 cells where they found fucoxanthinol concentration < 5 μM to be non-toxic to cells and reported significant decrease in cell survival at 10 μM concentration. Whereas, Maeda et al. (2015) have successfully used higher concentrations of FuOH in similar cell systems. Different concentrations (5, 10, 20, 50 μM) of SQ showed cell viability of 85–102 % with 10 μM showing maximum support for cell growth and this concentration was selected to study the combined effect of SQ with the xanthophylls.

Effect of Ax or FuOH in combination with SQ on the cells was also measured. On administering 10 μM SQ along with 5 and 10 μM Ax, cell viability was not significantly affected ($p > 0.01$) as compared to the effect of Ax or SQ alone in the media. Whereas, an increase in cell viability was observed on combining FuOH with SQ (Fig. 2). While 2 μM FuOH showed only a slight increase ($p > 0.05$) in the cell viability when in combination with 10 μM SQ, this effect was distinctly higher ($p < 0.01$) in the case of 5 μM and 10 μM of FuOH (approximately 4 and 2 fold increase respectively). This is an interesting result indicating that SQ has the potential to nullify the detrimental effect of higher concentrations of FuOH on the cells. Based on the results, 2, 5 and 10 μM of FuOH, Ax and

Fig. 3 Effect of astaxanthin (Ax), fucoxanthinol (FuOH) and squalene (SQ) - alone and in combination - on lipid hydroperoxide levels. Columns sharing a common alphabet are not significantly different ($p > 0.01$). ($n = 8$). [Control = Treated with LPS, 0.1 μg/ml media; Concentration of Ax, FuOH, SQ is in μM]

SQ, respectively, were employed to further study their effect on lipid peroxidation.

RAW 264.7 cells were stimulated by LPS (0.1 µg/ml, 6 h) and the lipid hydroperoxide levels (as DPPP in nmol/g lipid) on treatment with Ax, FuOH and SQ were measured. The DPPP level as a result of exposure of the cells to 0.1 µg/ml LPS for 6 h (control) was 49.1 nmol/g lipid (Fig. 3). Treatment with 10 µM SQ resulted in no change ($p > 0.05$) in the DPPP levels. Similar to our results, Moreno (2003) have reported that SQ did not exert any protective effect against oxidative stress in RAW 264.7 cells. However, Cardeno et al. (2015) have reported SQ to be a potent antioxidant in murine macrophages. Unlike SQ, Ax and FuOH exerted a strong ($p < 0.01$) suppressive effect as indicated by the decreased DPPP levels (37.2 and 39.9 nmol/g lipid, respectively). Li et al. (2013) and Nakajima et al. (2008) have reported decrease in H₂O₂ oxidative stress by Ax in ARPE-19 and RCG-5 cells respectively. Kim et al. (2009) have reported suppression of lipid peroxidation by Ax in proximal tubular epithelial cells. Heo et al. (2008) have reported the restorative and protective effects of fucoxanthin against H₂O₂ induced oxidative stress and associated damage. Ax and fucoxanthin (and its metabolites including FuOH) are known to exert antioxidant effect by scavenging superoxide anion and reducing active intermediates as well as up-regulating the activity of antioxidant enzymes in addition to inhibiting molecules such as NF-κB, NO, COX-2 (Augusti et al. 2008; Kim et al. 2009, Peng et al. 2011).

However, in combination with SQ, the DPPP levels were markedly decreased ($p < 0.01$) only in the case of Ax (>60 %) Previously, SQ and PUFA in combination were reported to suppress lipid peroxidation in rats (Dhandapani et al. 2007). In another study, 12.5 µM of Ax, EPA and DHA demonstrated synergistic antioxidant effect in HepG2-C8 cells (Saw et al. 2013). In the present study, although combination of SQ and FuOH resulted in decrease in lipid hydroperoxides (16 %), this was smaller compared to Ax, FuOH and Ax + SQ which exhibited greater antioxidant potential (24, 18 and 62 % compared to control). Hence, between the two xanthophylls, Ax was more potent (18.6 nmol/g lipid) as compared to FuOH (41.1 nmol/g lipid), in presence of SQ. The underlying mechanism for this is likely to involve regulation of molecules such as NF-κB, ERK, MAPK, iNOS, Cox-2, P13/Akt, as both Ax and SQ have been reported to exert antioxidant and anti-inflammatory effects by attenuating these (Kim et al. 2009, Li et al. 2013, Cardeno et al. 2015) and possibly other down-stream factors. Further studies are warranted to identify the underlying mechanisms for the synergistic antioxidant effect of Ax and SQ.

To conclude, Ax (5 µM), FuOH (2 µM) and SQ (10 µM) supported the growth of cells in media. In addition, when combined with SQ, viability of cells improved with higher concentrations of FuOH, demonstrating the ability of SQ to attenuate the toxic effects of higher concentration of FuOH. The results

thus also emphasize on the importance of controlled intake/dosage of antioxidants for desired beneficial results. Ax and FuOH suppressed lipid peroxidation. Additionally, combination of Ax or FuOH with SQ resulted in decreased lipid hydroperoxides and this effect was distinctly higher in the former. The combination of Ax and SQ hence resulted in marked decrease in lipid peroxidation. Therefore, the combination of squalene with the marine xanthophylls, Ax and FuOH, resulted in improved cell viability and decreased lipid hydroperoxides.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

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