

Astaxanthin Co-Crystallized With Dark Chocolate Causes a Dose-dependent Inhibition of Oxidation Markers in Middle-aged Volunteers

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Abstract Oxidative stress and antioxidant deficiency are contributing factors in aging, cardiovascular disease, diabetes and cancer. The effect of highly bioavailable polyphenols of lycosome-formulated dark chocolate (DC) containing co-crystallized astaxanthin (LF-DC-ASTX) on parameters of biological oxidation was investigated in this work. 94 healthy middle-aged volunteers (48 male, 46 female, 45 – 65 years old) were enrolled and randomized into four study groups. The 1st group were instructed to ingest conventional control DC (7.5 g). The 2nd group were asked to ingest capsules containing either 4 mg or 7 mg astaxanthin (ASTX), an algal antioxidant. The 3rd group were given DC bars (7.5 g) and 4 mg ASTX capsules for co-ingestion as two separate formulations. The 4th group were instructed to ingest LF-DC-ASTX containing different amounts of ASTX (1 mg, 2 mg, 4 mg or 7 mg) co-crystallized with 7.5 g of DC matrix. Each product was ingested once daily after breakfast for a period of 1 month. Serum levels of oxidized LDL (ox-LDL) and malonic dialdehyde (MDA) were measured after completion of 2 and 4 weeks of the study. No significant changes were observed in the values for ox-LDL or MDA concentrations in serum irrespective of cocoa content (70%, 72% or 85%) or duration of control DC intake. In contrast, ingestion of different doses of ASTX, as a single formulation, translated into reduction of oxidation markers without any dose-dependency. The inhibition of ox-LDL in volunteers following co-ingestion of DC and ASTX as two separate formulations was far greater than that seen for ASTX alone, revealing some additive effect of DC on lipid oxidation parameters. The inhibition of both oxidative markers was dose-dependent reflecting amount of ASTX in the DC matrix. These results may reflect greater bioavailability and improved pharmacokinetics for cocoa flavanols and ASTX following ingestion of LF-DC-ASTX. Therefore, nutraceutical formulations of DC fortified with ASTX can be successfully used for management of oxidative disorders associated with increased levels of ox-LDL.

Keywords: dark chocolate, astaxanthin, biological oxidation

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1. Introduction

The skin is the largest organ of the human body, responsible for the separation of internal tissues and cells from the external environment as well as sensory functions, temperature balance and certain important metabolic functions [1,2]. The constant interaction of the skin with environmental factors (sunlight, humidity, airflow, pollutants and temperature) predetermines the functional characteristics of the cells forming its external lining and their ability to withstand environmental challenges and undergo age-related transformation. Various intrinsic aspects (innate immunity, microcirculation, hormonal balance and intermediate

metabolism) form another decisive set of factors which predetermine the outcomes of skin interaction with environmental effects [3,4,5,6]. A growing body of recent scientific evidence suggests that free radical formation is a major axis mediating the interplay between environment and homeostasis responsible for skin function and aging [7,8]. Excessive free radical formation in the skin and subsequent oxidative stress are known to be associated with wrinkle formation and abnormal skin hydration and may lead to various skin diseases and cancer [9,10]. Lipids are a major source of free radicals in any biological system [7]. The ability of the skin to produce and secrete lipids and the presence of triglycerides, squalene, ceramides, cholesterol and fatty acids in the stratum corneum and sebum with their constant exposure to, and

interaction with, atmospheric oxygen leads to the production in the skin of reactive oxygen species (ROS) and the accumulation of different peroxidation products (superoxide, nitric oxide, hydroxyl radicals, peroxides and hydroperoxides). These compounds are known to reduce dermal collagen synthesis, promote collagen degradation and inhibit several enzymatic systems including metalloproteinases and elastase resulting altogether in accelerated skin aging and an enhanced risk of inflammation and neoplastic growth [11,12]. ROS formation in the skin is counteracted by a cutaneous antioxidant system represented by enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic pathways. Lipoic acid, tocopherols, ascorbic acid, melatonin, phenolic compounds and certain hormones (estradiol and estrogens) are major constituents of the non-enzymatic cutaneous antioxidant network [13,14]. Topical application of antioxidants is the most widely used approach in preventive dermatology and cosmetology. However, chemical instability, excessive hydrophilicity or lipophilicity, inability to be absorbed from the surface of the skin to its deeper layers and other physio-chemical factors are the main obstacles to efficient use of topical cosmetic ingredients [15].

There is a newly emerging generation of nutritional skin care products which can be defined as nutricosmetics or cosmeceuticals. The rationale behind their development lies in the basic principle of integrative dermatology which suggests that skin function as well as age-related skin transformations including skin aging reflect the overall status of intermediate metabolism in the human body [4]. Therefore, systemic nutritional interventions such as dietary changes and oral supplementation with specific nutraceuticals may be highly beneficial to skin health and may reverse age-related dermatological abnormalities. As we show below, ingestion of a nutricosmetical formulation of dark chocolate (DC) containing astaxanthin (ASTX), proposed for supporting skin health and the prevention of its aging (Lycotec Ltd, Cambridge, UK) can reduce markers of oxidative damage in the blood serum of middle-aged volunteers. This reduction occurs in a clear dose-dependent manner reflecting the amount of ASTX ingested.

2. Materials and Methods

Study Sites and Registration. The main goal of the study was to investigate the effect of lycosome-formulated dark chocolate (DC) containing ASTX on parameters of oxidative damage in healthy middle-aged volunteers after 1 month of daily ingestion. Discrimination between the independent antioxidant effects of DC and ASTX administered as either two separate formulations or co-delivered separately was also one of the main objectives of the study. The investigation was initiated and carried out by Lycotec Ltd (Cambridge, UK) at its facilities in Cambridge, UK, and at the Institute of Cardiology, the Ministry of Health of the Russian Federation (Saratov, Russian Federation). The study was planned as a part of a larger multi-arm trial under a protocol approved by the local Ethics Committee and registered (ACTRN12613000966796) at the World

Clinical Trial Registry. All volunteers were fully informed of the purpose of the study as well as its outcomes and gave signed written consent to their participation. All volunteers underwent physical and laboratory examinations. They were also asked about their medical history and socio-economic background and were fully informed of study protocol.

Study Groups. Ninety four (94) volunteers (48 male and 46 female) were divided into four major study groups:

1st group (28 volunteers). Ingestion of Dark Chocolate (DC) with different cocoa content (70%, 72% and 85% cocoa). Volunteers were instructed to ingest 7.5 g of DC daily.

2nd group (16 volunteers). Ingestion of ASTX alone with a daily dose of either 4 or 7 mg. Seven participants were instructed to ingest one capsule containing 4 mg of ASTX and the other half of the group 10 mg of ASTX.

3rd group (8 volunteers). Ingestion of DC and an ASTX capsule daily as two separate formulations. The participants were instructed to ingest one capsule containing 4 mg of ASTX along with 7.5 g of DC.

4th group (42 individuals). Ingestion of lycosome formulation of DC containing ASTX (LF-DC-ASTX, ESTHECHOC). The participants were instructed to ingest daily 10 g of DC containing different amounts of ASTX (1 mg, 2 mg, 4 mg or 7 mg) co-micellized with the dark chocolate matrix.

All interventions took place in the morning following breakfast for a period of 4 weeks.

All LF-DC-ASTX and control dark chocolate groups were double blind. The dark chocolate with astaxanthin capsule group was open label. The two control astaxanthin capsule groups were double blind.

Overall duration of the study for each group was 4 weeks. Oxidation markers were evaluated at the mid-point (2 weeks from the beginning of intervention) and at the end point of the study after 4 weeks.

Subjects. The study aimed to enrol healthy Caucasian volunteers (male and female) aged 45-65 years of age. All participants were asked preliminary questions regarding their family and socio-economic status, habits, dietary preferences and daily activities. After obtaining written consent the volunteers underwent basic physical (heart rate determination, blood pressure monitoring, respiratory function evaluation, body mass index measurement) and laboratory evaluations (total blood cell count, sedimentation rate measurements, liver enzyme determinations, CRP). Following screening versus inclusion/exclusion criteria, qualifying volunteers were selected and randomized into the study groups using a computerized randomization protocol. Similar physical and laboratory evaluations were performed at the mid-point (end of the second week) and the end point (end of the fourth week) of the study.

Inclusion criteria. Caucasian male or female subjects 45-65 years old, signed informed consent, non- or light to moderate smokers (≤ 10 cigarettes daily), serum positivity for markers of inflammation such as oxidized low density lipoprotein (ox-LDL ELISA ≥ 100 ELISA units) and oxidation (inflammatory oxidative damage, IOD ≥ 100 μ M MDA), willing and able to comply with the protocol for the duration of the study.

Exclusion criteria. Not willing to sign informed consent. Not able to comply with the protocol for the duration of

the study. Ejection fraction (EF) < 45%. Significant medical condition that would impact safety considerations (hepatitis, severe dermatitis, uncontrolled diabetes, cancer, severe GI disease, fibromyalgia, renal failure, recent cerebrovascular accident, pancreatitis, respiratory disease, epilepsy etc.). Intake of anti-hypertensive, lipid-lowering, anti-diabetic or cardiovascular drugs. Compulsive alcohol abuse (>10 drinks weekly) or regular exposure to other substances of abuse. Participation in other nutritional or pharmaceutical studies. Resting heart rate of >100 beats per minute or <45 beats per minute. Positive test for tuberculosis, HIV or hepatitis B. Intolerance to phlebotomy. Intolerance to dark chocolate. On a special diet in the 4 weeks prior to the study (e.g., liquid, protein, raw food, etc.).

All volunteers were asked to abstain from consumption of cocoa-containing and seafood products for the 10 days prior to the study and during the study itself, apart from the study products received under the protocol. After completion of the run-in period participants were given the study products.

Study products

Dark chocolate. Dark chocolate (DC) bars (7.5 g) with 70%, 72% or 85% cocoa from Master Martini (Milan, Italy) were used in the study. The chocolate was melted, treated, and tempered in precisely the same way for all groups of the study regardless of the addition of ASTX. Nutritional parameters are available from the manufacturer.

Astaxanthin. *Haematococcus* microalgae astaxanthin (ASTX) was purchased from Valensa, USA.

Lycosome-formulated Dark Chocolate containing co-crystallized ASTX (LF-DC-ASTX, ESTHECHOC) was prepared using lycosome technology (16) and provided to participants as 7.5g bars containing 1, 2, 4, or 7 mg of ASTX. Formation of ASTX lycosomes (coco-lycosomes) had been shown in our preliminary work to provide mutual protection of both cocoa flavanols and the ASTX from gastrointestinal enzymes and to increase their bioavailability and intestinal absorption rate [16,17].

Blood Collection. Whole blood samples were taken from the contralateral cubital vein in the morning following overnight fast. The serum was separated from the rest of the clotted mass by centrifugation then aliquots were stored at -80°C prior to analysis.

Oxidized LDL. Oxidized Low Density Lipoproteins (ox-LDL) were measured in serum as described previously [17].

Inflammatory Oxidative Damage (IOD). IOD values were calculated using serum concentration of malonic dialdehyde (MDA) which was measured spectrophotometrically (17) using kits from Cayman Chemical (MC, USA).

Statistics. Student's t-test was applied for both paired and unpaired samples. Data analysis was performed using Stata SE (College Station, TX) version 12.1. All statistical tests were two sided and statistical significance level alpha was set at 0.05 for the analysis.

3. Results

Dark Chocolate Alone. Table 1 shows the parameters of LDL oxidation and inflammatory oxidative damage in the serum of volunteers ingesting DC with different

concentrations of cocoa. No significant changes were observed in the values for ox-LDL concentration in the serum irrespective of DC cocoa content and duration of DC intake. No significant changes were observed for the IOD values either. However, analysis of average values suggests that ingestion of DC tends somewhat to decrease the mean parameters for IOD over the course of the observational period. Ingestion of DC with the highest cocoa content (85%) gave the most noticeable reduction in mean IOD value (-14.4 points) at the mid-point of the study. However, this reduction fell below the level of statistical significance set up in our study ($p=0.074$).

Astaxanthin Alone. Table 2 shows the serum ox-LDL concentrations and IOD values in volunteers ingesting ASTX alone. Although average values for both parameters tend consistently to decline at the mid-point of the interventional period (2nd week of the study) this tendency was not statistically meaningful. A statistically significant inhibition of oxidative markers with P values <0.05 took place in ASTX-treated volunteers only at the end point of the study. In particular, there were 50.7 % and 55.4 % reductions in mean ox-LDL for the 4 and 7 mg intake regimens respectively after 4 weeks of ASTX ingestion. Similarly, the end point of the study was manifested by a decline in IOD values of 54.1 % and 48.5% for the 4 mg and 7 mg intake regimens respectively. In quantitative terms the reduction of ox-LDL and IOD values during the course of ASTX ingestion was not dose-dependent. There was no statistically significant difference in the magnitude of ox-LDL or IOD reduction reflecting the amount of ASTX ingested ($P > 0.05$).

Co-ingestion of ASTX and DC. Table 3 shows the oxidative marker values following co-ingestion of 7.5 g of DC and 4 mg of ASTX given to volunteers as two separate formulations. As can be seen, the degree of ox-LDL decline at the end of the observational period was more noticeable in the case of ASTX and DC co-ingestion as compared to ASTX alone. A decline of 66.1% was seen following co-ingestion as opposed to 50.7% for 4.0mg ASTX alone. However, no such difference was seen in values reflecting MDA concentration. Overall, co-ingestion of ASTX and DC as two separate formulations affected the oxidation markers in a manner similar to intake of ASTX alone.

Lycosome-formulated DC. As can be seen from Table 4 there was a clear ASTX dose-dependency pattern in downregulation of serum ox-LDL after 2 weeks' ingestion of lycosome-formulated DC containing ASTX. Higher amounts of ASTX co-micellized with DC matrix (7 mg) caused a greater reduction in average ox-LDL values (by 48.4% from the control level), whereas lower ASTX content (1 mg) was associated with a less significant reduction in ox-LDL (by 32.3% from the pre-treatment level). Ingestion of lycosome-formulated DC with ASTX over a longer period (4 weeks) lead to an even greater decline in ox-LDL, manifested by a further reduction in mean values. However, the dose-dependency pattern was less obvious at the end point of the study. In particular, the greatest decline in ox-LDL was seen following daily ingestion of DC containing 2 mg of ASTX (by 78.7%) whereas DC with 7 mg of ASTX caused a less pronounced fall in ox-LDL (by 53.7% from the control level).

Table 1. PARAMETERS OF OXIDATION AFTER DAILY INGESTION OF DARK CHOCOLATE (DC) ALONE (7.5 g) (Mean with Standard Deviation)

Product	n	ox-LDL			IOD (MDA)		
		Day 0	2 weeks	4 weeks	Day 0	2 weeks	4 weeks
DC 70% COCOA	10	98.3±10.2	123.5±16.2	102.3±17.4	118.1±11.5	100.4±13.2	104.1±12.5
DC 72% COCOA	8	147.2±16.8	162.8±17.5	153.9±14.2	122.3±13.3	114.3±12.0	111.3±10.8
DC 85% COCOA	10	123.2±21.8	129.7±15.5	141.2±19.4	94.1±10.1	79.7±7.0	83.2±9.6

n – number of volunteers

Note. The volunteers were screened, enrolled and randomized into 3 groups. The parameters of oxidation were analyzed at “0” time point as well as after 2 and 4 weeks of observational period as described in the “Material and Methods” section.

Table 2. PARAMETERS OF OXIDATION AFTER DAILY INGESTION OF ASTAXANTHIN (ASTX) ALONE (Mean with Standard Deviation)

ASTX	n	ox-LDL			IOD (MDA)		
		Day 0	2 weeks	4 weeks	Day 0	2 weeks	4 weeks
4 mg	8	164.3±23.5	135.7±15.1	81.0±11.6*	105.4±12.9	92.1±11.6	48.3±7.2*
7 mg	8	159.0±18.8	124.0±13.7	70.9±9.3*	116.0±13.0	97.0±11.3	59.7±6.0*

n – number of volunteers; (*) – P<0.05

Note. The volunteers were screened, enrolled and randomized into 2 groups. The parameters of oxidation were analyzed at “0” time point as well as after 2 and 4 weeks of observational period as described in the “Material and Methods” section.

Table 3. PARAMETERS OF OXIDATION AFTER DAILY CO-INGESTION OF ASTAXANTHIN (ASTX) AND DARK CHOCOLATE (DC) (Mean with Standard Deviation)

Products	n	ox-LDL			IOD (MDA)		
		Day 0	2 weeks	4 weeks	Day 0	2 weeks	4 weeks
4 mg ASTX Alone	8	164.3±23.5	135.7±15.1	81.0±11.6*	105.4±12.9	92.1±11.6	48.3±7.2*
4 mg ASTX and 7.5 g DC	8	141.3±15.0	111.9±12.3	47.9±6.9*	107.5±11.4	89.0±9.6	64.3±8.3*

n – number of volunteers; (*) – P<0.05

Note. The volunteers were screened, enrolled and randomized into 2 groups. The parameters of oxidation were analyzed at “0” time point as well as after 2 and 4 weeks of observational period as described in the “Material and Methods” section.

Table 4. PARAMETERS OF OXIDATION AFTER DAILY INGESTION OF LYCOSOME-FORMULATED DARK CHOCOLATE CO-MICELLIZED WITH ASTX (ESTHECHOC) (Mean with Standard Deviation)

ESTHECHOC WITH:	n	ox-LDL			IOD (MDA)		
		Day 0	2 weeks	4 weeks	Day 0	2 weeks	4 weeks
1 mg ASTX	8	138.0±9.3	93.4±10.1	52.4±6.8*	113.0±9.5	84.6±9.1*	66.5±7.0*
2 mg ASTX	10	176.0±12.1	101.4±11.9*	37.4±7.4*	140.0±11.3	72.5±7.5*	40.3±8.0*
4 mg ASTX	16	155.8±16.3	92.0±9.8*	64.1±8.0*	171.4±18.3	77.7±7.5*	48.6±6.5*
7 mg ASTX	8	127.0±13.4	65.5±7.6*	58.8±6.0*	139.4±15.7	51.0±5.5*	20.1±4.3*

n – number of volunteers; (*) – P<0.05

Note. The volunteers were screened, enrolled and randomized into 4 groups. The parameters of oxidation were analyzed at “0” time point as well as after 2 and 4 weeks of observational period as described in the “Material and Methods” section.

A high degree of dose-dependency was also observed for changes in IOD parameters at the mid-point of the study following 2 weeks’ ingestion. Increasing amounts of ASTX fused with DC matrix (1, 2, 4 and 7 mg ASTX) gave a corresponding decline in IOD values (by 25.1, 48.2, 54.6 and 63.4% respectively). Similar results revealing a dose-dependent pattern were seen at the end point of the study following 4 weeks’ ingestion when IOD values were reduced even more significantly (by 41.1, 71.2, 71.6 and 85.5% respectively). Overall, the magnitude of reduction for IOD values was more pronounced in quantitative terms than the decline in ox-LDL parameters.

4. Discussion

The results reported above expand on our newly published data [17] revealing the antioxidant properties of

LF-DC-ASTX and provide some additional insight into the molecular mechanisms behind LF-DC-ASTX action as well as the nutritional physiology of DC and biopharmacology of ASTX. First of all, the results reported above show that continuous ingestion of ASTX taken as a singular formulation translates into a measurable decline in malonic dialdehyde and oxidized LDL in serum with no clear dose-dependency pattern. In contrast, DC ingested alone does not affect these parameters in a statistically significant manner. However, some antioxidant activity of DC was “unmasked” when DC and ASTX were ingested together as two separate formulations. This conclusion arises from the fact that the decline in ox-LDL observed in the serum of volunteers following co-ingestion of DC and ASTX as two separate formulations was far greater than the decline seen for ASTX alone. Nevertheless, the highest degree of ox-LDL and MDA decline was observed in volunteers following ingestion of the LF-DC-ASTX

formulation. Interestingly, this decline took place in a clear dose-dependent pattern reflecting the amount of ASTX co-crystallized with DC matrix. Such a dose-dependency pattern is likely to mirror the changes in pharmacokinetics of cocoa flavanols and ASTX upon ingestion of LF-DC-ASTX. As we have reported previously [17], serum levels of cocoa flavanols and ASTX reach their highest level following ingestion of LF-DC-ASTX as compared to the ingestion of DC and ASTX alone or their co-ingestion as two separate formulations. It can be assumed that the improved bioavailability of ASTX and cocoa flavanols alleviates individual patterns of intestinal antioxidant absorption and maximizes gradients in serum concentration of ASTX and cocoa flavanols, conferring thereby dose-dependent changes in markers of oxidation following ingestion of LF-DC-ASTX.

The medicinal properties of dark chocolate (DC) and its active ingredients (cocoa polyphenols, flavanols and catechins) are the subject of extensive research and discussion. The effects of DC on the cardio-vascular and nervous systems as well as on lipid profile have been reported by many researchers despite the questionable reproducibility of certain results [18]. However, the concentration of bioactive ingredients in commercial brands of DC is highly variable due to differences in manufacturing and this is rarely taken into consideration for clinical studies. Moreover, cocoa flavanols are highly hydrophobic compounds with a poor intestinal absorption rate and low bioavailability [19]. Therefore, even the ingestion of DC brands with high cocoa content does not necessarily translate into an increase in circulating cocoa flavanols and a biological effect. This was exemplified in our study by the unchanged level of oxidation markers following regular DC intake. Thus, there is a need for new nutraceutical formulations of DC with increased bioavailability of flavanols as well as formulations of DC fortified with other bioactive compounds [20].

It should be clarified that LF-DC-ASTX was originally developed as a nutricosmetical product with enhanced bioavailability of both cocoa flavanols and ASTX aimed at supporting skin health and slowing down the age-associated changes resulting primarily from antioxidant deficiency and oxidative damage such as decreased skin firmness, elasticity, wrinkle formation and hypohydrosis [21,22]. Since the anti-oxidative effect of DC is highly conditional and depends on the concentration of flavanols in the DC, amount of DC ingested and duration of DC intake, ASTX, an algae-derived antioxidant, was included in the LF-DC-ASTX formulation and was the major rationale behind the design of LF-DC-ASTX. Astaxanthin is the carotenoid with the greatest antioxidant activity - up to 550 times stronger than vitamin E, 11 times more potent than beta-carotene and 5 times more potent than lutein, with a proven impact on risk reduction for various disorders in both clinical and experimental settings [23,24]. ASTX possesses a unique molecular structure with alternating polar-nonpolar loci and 13 carbon-carbon conjugated double bonds conferring the ability to quench free radicals and atoms with unpaired valence electrons including oxygen species, nitrogen, sulfur and carbon during biological oxidation [24,25]. Moreover, the relatively long amphiphilic molecule of ASTX is essential for the assembly and stability of coco-lycosome particles.

The profound and systemic effect of LF-DC-ASTX on biological oxidation parameters reported above may create a rationale for the use of LF-DC-ASTX in the prevention and treatment of oxidative disorders far beyond dermatological applications, including various diseases associated with oxidative stress (cardiovascular disease, type 2 diabetes mellitus and cancer). Additional studies would be required to evaluate the therapeutic potential of DC formulations fortified with ASTX.

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Conflict of Interests Disclosure

The authors declare no conflict of interests involved.

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