

Modulatory effects of an algal extract containing astaxanthin on UVA-irradiated cells in culture

Nicole M. Lyons, Nora M. O'Brien *

Department of Food Science, Food Technology and Nutrition, University College Cork, Cork, Ireland

Received 11 March 2002; received in revised form 27 May 2002; accepted 29 May 2002

Abstract

UV radiation from sunlight is the most potent environmental risk factor in skin cancer pathogenesis. In the present study the ability of an algal extract to protect against UVA-induced DNA alterations was examined in human skin fibroblasts (1BR-3), human melanocytes (HEMAc) and human intestinal CaCo-2 cells. The protective effects of the proprietary algal extract, which contained a high level of the carotenoid astaxanthin, were compared with synthetic astaxanthin. DNA damage was assessed using the single cell gel electrophoresis or comet assay. In 1BR-3 cells, synthetic astaxanthin prevented UVA-induced DNA damage at all concentrations (10 nM, 100 nM, 10 μ M) tested. In addition, the synthetic carotenoid also prevented DNA damage in both the HEMAc and CaCo-2 cells. The algal extract displayed protection against UVA-induced DNA damage when the equivalent of 10 μ M astaxanthin was added to all three-cell types, however, at the lower concentrations (10 and 100 nM) no significant protection was evident. There was a 4.6-fold increase in astaxanthin content of CaCo-2 cells exposed to the synthetic compound and a 2.5-fold increase in cells exposed to algal extract. In 1BR-3 cells, exposure to UVA for 2 h resulted in a significant induction of cellular superoxide dismutase (SOD) activity, coupled with a marked decrease in cellular glutathione (GSH) content. However pre-incubation (18 h) with 10 μ M of either the synthetic astaxanthin or the algal extract prevented UVA-induced alterations in SOD activity and GSH content. Similarly, in CaCo-2 cells a significant depletion of GSH was observed following UVA-irradiation which was prevented by simultaneously incubating with 10 μ M of either synthetic astaxanthin or the algal extract. SOD activity was unchanged following UVA exposure in the intestinal cell line. This work suggests a role for the algal extract as a potentially beneficial antioxidant. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Algal extract; Astaxanthin; UVA radiation; Comet assay; Cellular antioxidant status

1. Introduction

Skin cancers are among the most common human cancers. Indeed, in the United States, reported cases of skin cancer exceed all other human cancers combined [1]. Furthermore, skin cancer incidence is increasing. An etiological link

* Corresponding author. Tel.: +353-21-490-2884; fax: +353-21-427-0244

E-mail address: nob@ucc.ie (N.M. O'Brien).

between skin cancer development (basal cell carcinoma, squamous carcinoma or malignant melanoma) and UV radiation has been firmly established [2,3]. Over exposure to UV radiation from sunlight is currently believed to be the primary causative agent in skin tumour pathogenesis. Dermal photo-ageing is accompanied by wrinkling, loss of elasticity, increased fragility and slow wound healing [4]. Radiation from the UV spectrum encompasses UVA (320–400 nm), UVB (280–320 nm) and UVC (100–290 nm). However, as UVC is, for the most part, filtered out by atmospheric ozone, both UVA and UVB radiation play a more significant role in the initiation of photo-carcinogenesis [4].

UVA and UVB radiation have previously been shown to induce mutations, some of which may degenerate into malignant transformations [5,6]. At a molecular level, UVA and UVB differ in their sites of action in the generation of pre-mutagenic lesions. UVB radiation is site specific and is absorbed directly by cellular DNA. The most frequent photo-lesions resulting from UVB-induced DNA alterations are cyclobutane pyrimidine dimers (CPDs) capable of interfering with DNA replication. CPDs have been shown to be instrumental in photo-induced melanogenesis and immunosuppression [7,8]. However these can be removed by several repair mechanisms including excision repair. Conversely, UVA radiation does not attack the DNA directly but is absorbed by intracellular chromophores such as riboflavin and membrane bound enzymes. This results in an altered cellular redox potential via the generation of reactive oxygen species (ROS) and/or nitric oxide causing photosensitization. Two types of reaction may occur, ROS may: (a) react directly with the DNA via the Fenton reaction generating superoxide (O_2^-) or the hydroxyl radical (OH^\bullet) leading to the development of single strand breaks or (b) induce oxidised base formation in DNA via singlet oxygen (1O_2) production [9].

UVB radiation accounts for $\leq 5\%$ of total UV radiation reaching the earth's surface. Of this only 10% UVB can penetrate the dermal layers of the skin. UVB is much more damaging to skin than UVA if equal exposures are carried out, however the greater ubiquity of UVA coupled with its

ability to penetrate the dermis more deeply would implicate UVA as the major contributory factor in photocarcinogenesis [4]. Also, because UVB only penetrates the epidermis, it is unlikely to be responsible for the more serious melanotic mutations which gives rise to malignant melanomas in the dermis of the skin. Therefore, in this study we employed only UVA radiation.

It is now believed that antioxidants may play a crucial role in ameliorating or indeed preventing photobiologic damage (phototoxicity, photoageing and cancers) *in vivo*. Previous studies have focused on α -tocopherol and β -carotene as potential photo-protective agents [10,11]. However, despite the fact that β -carotene only accounts for 10–15% of the total plasma carotenoid pool [12], the function of other carotenoids in the prevention of skin erythema is sparsely documented.

Astaxanthin (3'3 dihydroxy-4'4' diketo- β -carotene; [AST]) is a red pigment found in marine fish, algae and crustaceans. It is a lipophilic xanthophyll carotenoid, which is structurally similar to β -carotene but possesses an additional hydroxyl and ketone group on each β -ionone ring. AST is reported to be more effective than β -carotene in preventing lipid peroxidation in solution and various biomembrane systems such as egg yolk phosphatidylcholine liposomes and rat liver microsomes [13–16]. To gain a better understanding of why the antiperoxidation effect of AST is greater than that of β -carotene Goto et al. [17] examined the effects of these two carotenoids on ADP/ Fe^{2+} induced lipid peroxidation of liposomes under various conditions. The authors demonstrated, for the first time, that AST trapped radicals not only at the conjugated polyene chain but also in the terminal ring moiety in which the hydrogen atom at the C3 methine was suggested to be the radical trapping site. Other versatile radical scavengers such as flavonoids and alkaloids also have an active methine moiety [18,19]. Naguib [20] reported that AST exhibited the highest antioxidant activity toward peroxy radicals compared to a range of other carotenoids assessed using novel fluorometric assays. The relative reactivities of AST, α -carotene, lutein, β -carotene and lycopene were determined to be 1.3, 0.5, 0.4, 0.2 and 0.4 respectively. Earlier work from our laboratory

indicated that AST was very effective in protecting primary chicken embryo fibroblasts against paraquat-induced, and rat kidney fibroblasts against UVA-induced, alterations in cellular antioxidant enzymes [21,22]. AST accumulation by green microalgae is a natural phenomenon known as 'red snows' or 'blood rains'. Under conditions of high stress *Haematococcus Pulvaris* synthesise AST via the oxidation, or the cytochrome P 450 mediated hydroxylation, of β -carotene [23].

The aim of the present study was to evaluate the potential of a natural algal extract containing AST to protect against UVA induced genotoxicity in three different cell lines. Human skin fibroblasts (1BR-3) and the human epidermal melanocytes (HEMAc) were exposed to a fixed dose of UVA radiation for a defined time period in either the presence or absence of synthetic AST or the algal extract. Synthetic AST was obtained from the Sigma Chemical Company and used as a positive control throughout the study. The majority of less serious cancers such as basal cell and squamous carcinomas occur in the keratinocytes of the epidermis [24]. However, in this study we were more interested in the possible photo-protective effects of the algal extract following deeper UV penetration, into the dermis. Therefore 1BR-3 cells and the HEMAc cells were chosen as a more representative model. The ability of the test compounds to protect at an intestinal level was also determined using the human colonic adenocarcinoma (CaCo-2) cell line as a model. Following exposure to UVA radiation, DNA damage (single strand breaks, SSBs) was measured using the single cell gel electrophoresis assay. To determine if the algal extract conferred a protective effect on cellular antioxidant status, superoxide dismutase (SOD) activity and glutathione (GSH) content were estimated in 1BR-3 and CaCo-2 cells following UVA-irradiation of supplemented and unsupplemented cells.

2. Materials and methods

2.1. Materials

Tissue culture reagents and chemicals were purchased from Sigma-Aldrich Ireland, (Dublin,

Ireland) unless stated otherwise. Cell culture plastics were supplied by Costar (Cambridge, MA). The CaCo-2 and 1BR-3 cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) while the HEMAc cell line was purchased from TCS Cell Works (Buckinghamshire, UK). Information on the purity of the synthetic AST (purity > 98%), was supplied by Sigma-Aldrich. The algal extract product (proprietary algal extract, AST-101) was supplied by SPI- Swiss Pharmaceutical Industries SA, Switzerland and contained a minimum of 14% AST. Throughout the rest of the manuscript these compounds will be referred to as synthetic (Sigma-Aldrich) or algal (AST-101) AST. The carrier vehicle for compound delivery to the cells was either di-methyl sulfoxide (DMSO) or methyl ethyl ketone (MEK) depending on the concentration of AST used. The final concentration of solvent in the culture medium did not exceed 1% (v/v).

2.2. Cell maintenance

Cell lines were grown in a humidified atmosphere of 5% CO₂:95% air, at 37 °C in the absence of antibiotics and screened routinely for mycoplasma using the Hoechst staining method previously described [25]. CaCo-2 and 1BR-3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% (v/v) non-essential amino acids (NEAA), 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamine (w/v) and were grown for 4 and 8 days respectively, until approximately 80% confluence was achieved. HEMAc cells were grown in epidermal melanocyte basal medium containing 1% (v/v) growth supplement for a minimum of 8–10 days until 60–80% confluent.

2.3. Cell treatments

CaCo-2 and 1BR-3 cells were seeded at a density of 2×10^4 cells/cm² and HEMAc cells were seeded at 5×10^4 cells/cm² in 6 well dishes for all experimental conditions. The seeding density of HEMAc cells was increased because of the low plating density of this cell line. It was necessary to use different experimental conditions for the

intestinal (CaCo-2) and epithelial (skin) cell lines due to their different population doubling times and growth requirements. CaCo-2 cells were incubated with 10 μM (determined spectrophotometrically at 468 nm) synthetic or algal AST and immediately irradiated. 1BR-3 and HEMAc cells were incubated with media containing 10 nM, 100 nM or 10 μM (synthetic or algal) AST for 18 h prior to UVA exposure. AST (synthetic and algal) was delivered to the cells in DMSO, with the exception of the higher concentration of 10 μM where it was necessary to use MEK to increase the solubility of these compounds. All AST manipulations and extractions were performed in an amber-lit laboratory, glass vessels were blown through with $\text{N}_2(\text{g})$ before use, and samples were handled under $\text{N}_2(\text{g})$.

2.4. Preparation of cells for the comet assay and exposure to UVA radiation

Following treatment with or without the synthetic or algal AST, cells were prepared for the alkaline single cell gel electrophoresis (comet) assay according to the method of Woods et al. [26]. Briefly, 1% normal gelling agar was smeared onto frosted slides and allowed to dry. A single cell suspension was created for each cell line. Cells (30 μl) were then mixed with low melting point agarose (70 μl) and embedded in the centre layer of an agar gel sandwich. The microgel was covered with a coverslip and cooled on ice for 5 min. Immediately prior to irradiation the coverslips were removed and slides were placed in irradiation dishes containing 10 ml pre-warmed PBS. The dishes were then anchored in a water bath at a fixed temperature of 37 °C. Cells were exposed to polychromatic light from a 2 tube fluorescent sunlamp that emitted UV radiation of low fluences between the wavelengths of 320–400 nm. A UVX radiometer was used to determine the irradiance and this was fixed at 5.6 mW/cm^2 . Slides were irradiated through glass filters in 10 ml PBS at 37 °C. The glass filter was 4.5 mm thick with a diameter of 37 mm which was sufficient to completely cover all slides. A thickness of ≥ 3 mm has previously been determined to be sufficient to block 99.8% total UVB. Cell cytotoxicity

was examined following exposure to UVA using the neutral red uptake assay (NRUA). Control slides were 'sham irradiated'. Immediately after irradiation slides were immersed in lysis buffer for 1.5 h in the dark at 4 °C, followed by electrophoresis buffer for 30 min and then subjected to electrophoresis at 25 V/cm^2 (300 mA) for 25 min. Slides were removed, washed with neutralisation buffer ($\times 3$) and stained with 70 μl ethidium bromide (0.5 mg/ml). Slides were analysed using fluorescence microscopy (green fluorescent light from a Nikon microscope-100 \times magnification). For each experiment randomly selected comets (25) were scored with duplicate slides per condition. Slides were viewed using a KOMET compatible version of Windows NT, and analysis of genotoxic damage was conducted using KOMET 4.0 Image Analysis system (Kinetic Imaging, Liverpool, UK). The arbitrary units of measurement chosen in this study were tail DNA (the quantity of DNA present in the tail) and the olive tail moment (tail length \times tail intensity or percentage migrated DNA).

2.5. Evaluation of cellular antioxidant status

To assess effects on cellular GSH content and SOD activity, CaCo-2 and 1BR-3 cells were incubated for 2 and 18 h, respectively, with AST (synthetic or algal) prior to UVA irradiation. The activity of total cellular SOD was determined using the method previously outlined [27]. SOD was expressed relative to protein content estimated by the Lowry method for protein assessment [28]. The cellular GSH content was measured according to the method of Hissin and Hilf [29]. GSH content was expressed as nmol/mg protein.

2.6. HPLC analysis

Uptake of AST by CaCo-2 cells was measured using a modification of the method of Nells and De Leenheer [30]. Briefly, cells were exposed to either 10 μM synthetic or algal AST for 24 h prior to extraction. Cells (7.2×10^6) were washed ($\times 3$) and harvested by centrifugation. A recovery standard (25 mM α -tocopherol acetate in ethanol,

0.05% butylated hydroxytoluene) was added to the cell pellets, and the samples were extracted twice in ethyl acetate followed by a final hexane extraction. Individual fractions were pooled and dried under N₂, reconstituted in mobile phase (dichloromethane:acetonitrile:methanol [1:7:2]) and analysed by HPLC. Samples were injected onto a Shimadzu SCL-10A model HPLC, (equipped with two 150 mm × 4.6 mm C18 columns) and were eluted with mobile phase. The retention time for AST was 3.1 min and peaks were detected at 450 nm using a Shimadzu SPD-10AV UV-visible detector. Results were collected and analysed using Millennium Chromatography Manager data collection software (Waters Corporation, Milford, MA).

2.7. Statistical analysis

Where appropriate, data were analysed using one way analysis of variance (ANOVA) followed by Dunnett's test. Unless otherwise stated, results are presented as the mean ± standard error (S.E.) of duplicate cultures from 3 independent experiments.

3. Results

3.1. UVA-induced DNA damage in three cell lines

UVA irradiation of CaCo-2 and 1BR-3 cells for 2 h and HEMAc cells for 45 min, resulted in a significant increase in DNA damage in all three cell lines (Figs. 1–3).

3.1.1. 1BR-3 cells

1BR-3 cells were pre-treated for 18 h with increasing concentrations (10 nM, 100 nM, 10 μM) of either the synthetic (Fig. 1A) or algal AST (Fig. 1B) prior to irradiation with UVA. Samples were processed onto slides in preparation for the comet assay and subsequently irradiated for 2 h at 37 °C. A highly significant ($P < 0.01$) increase in olive tail moment (an arbitrary measure of single strand breaks) was observed in cells exposed to UVA only. However pre-enrichment with synthetic AST maintained the olive tail moment

similar to untreated control values at all concentrations (Fig. 1A). When cells were pre-treated with the algal AST and subsequently exposed to UVA light, both the 10 and 100 nM concentrations failed to protect against an increase in olive tail moment with values differing significantly ($P < 0.01$, $P < 0.05$, respectively), from untreated controls. Only the 10 μM concentration of algal AST was capable of maintaining olive tail moment analogous to untreated control values and thus, significantly inhibiting UVA-induced DNA damage in 1BR-3 cells (Fig. 1B).

3.1.2. CaCo-2 cells

Following a 2-h exposure to UVA radiation, a highly significant ($P < 0.01$) increase in CaCo-2 cell DNA olive tail moment was observed relative to untreated controls (Fig. 2). However, when cells were incubated immediately prior to irradiation with either synthetic or algal AST (10 μM) and subsequently exposed to UVA light, the olive tail moment decreased significantly and did not differ from controls.

3.1.3. HEMAc cells

HEMAc cells were pre-enriched for 18 h with both the synthetic and algal AST at 10 μM (Fig. 3). Cells were irradiated with UVA light for a shorter time-scale of 45 min. As has been reported previously [31], human melanocytes were found to display very heterogeneous comets with differing frequency, intensity and shape even within the same sample. Fig. 3 shows a significant ($P < 0.05$) increase in Tail DNA in cells exposed to UVA only, relative to untreated control cells. Treatment with both the synthetic and algal AST (10 μM) significantly inhibited UVA-induced DNA migration from head to tail.

3.2. Modulation of cellular antioxidant status by UVA radiation-protection by the test compounds

Cellular GSH content and SOD activity were assessed following exposure to UVA both in the absence and presence of synthetic or algal AST. Two different experimental approaches were used. Firstly, 1BR-3 cells were pre-enriched with the either synthetic or algal AST for 18 h prior to

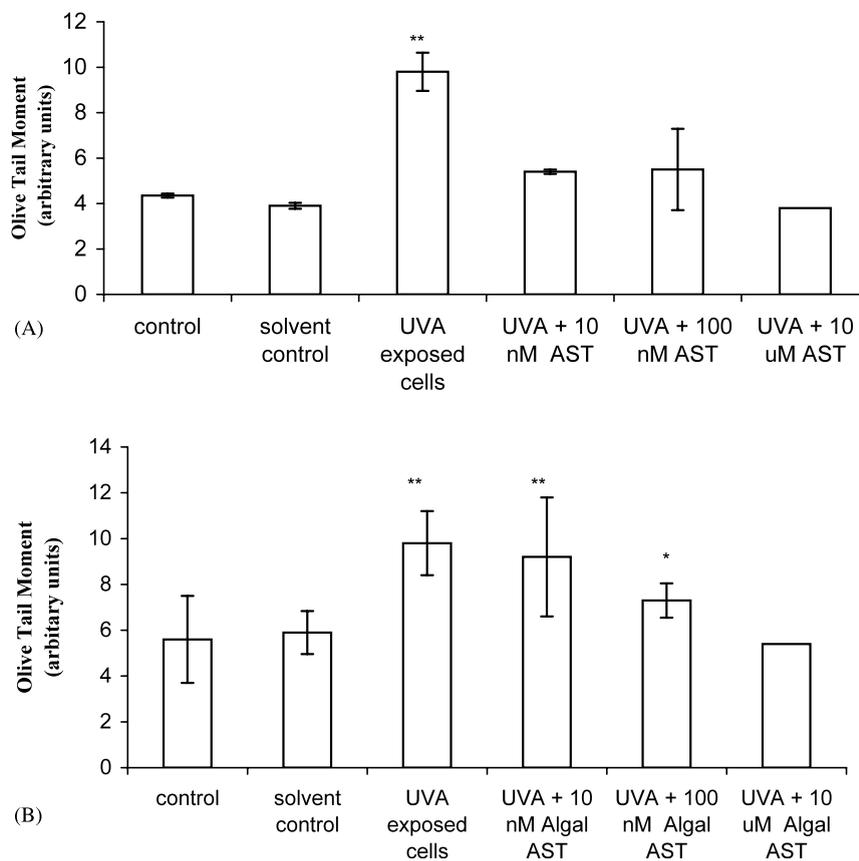


Fig. 1. Ability of synthetic astaxanthin (AST) (A) and an algal extract containing astaxanthin (Algal AST) (B) to protect against UVA-induced DNA damage in 1BR-3 cells. Cells were incubated with 10 nM, 100 nM or 10 μ M synthetic AST or algal extract containing AST for 18 h and subsequently irradiated with UVA for 2 h. DNA damage was assessed using the alkaline single cell gel electrophoresis or comet assay and the results were expressed as olive tail moment. Control cells represent unexposed, untreated cells, while solvent controls represent unexposed cells treated with the solvent carrier vehicle [di-methyl sulfoxide (DMSO) or methyl ethyl ketone (MEK)]. Data are means \pm S.E. of duplicate cultures from $n = 3$ independent experiments. Values differed significantly (** $P < 0.01$; * $P < 0.05$) from untreated controls as measured by ANOVA followed by Dunnett's test.

photo-treatment. The medium was then replaced with fresh medium and cells were exposed to UVA radiation for 2 h at 37 °C. In the second model, CaCo-2 cells were incubated with either synthetic or algal AST and immediately exposed for 2 h to UVA light. Exposure of 1BR-3 cells to UVA light resulted in a highly significant ($P < 0.01$) induction of SOD activity, coupled with a significant ($P < 0.05$) decline in GSH content, compared to untreated controls. However, when cells were pre-treated (18 h) with either the synthetic or algal

forms (10 μ M) of AST and subsequently irradiated, SOD activity returned to control values. GSH content was also maintained similar to untreated control value in AST enriched samples (Table 1). SOD activity in CaCo-2 cells was unaffected by exposure to UVA radiation in the presence or absence of either form of AST with none of the samples differing significantly from untreated controls (Table 2). However a significant ($P < 0.05$) decline in GSH content was observed following UVA-exposure. GSH deple-

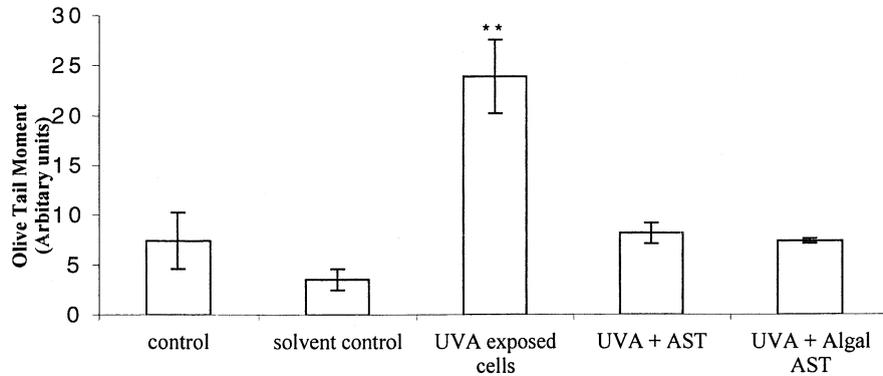


Fig. 2. Ability of synthetic astaxanthin (AST) and an algal extract containing astaxanthin (Algal AST) to protect against UVA-induced DNA damage in CaCo-2 cells. CaCo-2 cells were incubated with 10 μM synthetic AST or an algal extract containing AST and irradiated with 22 J/cm^2 UVA for 2 h. DNA damage was measured using the alkaline single cell gel electrophoresis or comet assay. Control cells represent unexposed, untreated cells, while solvent controls represent unexposed cells treated with the solvent carrier vehicle (methyl ethyl ketone) only. Data are means \pm S.E. of duplicate cultures from $n = 3$ independent experiments. Values differed significantly (** $P < 0.01$) from untreated controls as measured by ANOVA followed by Dunnett's test.

tion was prevented in synthetic AST treated samples. A similar trend was observed in algal treated samples (Table 2).

3.3. Cellular incorporation of synthetic and algal AST by CaCo-2 cells

CaCo-2 cells were incubated with synthetic or algal AST for 24 h and the carotenoid fraction was subsequently extracted to determine cellular uptake. There was a significant ($P < 0.05$) incorporation of AST in cells exposed to the synthetic

compound, with a 4.6-fold increase in the carotenoid content relative to untreated control cells (Table 3). CaCo-2 cells exposed to algal AST were also shown to accumulate the carotenoid but here the fold increase was 2.5 relative to controls (Table 3).

4. Discussion

The induction of cellular phototoxicity in response to UVA radiation has previously been

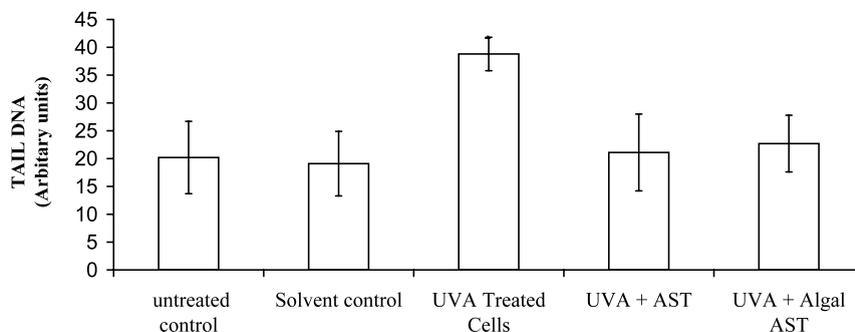


Fig. 3. Ability of synthetic astaxanthin (AST) and an algal extract containing astaxanthin (Algal AST) to protect against UVA-induced DNA damage in HEMAc cells. HEMAc cells were pre-enriched with 10 μM synthetic AST or an algal extract containing AST for 18 h. Samples were subsequently irradiated with 22 J/cm^2 UVA for 45 mins. Control cells represent unexposed, untreated cells, while solvent controls represent unexposed cells treated with the solvent carrier vehicle (methyl ethyl ketone) only. Data are means \pm S.E. of duplicate cultures from $n = 3$ independent experiments. Values differed significantly (* $P < 0.05$) from untreated controls as measured by ANOVA followed by Dunnett's test.

Table 1

Superoxide dismutase (SOD) activity and glutathione (GSH) concentration in UVA-exposed 1BR-3 cells pre-treated for 18 h with synthetic astaxanthin (AST: 10 μ M) or an algal extract containing astaxanthin (algal AST: 10 μ M)

Treatment	SOD activity		GSH content nmols/mg protein
	U/mg protein		
Untreated cells	6.15 \pm 2.2	37.39 \pm 1.9	
Solvent control	4.3 \pm 3.5	43.3 \pm 7.1	
UVA Treated	18.4 \pm 4.2**	21.1 \pm 4.9*	
UVA + AST	2.8 \pm 0.8	38.3 \pm 0.3	
UVA + Algal AST	7.4 \pm 2.4	33.4 \pm 4.5	

1BR3 cells were pre-enriched with 10 μ M of either of the test compounds for 18 h. Samples were subsequently irradiated for 2 h with 22 J/cm² UVA. Untreated cells were not exposed to UVA or either of the test compounds. Solvent controls represent cells exposed to the carrier vehicle (methyl ethyl ketone) only. UVA treated cells were exposed to UVA radiation only. Data are the means \pm S.E. of duplicate cultures from $n = 3$ independent observations.

** Samples were determined to be highly ($P < 0.01$) significantly different, or;

* Significantly ($P < 0.05$) different from untreated control values as measured by ANOVA followed by Dunnett's test.

Table 2

Superoxide dismutase (SOD) activity and glutathione (GSH) concentration in UVA-exposed CaCo-2 cells treated with synthetic astaxanthin (AST: 10 μ M) or an algal extract containing astaxanthin (algal AST: 10 μ M)

Treatment	SOD activity		GSH content nmols/mg protein
	U/mg protein		
Untreated cells	2.0 \pm 0.5	32.1 \pm 1.1	
Solvent control	2.8 \pm 0.85	34.1 \pm 1.1	
UVA Treated	2.1 \pm 0.5	24.9 \pm 0.9*	
UVA + AST	2.37 \pm 0.5	33.0 \pm 0.8	
UVA + Algal AST	1.95 \pm 0.2	27.6 \pm 1.4	

CaCo-2 cells were incubated with 10 μ M of either of the test compounds and immediately irradiated for 2 h with 22 J/cm² UVA. Untreated cells were not exposed to UVA or either of the test compounds. Solvent controls represent cells exposed to the carrier vehicle (methyl ethyl ketone) only. UVA treated cells were exposed to UVA radiation only. Data are the means \pm S.E. of duplicate cultures from $n = 3$ independent observations.

* Samples were significantly ($P < 0.05$) different from untreated control values as measured by ANOVA followed by Dunnett's test.

Table 3

Concentration of astaxanthin in CaCo-2 cells following supplementation for 24 h with the synthetic carotenoid (AST) or algal extract containing astaxanthin (Algal AST)

Treatment	Astaxanthin g/mg protein	
	Mean	S.E.
Untreated cells	5.44 $\times 10^{-7}$	0.2 $\times 10^{-7}$
Media only	2.73 $\times 10^{-7}$	0.03 $\times 10^{-7}$
Cells + AST	2.36 $\times 10^{-6}$ *	0.24 $\times 10^{-6}$ *
Cells + Algal AST	1.44 $\times 10^{-6}$ *	0.23 $\times 10^{-6}$ *

CaCo-2 cells were supplemented with either synthetic AST or the algal extract containing astaxanthin (algal AST) for 24 h. The carotenoid fraction was extracted and cellular incorporation of AST was measured using HPLC. Untreated media was also extracted to determine baseline carotenoid level. Data are means \pm S.E. of triplicate samples.

* Samples differed significantly ($P < 0.05$) from untreated control cells.

demonstrated in a variety of cell lines including the dermal layers of human skin [6,32,33]. Human skin fibroblasts and human melanocytes are particularly susceptible to UVA-induced cellular alterations, including the distortion of cellular antioxidant status and the induction of strand breaks within the DNA molecule. These physiological alterations are believed to occur as a result of altered cellular redox potential via the production of ROS during photosensitization [34]. Antioxidants are well established for their ability to quench ROS. However the role of antioxidants in UV-induced photo-protection is complex and evidence for a protective effect conferred by carotenoids, in particular, against UVA-induced cellular alterations is conflicting in the literature.

In the present study we examined the ability of a natural algal extract containing AST to protect against UVA-induced DNA damage in three cell lines (CaCo-2, 1BR-3 and HEMAc cells) using the comet assay. The comet assay is a sensitive, reliable and effective method to detect single strand breaks within the DNA molecule [35]. In the assay, irradiated cells are embedded in agarose gel and lysed by detergents at high salt concentration. Subsequently, the microgel is placed in an alkaline electrophoresis buffer for DNA unwinding. The presence of strand breaks within the highly supercoiled DNA increases the electro-

phoretic mobility of the DNA fragments. During electrophoresis these single strand DNA fragments migrate towards the anode so the cells resemble a comet composing of a bright fluorescent head with a tail streaming away from it [26,36].

CaCo-2 cells were used in conjunction with skin cells to investigate the toxic effects of UVA radiation, the possible protective effects conferred by AST, and also to examine the efficacy with which the two different forms of AST are incorporated into human cells. The premise for choosing the CaCo-2 cell line was two-pronged. Firstly, preliminary work in our laboratory identified similar cytotoxic effects occurring in CaCo-2 cells relative to human skin fibroblast (1BR-3) cells. Because CaCo-2 cells are a finite cell line they are significantly less troublesome to work with and thus a greater number of experiments could be performed. Secondly, the CaCo-2 cell line is a well-established model for examining the cytotoxicity of various toxic stimuli. Because this cell line represents the best in vitro model of the intestinal mucosa, CaCo-2 cells are ideal for providing information on the uptake of AST by the 'gut'.

In all three cell lines, irradiation with a physiologically relevant dose (22 J/cm^2) of UVA, resulted in a time dependant increase in single strand breaks (data not shown) with the order of damage equivalent to HEMAc > 1BR3 > CaCo-2, respectively. Synthetic AST (99.5% purity) was included as a positive control and it was observed that while this form offered significant protection at all concentrations chosen, only the highest concentration of the algal AST could significantly reduce the induction of single strand breaks in 1BR-3 and HEMAc cells. Similar results were seen in the CaCo-2 cell model.

Human fibroblasts are known to contain millimolar and nanomolar concentrations of GSH and SOD respectively [4]. UV radiation has previously been shown to reduce cellular GSH content in human fibroblasts and SOD activity in rat kidney fibroblasts [22,37]. In the present study we examined the effect of UVA radiation on cellular antioxidant status (SOD and GSH) in CaCo-2 and 1BR-3 cells in both the presence and absence of algal AST. While SOD activity was unchanged

in CaCo-2 cells exposed to UVA, a significant reduction in GSH content was observed in irradiated cells (Table 2). However by adding either synthetic or algal AST ($10 \mu\text{M}$), depletion of cellular GSH was prevented with irradiated samples expressing similar GSH values to untreated controls (Table 2). Similar results were obtained in 1BR-3 cells, however the effect on antioxidant status was more marked with a significant ($P < 0.05$) reduction in GSH content in UVA-exposed cells. This was accompanied by a highly significant ($P < 0.01$) enhancement of SOD activity following UVA-irradiation.

The mechanism of UVA-induced chromophore excitation is unclear but is believed to involve the generation of various ROS possibly including superoxide anion. Indeed, in vitro photosensitization is non-specific and therefore there may be several possible sites of chromophore excitation. For example the DNA itself may become 'excited' at wavelengths $\leq 320 \text{ nm}$ resulting in the formation of thymine photo-dimers [38]. However more usually UVA photons do not attack the DNA directly but are absorbed by intracellular components such as riboflavin, porphyrins, nicotinamide and certain membrane bound enzymes [39]. Chromophore photosensitization in skin cells may also result in the activation of a wide range of prostaglandins and histamine resulting in an inflammatory response [40] and the photo-degradation of tryptophan [41].

Superoxide anion (O_2^-) is highly reactive and may attack cellular DNA resulting in altered DNA bases, pyrimidine dimers and single strand breaks. SOD, a primary antioxidant, is specific for superoxide anion. It is present at a basal level in all cells however its activity increases in response to increased superoxide production (oxidative stress). SOD activity has previously been reported to increase following irradiation with UVA [42,43]. In the present study the UVA-induced induction of SOD activity did not occur in AST treated 1BR-3 cells (Table 1). AST is a very efficient antioxidant due to the unique structure of the terminal ring moiety [17]. It is therefore feasible that AST has an affinity for the superoxide free radical and thus may act as a sacrificial antioxidant, ultimately preventing an increase in basal SOD activity.

Indeed, induction of cellular SOD following UVA exposure has previously been shown to be inhibited by quercetin [44], however the mechanism involved remains to be elucidated. The ability of both the synthetic and algal AST to protect against GSH depletion and the induction of SOD activity was more notable in 1BR-3 cells (Table 1).

Our results corroborate with previous observations citing a decrease in cellular GSH in parallel with an increase in cellular antioxidant enzyme activity as a consequence of UVA exposure. In vivo, a decrease in dermal GSH was observed in rats treated with 5 J/cm² UVA, with maximal GSH depletion occurring between 24 and 48 h post irradiation [45]. A substantial up-regulation of glutathione peroxidase in conjunction with a concomitant induction of SOD activity following exposure of human skin fibroblasts with repeated low doses of UVA radiation has also been reported [43]. Similarly, an up-regulation of SOD and glutathione peroxidase at mRNA level was observed in human fibroblasts following exposure to low-moderate physiologic doses of radiation [46]. Both authors suggest the up-regulation of cellular antioxidant defence enzymes to be associated with enhanced photo-protection in the event of further UVA exposure.

In conclusion, we have shown a protective effect conferred by an algal extract containing AST in the reduction of DNA damage and maintenance of cellular antioxidant status in UVA-irradiated human cells in culture. AST may be the active ingredient in the algal extract, however this remains to be confirmed. The lower protective efficiency of the algal extract compared to the synthetic AST may be accounted for by the decreased bioavailability of the carotenoid from the extract (Table 3). It is interesting to speculate that other components of the extract may interfere with uptake of AST. Further studies are required to investigate this possibility. In summary, the potential chemo-therapeutic properties of this algal extract could possibly be exploited for the development of topical skin-care products or indeed as a natural dietary supplement to minimise the effects of UVA radiation in vivo.

Acknowledgements

This work was part-supported by a grant from Enterprise Ireland, Dublin, Republic of Ireland.

References

- [1] Martinez JC, Otley CC. The management of melanoma and nonmelanoma skin cancer: a review for the primary care physician. *Mayo Clin Proc* 2001;76:1253–65.
- [2] Setlow RB, Grist E, Thompson K, Woodhead AD. Wavelengths effective in the induction of malignant melanoma. *Proc Natl Acad Sci USA* 1993;90:6666–70.
- [3] England DR, Armstrong BK, Kricger A, Fleming C. Sunlight and cancer. *Cancer Causes Control* 1997;8:271–83.
- [4] Halliwell B, Gutteridge JMC. *Insults on the skin*. In: *Free Radicals in Biology and Medicine*. New York: Oxford University Press, 1998:530–7.
- [5] Robert C, Muel B, Benoit A, Dubertret L, Sarsin A, Stary A. Cell survival and shuttle vector mutagenesis induced by ultra violet A and ultra violet B radiation in a human cell line. *J Invest Dermatol* 1996;107:721–8.
- [6] De Leeuw SM, Smit NPM, Van Veldhaven M, Pennings EM, Pavel S, Simons JWIM, Chothorst AA. Melanin content of cultured human melanocytes and UV-induced cytotoxicity. *J Photochem Photobiol* 2001;61:106–13.
- [7] Eller MS, Ostrom K, Gilchrest BA. DNA damage enhances melanogenesis. *Proc Natl Acad Sci USA* 1996;93:1087–92.
- [8] Damian DL, Barneston RS, Halliday GM. Effects of low dose ultra violet radiation on in vivo human cutaneous recall responses. *Australas J Dermatol* 2001;42:161–7.
- [9] Brimer LH. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Mol Carcinog* 1990;3:188–97.
- [10] Kondo S, Mamada A, Yamaguchi J, Fukuro S. Protective effect of dl- α -tocopherol on the cytotoxicity of ultraviolet B against human skin fibroblasts in vitro. *Photodermatol Photoimmunol Photomed* 1990;7:173–7.
- [11] Greenberg ER, Baron JA, Stukel TA, Stevens MM, Mandel JS, Spencer SK, Elias PM, Lowe N, Nierenberg DW, Bayrd G, Vance JC, Freeman DH, Clendenning WE, Kwan T. The skin cancer prevention study group. A clinical trial of β -carotene to prevent basal-cell and squamous cell cancers of the skin. *N Engl J Med* 1990;323:789–95.
- [12] Thurnham DI. Carotenoids: functions and fallacies. *Proc Nutr Soc* 1994;53:77–87.
- [13] Terao J. Antioxidant activity of β -carotene related carotenoids in solution. *Lipids* 1989;24:659–61.
- [14] Miki W. Biological functions and activities of animal carotenoids. *Pure Appl Chem* 1991;63:141–6.

- [15] Palozza P, Krinsky NI. Astaxanthin and cantaxanthin are potent antioxidants in a membrane model. *Arch Biochem Biophys* 1992;292:184–7.
- [16] Lim BP, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K. Antioxidant activity of xanthophylls on peroxy radical-mediated phospholipid peroxidation. *Biochem Biophys Acta* 1992;1126:178–84.
- [17] Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E, Terada H. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochem Biophys Acta* 2001;1512:251–8.
- [18] Cizak E, Cody V, Luft J, Kempton RJ, Kesler BS. Flavonoid conformational analysis: comparison of the molecular structures of (Z)-4,4'6-triacetaurone and (Z)-3',5'-dibromo-2',4,4'6-tetrahydroxyaurone monohydrate by crystallographic and molecular orbital methods. *J Mol Std Thechem* 1991;251:345–57.
- [19] Kogure K, Goto S, Abe K, Ohiwa C, Akasu M, Terada H. Potent antiperoxidation activity of the bisbenzylisoquinoline alkaloid cepharanthine: the amine moiety is responsible for its pH-dependent radical scavenge activity. *Biochem Biophys Acta* 1999;1426:133–42.
- [20] Naguib YMA. Antioxidant activities of astaxanthin and related carotenoids. *J Agric Food Chem* 2000;48:1150–4.
- [21] Lawlor SM, O'Brien NM. Astaxanthin: antioxidant effects in chicken embryo fibroblasts. *Nutr Res* 1995;15:1695–704.
- [22] O'Connor I, O'Brien NM. Modulation of UVA-induced oxidative stress by β -carotene, lutein and astaxanthin in cultured fibroblasts. *J Dermatol Sci* 1998;16:226–30.
- [23] Schoefs B, Rmiki N-E, Rachadi J, Lemoine Y. Astaxanthin accumulation in *Haematococcus* requires a cytochrome P450 hydrolase and an active synthesis of fatty acids. *FEBS Letts* 2001;500:125–8.
- [24] Magnus K. The Nordic profile of skin cancer incidence: a comparative epidemiological study of the three main types of skin cancer. *Int J Cancer* 1991;47:12–9.
- [25] Mowles JM. Mycoplasma detection. In: Pollard JW, Walker JM, editors. *Methods in Molecular Biology. Animal Cell Culture*, vol. V. New Jersey: Humana Press, 1990:65–74.
- [26] Woods JA, O'Leary KA, McCarthy RP, O'Brien NM. Preservation of comet assay slides: comparison with fresh slides. *Mutat Res* 1999;429:181–7.
- [27] Misra H, Fridovich I. Superoxide dismutase: a photochemical augmentation assay. *Arch Biochem Biophys* 1977;181:308–12.
- [28] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin–Phenol reagents. *J Biol Chem* 1951;193:267–75.
- [29] Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 1976;74:214–26.
- [30] Nells HJCF, De Leenheer AP. Isocratic nonaqueous reversed-phase liquid chromatography of carotenoids. *Anal Chem* 1983;55:270–5.
- [31] Marrot L, Belaidi JP, Meunier JR, Perez P, Agapakis-Causse C. The human melanocyte as a particular target for UVA radiation and an endpoint for photoprotection assessment. *Photochem Photobiol* 1999;69:686–93.
- [32] Alapetite C, Wachter T, Sage E, Moustacchi E. Use of the alkaline comet assay to detect DNA repair deficiencies in human fibroblasts exposed to UVC, UVB, UVA and gamma rays. *Int J Radiat Biol* 1996;69:359–69.
- [33] Marrot L, Belaidi JP, Chaubo C, Meunier JR, Perez P, Agapakis-Causse C. An in vitro strategy to evaluate the phototoxicity of solar UV at the molecular and cellular level: application to photo-protection assessment. *Eur J Dermatol* 1998;8:403–12.
- [34] Didier C, Pouget J-P, Cadet J, Favier A, Beani J-C, Richard M-J. Modulation of endogenous levels of thiorodoxin in human skin fibroblasts preventing damaging effect of ultraviolet A radiation. *Free Rad Biol Med* 2001;30:537–46.
- [35] Rojas E, Lopez MC, Valerde M. Single cell gel electrophoresis assay: methodology and application. *J Chromatogr B* 1999;722:225–54.
- [36] Lehmann J, Pollet D, Peker S, Steinkrus V, Hoppe U. Kinetics of DNA strand breaks and protection by antioxidants in UVA- or UVB-irradiated HaCaT keratinocytes using the single cell gel electrophoresis assay. *Mutat Res* 1998;407:97–108.
- [37] Schmitz S, Garbe C, Jimbow K, Wulff A, Daniels H, Eberle J, Orfanos CE. Photodynamic action of ultraviolet A: induction of hydroperoxides: recent results. *Cancer Res* 1995;139:43–55.
- [38] Kochevar IE. Action spectrum and mechanisms of UV radiation-induced injury in lupus erythematosus. *J Invest Dermatol* 1985;85:140s–3s.
- [39] Carbonare DM, Pathak MA. Skin photosensitizing agents and the role of reactive oxygen species in photoageing. *J Photochem Photobiol B* 1992;14:105–24.
- [40] Gonzalez S, Pathak MA. Inhibition of ultraviolet-induced formation of reactive oxygen species, lipid peroxidation, erythema and skin photosensitization by polypodium leucotomos. *Photodermatol Photoimmunol Photomed* 1996;12:45–56.
- [41] De La Rochette A, Silva E, Birlouez-Aragon I, Mancini M, Edwards AM, Morliere P. Riboflavin photodegradation and photosensitizing effects are highly dependent on oxygen and ascorbate concentrations. *Photochem Photobiol* 2000;72:815–20.
- [42] Poswig A, Wenk J, Brenneisen P, Wlaschek M, Hommel C, Quel G, Faisst K, Dissemmond J, Briviba K, Kreig T, Scharffetter-Kochanek K. Adaptive antioxidant response of manganese-superoxide dismutase following repetitive UVA irradiation. *J Invest Dermatol* 1999;112:13–8.
- [43] Meewes C, Brenneisen P, Wenk J, Kuhr L, Ma W, Alikoski J, Poswig A, Kreig T, Scharffetter-Kochanek K. Adaptive antioxidant response protects dermal fibroblasts

- from UVA-induced phototoxicity. *Free Rad Biol Med* 2001;30:238–47.
- [44] Erden IM, Kahraman A, Koken T. Beneficial effects of quercetin on oxidative stress induced by ultraviolet A. *Exp Clin Dermatol* 2001;26:536–9.
- [45] Wheeler LA, Aswad A, Connor MJ, Lowe N. Depletion of cutaneous glutathione and the induction of inflammation by 8-methoxypsoralen plus UVA radiation. *J Invest Dermatol* 1986;87:658–62.
- [46] Leccia MT, Yaar M, Allen N, Gleason M, Gilvchrest BA. Solar stimulated irradiation modulates gene expression and activity of antioxidant enzymes in cultured human dermal fibroblasts. *Exp Dermatol* 2001;10:272–9.