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Effects of macular xanthophyll supplementation on brain-derived neurotrophic factor, pro-inflammatory cytokines, and cognitive performance

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ABSTRACT

Purpose: Oxidative and inflammatory processes play a major role in stress-induced neural atrophy. There is a wide body of literature linking oxidative and inflammatory stress with reductions in neurotrophic factors, stress resilience, and cognitive function. Based on their antioxidant and anti-inflammatory capacity, we investigated the effect of the dietary carotenoids lutein and zeaxanthin, along with the zeaxanthin isomer meso-zeaxanthin (collectively the "macular xanthophylls" [MXans]) on systemic brain-derived neurotrophic factor (BDNF) and anti-oxidant capacity (AOC), and the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β. To investigate higher-order effects, we assessed cognitive performance.

Methods: 59 young (18–25 yrs.), healthy subjects participated in a 6-month, double-blind, placebo-controlled trial to evaluate the effects of MXan supplementation on the aforementioned serum parameters and cognitive performance. Subjects were randomly assigned to one of three groups: placebo, 13 mg, or 27 mg/day total MXans; all measures were taken at baseline and 6 months. Blood was obtained via fasting blood draw, and MXan concentration in the retina (termed macular pigment optical density [MPOD]) was measured via customized heterochromatic flicker photometry. Serum BDNF and cytokines were assessed via ELISA. Serum antioxidant capacity (AOC) and serum MXan concentrations were quantified via colorimetric microplate assay, and high-performance liquid chromatography, respectively. Cognitive performance was measured via a computer-based assessment tool (CNS Vital Signs).

Results: BDNF, MPOD, serum MXans, and AOC all increased significantly versus placebo in both treatment groups over the 6-month study period ($p < .05$ for all). IL-1β decreased significantly versus placebo in both treatment groups ($p = .0036$ and $p = .006$, respectively). For cognitive measures, scores for composite memory, verbal memory, sustained attention, psychomotor speed, and processing speed all improved significantly in treatment groups ($p < .05$ for all) and remained unchanged in the placebo group. Several measures were found to be significantly associated in terms of relational changes over the course of the study. Notably, change in BDNF was related to change in IL-1β ($r = -0.47$; $p < .001$) and MPOD ($r = 0.44$; $p = .0086$). Additionally, changes in serum MXans were strongly related to AOC ($r = 0.79$ & 0.61 for lutein and zeaxanthin isomers respectively; $p < .001$). For cognitive scores, change in BDNF was correlated to change in composite memory ($r = 0.32$; $p = .014$) and verbal memory ($r = 0.35$; $p = .007$), whereas change in MPOD was correlated with change in both psychomotor speed ($r = 0.38$; $p = .003$), and processing speed (r = 0.35; $p = .007$). Change in serum lutein was found to be significantly correlated to change in verbal memory ($r = 0.41$; $p < .001$), composite memory ($r = 0.31$; $p = .009$), and sustained attention ($r = 0.28$; $p = .036$). Change in serum zeaxanthin isomers was significantly correlated with change in verbal memory ($r = 0.33$; $p = .017$). Lastly, change in AOC was significantly associated with verbal memory ($r = 0.34$; $p = .021$), composite memory ($r = 0.29$; $p = .03$), and sustained attention ($r = 0.35$; $p = .016$). No significant relational changes in any cognitive parameter were found for the placebo group.

Conclusions: Six months of daily supplementation with at least 13 mg of MXans significantly reduces serum IL-1β, significantly increases serum MXans, BDNF, MPOD, and AOC, and improves several parameters of cognitive performance. Findings suggest that increased systemic antioxidant/anti-inflammatory capacity (and not necessarily deposition of the carotenoids in neural tissues), may explain many of the effects determined in this

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study. The significant relationship between change in BDNF and IL-1β over the course of the study suggests that regular consumption of MXans interrupts the inflammatory cascade that can lead to reduction of BDNF. Changes in MPOD and BDNF appear to account for enhancement in cognitive parameters that involve speed of processing and complex processing, respectively.

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1. Introduction

Oxidative stress occurs when oxidation caused by reactive oxygen species (ROS) outpaces the body's endogenous and exogenous antioxidant capacity. Prolonged, excessive oxidative stress can cause devastating, often irreversible damage to neural tissues such as the retina (e.g. [5]) and brain (e.g. [23]). Moreover, prolonged oxidative stress promotes pro-inflammatory responses [42], which can exacerbate damage and result in the creation of more ROS [1]. This positive feedback loop can result in the cumulative damage that is manifest in several age-related diseases, such as age-related macular degeneration [38], atherosclerosis [8], and certain forms of cancer [62]. At relatively low levels, however, ROS can promote optimal physiological function via their role in glial and neuronal signaling pathways [63]. Additionally, ROS such as superoxide radicals (at low/moderate levels) can protect cells by mounting a defense against infectious agents [43]. The body's ability to maintain an advantageous "redox homeostasis" therefore appears to be crucial to protecting tissues and promoting optimal function [10,19].

Endogenous antioxidant agents (e.g. glutathione and superoxide dismutase) have been shown to be inadequate in maintaining this homeostatic balance [63]. Moreover, corticosteroids generated from the stress response decrease the effectiveness of endogenous antioxidant systems [41]. Exogenous, diet-derived antioxidants, such as vitamins C and E and the carotenoids lutein and zeaxanthin, supplement the endogenous antioxidant system and play a key role in maintaining this delicate equilibrium. In fact, the two systems act synergistically to re-establish oxidative homeostasis; an example of this is the regeneration of Vitamin E by glutathione to prevent lipid peroxidation [9].

Cellular damage and the potential for development of age-related disease are but two of several negative outcomes of oxidation and inflammation. Compromised neuroplasticity, via impaired synaptogenesis and reduced dendritic spine density [12,13], is another deleterious result of oxidative stress and inflammation. Brain-derived neurotrophic factor (BDNF), a neurotrophin widely reported to be associated with neuroplasticity (e.g. [11,16]), exhibits an inverse relationship with inflammatory status [49]. The pro-inflammatory cytokines, IL-6 and TNF-α, which are derived centrally by microglia (in addition to peripheral sources), have been shown specifically to attenuate neuroplasticity via reduction of BDNF [24]. For example, exposure to uncontrollable stress leads to a chronic elevation in brain and serum levels of these pro-inflammatory cytokines [32], which in turn can cause reduction in BDNF [24,46], and compromised cognitive capacity [2]. Additionally, in rats injected with IL1-β, BDNF mRNA levels were significantly reduced in the hippocampus [37]. The hippocampus, given its roles in learning and memory and its capacity for neurogenesis [2] is a primary site of adult neuroplasticity [55]. The finding that neuroinflammatory status can directly impact BDNF levels within the hippocampus illustrates the essential role of neuroplasticity in cognition and neural health.

As noted above, one way that the body promotes neural health and optimal neural efficiency is through the regulation of endogenous and exogenous antioxidant systems. Of relevance to the present study is the potential for exogenous, diet-derived antioxidants to enhance BDNF levels and cognitive function via reduction of oxidative stress and inflammation. The diet-derived xanthophyll carotenoids lutein and zeaxanthin are exceptional antioxidants [36], and exhibit meaningful anti-inflammatory capability [22,61]. Indeed, in vitro experiments reveal that lutein decreases microglia-derived IL1- β and TNF-α, and that this anti-inflammatory action involves inhibition of the intracellular transcription factor NFκ-B [71]. The reduction of inflammation associated with increased lutein could serve to moderate the many negative effects of neuroinflammation, including a reduction in BDNF production. As a preliminary basis for this hypothesis, in a rat model of diabetic retinopathy, administration of lutein resulted in maintenance of BDNF levels in the retina; those rats receiving only vehicle showed significant deficits in BDNF [53].

Lutein and zeaxanthin accumulate in the macula of the retina in primates as macular pigment ([57]); concentrations are referred to as macular pigment optical density (MPOD) due to their collective ability to absorb short-wavelength (blue) light. Lutein and zeaxanthin also accumulate in the brain ([17,35,65,66], where levels are positively associated with MPOD [64]. Additionally, MPOD has been reported to be associated with cognitive performance in a number of age groups, across the lifespan: In pre-adolescent children [51], middle-aged individuals [21] and the elderly [33,65,66]. Also, in the brain tissues of decedents aged over 80 years, lutein concentrations are shown to be significantly correlated with cognitive function prior to death [35].

Meso-zeaxanthin, a third macular xanthophyll (MXan) that arises from conversion of lutein within the retina [73]) exhibits exceptional antioxidant and anti-inflammatory capability. In fact, when compared to lutein and zeaxanthin, meso-zeaxanthin maintains the highest antioxidant capacity [7]. Although not typically derived from the diet (although see Nolan et al. [74]), meso-zeaxanthin is readily absorbed into circulation and deposited in the retina when taken in supplement form (e.g., [40]). Supplementation with MXans has been shown to improve aspects of cognitive performance in healthy young adults [50], middle-aged adults [47], and older adults [28].

Although many of the details are still unknown, transport of the MXans across the blood-retina barrier (i.e. the retinal pigmented epithelium) appears to be facilitated by their binding to LDL and HDL cholesterol molecules, and subsequent delivery to the retina via LDL receptor- and scavenger receptor B1-dependent mechanisms [30]. After reaching the retinal space, the MXans bind to specific xanthophyll-binding proteins such as glutathione S-transferase P1 and steroidogenic acute regulatory domain (STARD) proteins, which likely serve in their intracellular transfer, deposition, and metabolism [6,31]. It is assumed that the MXans cross the blood-brain barrier via similar mechanisms and accumulate in brain regions that maintain relatively high metabolism (e.g. frontal and occipital lobes, and hippocampus). Given that neural tissues almost exclusively use oxidative metabolism to generate ATP for energy, areas of very high metabolic rate (e.g. the foveal retina and hippocampus) are at higher risk for oxidative stress and inflammation [25]. As noted above, MPOD is significantly correlated with brain levels of lutein and zeaxanthin [64], supporting the idea that there is preferential deposition of these robust antioxidants/anti-inflammatories in neural tissues that maintain high metabolism and potential for oxidative stress and inflammation. If these molecules reduce inflammation locally in structures such as the hippocampus, supplementation with MXans may serve to reduce local inflammation and potentially enhance BDNF levels. Notably, lutein and zeaxanthin are obtained solely via diet, and recent data suggest that Americans consume very little of the foods that contain these carotenoids [14,34]. Supplementation with even a modest amount of MXans may therefore have acute, meaningful effects on oxidative, inflammatory, and BDNF

status. By extension, cognitive performance may also be positively influenced by the biochemical changes produced by MXan supplementation. To test these hypotheses, we supplemented our participants with MXans (lutein, zeaxanthin, and meso-zeaxanthin; versus placebo) for 6 months, and examined the effect on MPOD, BDNF, systemic antioxidant capacity, pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, and cognitive performance.

2. Materials and methods

Fifty-nine subjects participated in a double-blind, randomized, placebo-controlled macular carotenoid supplementation trial. Subjects were healthy, college-aged (18–25, mean = 21.5 yrs.; 27 male/32 female) non-smokers with a BMI between 18.5 and 27. Subjects were instructed to maintain their current diet. In consideration of macular pigment testing, all subjects had uncorrected or contact lens-corrected visual acuity of 20/20 or better in the test (right) eye and had no self-reported current or previous history of ocular pathology. Current or previous supplementation with lutein and/or zeaxanthin was an exclusionary criterion. Subjects were recruited from the population of students at the University of Georgia in Athens, Georgia. Informed consent was obtained from each subject and the study adhered to the tenets of the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University of Georgia.

2.1. Macular xanthophyll (MXan) supplementation

Subjects were randomly assigned to one of three groups: placebo (n = 10), ~13 mg total MXans/day group (n = 24), or ~27 mg total MXans/day (n = 25). Pills were brown colored, soft gelatin capsules, with lutein, zeaxanthin, and meso-zeaxanthin suspended in safflower oil. Independent analysis indicated that the 13-mg supplement contained 10.86 mg lutein/2.27 mg zeaxanthin isomers (both zeaxanthin and meso-zeaxanthin; chiral analysis was not independently determined), and the 27-mg supplement contained 22.33 mg lutein/4.70 mg zeaxanthin isomers. Placebos contained no lutein or zeaxanthin isomers. All reported values were within +/− 5% variability. Subjects were instructed to ingest one pill with a meal every day. Compliance was ensured with weekly phone calls and pill counts.

2.2. Dietary assessment of MXan consumption

A short (7-question) dietary assessment was administered at baseline and 6 months to evaluate subjects' consumption of foods that contain MXans, and to determine any dietary changes during the study that may have influenced results. The purpose of this questionnaire was not to accurately quantify absolute consumption of foods that contain lutein and zeaxanthin, but rather to simply determine if changes occurred over the 6-month study period.

2.3. Measurement of macular pigment optical density (MPOD)

The concentration of macular carotenoids in the central retina (MPOD) was assessed with a non-invasive, perceptual task called heterochromatic flicker photometry (HFP). A densitometer (Macular Metrics Corp., Rehoboth, MA) described by Wooten et al. [69] was used for this purpose. The apparatus, procedures, and the principle of HFP have been fully described in earlier publications (e.g. [58,70]). Briefly, subjects are presented with two narrowband, superimposed lights (460 nm and 550 nm, respectively) that are temporally alternated in square-wave counterphase. This arrangement gives the subject the impression of a flickering disc of light. The 550 nm light spectrally bypasses the absorption of MP, and the other (460 nm) is strongly absorbed by MP. The subject's task is to adjust the relative radiance of the two lights until a perception of no flicker (i.e. equiluminance) is achieved. All other factors being equal, a subject that requires more short-wave (i.e., 460 nm) relative to middle-wave (i.e., 550 nm) light to achieve null flicker has higher MPOD. This task is performed for the locations of interest within the fovea, which presumably contain macular pigment, and for a reference location in the parafovea that does not (about 7° eccentricity). To obtain a measure of MPOD at a given test locus, the logarithmic ratio of short-wave: middle-wave radiance (at null flicker) for the reference location (i.e. 7° eccentricity) is subtracted from the corresponding logarithmic ratio found at the test locus. For the present study, we based all analyses involving MPOD on the standard, 0.5° retinal eccentricity measure. Measurements of MPOD were taken at baseline and 6 months.

2.4. Blood collection

Fasting blood was collected between 9 am–11 am, by a licensed phlebotomist, at baseline and 6-month visits. Subjects' whole blood was collected into a serum separator vacutainer tube (SST) via venipuncture. Blood was allowed to clot for 30 min at room temperature before centrifugation for 15 min at 1000 xg. Serum was then removed and stored in microvials at −20C until analysis.

2.5. Lutein and Zeaxanthin isomer measurement: high-performance liquid chromatography (HPLC)

Sample extractions and analyses were completed under yellow light. Serum proteins were precipitated with an equal volume of ethanol (1% BHT), containing the internal standard, trans-β-apo-8′-carotenal. After centrifugation, samples were extracted three times with n-hexanes, mixing, and centrifugation. Organic layers were pooled and evaporated to dryness with nitrogen and re-suspended in the mobile phase. An Agilent 1200 series HPLC system, consisting of a quaternary pump with degasser, autosampler, thermostated column compartment, UV–vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA, USA), was employed for the chromatography. A reversed-phase YMC C30 column (4.6 × 250 mm, 5-μm particle size) was utilized. A stepwise elution consisting of mobile phase A (95% methanol) and mobile phase B (methyl tertbutyl ether) from 15 to 85% B over a 27-min period at a flow rate of 1 mL/min was employed. A volume of 100 μL was injected for each of the serum samples. Detection wavelengths were λ = 447 nm (L) and 450 nm (Z isomers).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Concentration of BDNF, IL-6, IL-1β, and TNFα, in serum obtained from baseline and 6 month visits, was determined via solid-phase sandwich ELISA. All wells for each marker were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc., Santa Monica CA, USA), averaged across duplicates, and a curve of best fit was used to calibrate to standards. All coefficient of variability values were under 15%. Specific methods for each serum marker were as follows: BDNF, IL-6, IL-1β, and TNF-α levels were analyzed by Quantikine ELISA kits (R &D Systems, Minneapolis MN, USA) following dilution of serum and processing according to the manufacturer's instructions. Concentrations are reported as pg/ml.

2.7. Colorimetric microplate assay

Total antioxidant capacity of serum obtained from baseline and 6-month visits, was determined via colorimetric microplate assay. Serum was diluted and processed according to the manufacturer's instructions for the Colorimetric Microplate Assay for total antioxidant power (Product No. 430710, Neogen Corporation, Lexington KY, USA). Microplate wells were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc., Santa Monica CA, USA), averaged across duplicates, and a curve of best fit was used to calibrate to standards. All coefficient

of variability values were under 15%. Antioxidant capacity data are reported in Trolox equivalents.

2.8. Cognitive assessment

Cognitive function was measured using a computerized test battery (CNS Vital Signs; Morrisville, NC) at baseline and 6 months. This assessment has been shown to be a reliable and valid test of neurocognitive function [26], with scores normed to an average of 100 and a standard deviation of 15 points. Participants were tested in a well-lit, quiet room. The researcher introduced the participant to the testing system and explained all aspects of the testing procedure (e.g., which buttons on the keyboard would be used for responding). To familiarize participants with the general flow of the cognitive testing procedure, a short practice session was provided. Table 1 lists the cognitive domains assessed during the battery, and a brief description of each for the reader's reference.

2.9. Statistical analysis, blinding procedure

The statistical and graphing program OriginPro 9.3 (Northampton, MA, USA) was used to calculate repeated-measures ANOVA, Pearson product-moment correlations, and to generate figures for the manuscript. Assuming a placebo group of $n = 10$, an a priori power calculation using a 20% change in BDNF in treatment groups, coupled with a standard deviation of 20%, and $\alpha = 0.05$ indicated that both 13 and 27 mg MXan groups required 25 subjects to detect effects (if present). We assumed an attrition rate of roughly 20%, and therefore enrolled 75 subjects. As noted above, 59 completed the trial.

The randomization sequence was generated by the study coordinator (JMS), who performed random allocation to the three study groups. The study investigator (NTS) received a box of supplements labeled only with the participant identification number. Upon completion of the study, the randomization sequence was revealed, and data analysis ensued.

3. Results

3.1. MPOD/Serum parameters

As confirmation that our experimental and control groups were reasonably homogeneous, one-way ANOVAs determined no differences among groups at baseline with regard to any of the variables of interest ($p > .40$ for all). Additionally, scores from the short MXan dietary questionnaire, which indicated an average consumption of only 0.4 servings/day of foods that contained MXans, did not change over the course of the study ($t = 0.343$; $p = .733$). As noted above, due to similar average MXan responses in terms of both serum and MPOD, the supplement groups were combined and treated as one group for comparative analyses (i.e. MXan vs. placebo). It should be noted that, due to the increased power gained by combining the supplement groups, statistical significance was obtained for otherwise marginally significant relationships/changes seen when the groups were analyzed individually. Table 2 presents values for all serum parameters, for both supplement groups (combined), and the placebo group, at baseline and 6 months. A graphic representation of percent changes for these parameters and also serum L and Z for all groups can be seen in Figs. 2 and 3.

Table 2
Absolute values for serum markers obtained at baseline and 6 months, for pooled MXan supplement and placebo groups.

Serum marker	Mean (baseline)	SD	Mean (6 months)	SD
Pooled active supplement groups				
TNF-α	2.70 pg/mL	1.1	2.83 pg/mL	1.22
IL-6	4.6 pg/mL	1.87	4.452 pg/mL	1.96
IL-1β	0.659 pg/mL	0.54	0.498 pg/mL*	0.46
BDNF	14,672 pg/mL	3576	16,017 pg/mL*	3361
AOC	0.552 pg/mL	0.103	0.663 pg/mL*	0.111
Lutein	0.21 μg/mL	0.111	1.25 μg/mL*	0.684
Zeaxanthin isomers	0.04 μg/mL	0.025	0.192 μg/mL*	0.131
Placebo group				
TNF-α	2.495 pg/mL	0.95	2.82 pg/mL[a]	1.05
IL-6	5.53 pg/mL	2.17	5.66 pg/mL	2.42
IL-1β	0.529 pg/mL	0.44	0.518 pg/mL	0.71
BDNF	15,101 pg/mL	3819	14,648 pg/mL	3699
AOC	0.577 pg/mL	0.174	0.568 pg/mL	0.166
Lutein	0.237 μg/mL	0.141	0.222 μg/mL	0.132
Zeaxanthin isomers	0.052 μg/mL	0.034	0.050 μg/mL	0.055

Asterisks denote statistically significant change ($p < .05$) vs. placebo.
[a] Significant change from baseline measure.

Although absolute serum values for the markers assessed in this study can vary by test kit, species tested, age, and health status, our obtained serum values were reasonably consistent with previous findings in healthy subjects (e.g., BDNF [3]; TNF-α, IL-6, and IL1-β [4]).

At baseline, significant correlations were found between all inflammatory cytokines (see Table 3).

Additionally, baseline BDNF was found to correlate significantly to baseline MPOD (Table 2; r = 0.27; $p = .038$). Serum lutein was significantly correlated with serum zeaxanthin ($r = 0.77$; $p < .001$) and serum AOC ($r = 0.27$; $p = .032$). A one-way ANOVA determined that

Table 1
Brief description of the cognitive domains assessed during the computerized cognitive functional test battery.

Cognitive domain	Description
Composite memory	How well subject can recognize, remember, and retrieve words and geometric figures
Verbal memory	How well subject can recognize, remember, and retrieve words
Visual memory	How well subject can recognize, remember and retrieve geometric figures
Working memory	How well a subject can perceive and attend to symbols using short-term memory processes
Psychomotor speed	How well a subject perceives, attends, responds to complex visual-perceptual information and performs simple fine motor coordination
Reaction time	How quickly the subject can react, in milliseconds, to a simple and increasingly complex direction set
Complex attention	Ability to track and respond to a variety of stimuli over lengthy periods of time and/or perform complex mental tasks requiring vigilance quickly and accurately
Cognitive flexibility	How well subject is able to adapt to rapidly changing and increasingly complex set of directions and/or to manipulate the information
Processing speed	How well a subject recognizes and processes information i.e., perceiving, attending/responding to incoming information, motor speed, fine motor coordination, and visual-perceptual ability
Executive function	How well a subject recognizes rules, categories, and manages or navigates rapid decision making
Simple attention	Ability to track and respond to a single defined stimulus over lengthy periods of time while performing vigilance and response inhibition quickly and accurately to a simple task
Motor speed	Ability to perform simple movements to produce and satisfy an intention towards a manual action and goal
Social acuity	How well a subject can perceive, process, and respond to emotional cues
Reasoning	How well is subject able to recognize, reason and respond to non-verbal visual-abstract stimuli
Sustained attention	How well a subject can direct and focus cognitive activity on specific stimuli

Table 3
Baseline correlation matrix for serum markers and MPOD.

Measure	BDNF	IL-6	IL-1β	TNF-α	AOC	Lutein	Zeaxanthin isomers	MPOD
BDNF	1	–	–	–	–	–	–	–
IL-6	−0.23	1	–	–	–	–	–	–
IL-1β	−0.061	0.34*	1	–	–	–	–	–
TNF-α	−0.081	0.418*	0.378*	1	–	–	–	–
AOC	−0.023	−0.131	0.075	−0.106	1	–	–	–
Lutein	0.12	−0.12	−0.08	−0.14	0.27*	1	–	–
Zeaxanthin isomers	0.09	−0.13	−0.06	−0.15	0.21	0.77*	1	–
MPOD	0.27*	−0.16	−0.19	−0.093	−0.173	0.19	0.18	1

Values are Pearson correlation coefficients, and asterisks denote statistical significance ($p < .05$).

MPOD increased significantly (F = 3.55; $p = .023$) versus placebo in the 13 mg/day MXan (0.106 OD increase) and the 27 mg/day MXan (0.12 OD increase) – see Fig. 1. The two supplement groups did not differ statistically from each other in terms of MPOD increase. Fig. 2 presents a graphical representation of the changes in all serum measures (save MXans) obtained over the 6-month study period, for each of the study groups. In both the 13 mg/day MXan and 27 mg/day MXan groups, paired-samples *t*-tests showed that BDNF increased significantly after the 6-month supplementation period ($p = .014$, and $p = .04$ respectively). Antioxidant capacity was found to increase significantly in 13 mg/day MXan group ($t = -3.41$; $p = .0024$) and the 27 mg/day MXan group ($t = -3.66$; $p = .00124$ – see Fig. 2). In terms of the cytokines tested, IL-1β was found to significantly decrease in both the 13 mg/day MXan ($t = 3.25$; $p = .0036$) and 27 mg/day MXan groups ($t = 2.99$; $p = .006$ – see Fig. 2). TNF-α was found to increase slightly over the 6-month study period in both treatment groups, and significantly for the placebo group (see Fig. 2). The differences between changes in TNF-α found for placebo and treatment groups, respectively, were markedly different, albeit not statistically significant. Although these differences failed to reach statistical significance ($p = .13$), they are noteworthy due not only to the differences between placebo and both treatment groups, but also the similarity in pattern and scale with regard to placebo/treatment group changes in the other variables. No statistically significant changes were determined for IL-6, although there was a general trend of decrease with increasing MXan dose. Not surprisingly, in the treatment groups serum MXans were found to increase substantially after 6 months of supplementation (see Fig. 3). These changes were statistically significant ($p < .001$ for both) compared to placebo, which was found to decrease very slightly (see Fig. 3).

Table 3 presents the correlation matrix for change in variables over the 6-month study period. Significant correlations were found between change in BDNF and change in IL-1β ($r = -0.47$; $p < .001$), MPOD ($r = 0.44$; $p = .0086$), serum lutein ($r = 0.38$; $p = .011$), serum zeaxanthin ($r = 0.33$; $p = .01$), and AOC ($r = 0.31$; $p = .014$). Change in IL-1β was also correlated with change in serum lutein ($r = 0.51$; $p < .001$), serum zeaxanthin ($r = 0.412$; $p = .0019$), MPOD (-0.37; $p = .026$), AOC (-0.283; $p = .033$), and IL- 6 ($r = 0.26$; $p = .048$). Changes in serum lutein and zeaxanthin were also correlated with each other ($r = 0.81$; $p < .001$), with AOC ($r = 0.79$; $p < .001$, $r = 0.61$; $p < .001$, respectively), and with MPOD ($r = 0.62$ and 0.66, respectively; $p < .001$ for both). Lastly, change in MPOD correlated with change in AOC ($r = 0.43$; $p < .001$).

3.2. Cognitive performance

At baseline, MPOD was related significantly to reaction time (r = 0.26; $p = .044$), and BDNF was significantly related to reasoning ($r = 0.25$; $p = .046$). Additionally, serum lutein was found to be significantly related to verbal memory ($r = 0.28$; $p = .41$). Several parameters of cognitive performance improved significantly in the MXan supplementation groups. As noted earlier, due to their similar responses in terms of serum lutein/zeaxanthin and MPOD, the supplement groups were combined and compared to the placebo group via repeated-measures ANOVA. For the treatment group, composite memory (an overall metric of memory performance; $p = .023$), verbal memory ($p = .011$), sustained attention ($p = .038$), psychomotor speed ($p = .044$), and processing speed ($p = .0087$) all improved significantly versus placebo after 6 months (see Fig. 4). For both placebo and pooled MXan supplement groups, the other areas of cognitive performance not reported remained virtually unchanged (not exceeding +/− 1.75 point change) over the 6-month trial.

Analysis of relational changes over 6 months among MPOD, serum parameters, and cognitive metrics revealed that change in BDNF was significantly correlated with change in both composite memory ($r = 0.32$; $p = .014$) and verbal memory ($r = 0.35$; $p = .007$), whereas change in MPOD was correlated with change in both psychomotor speed ($r = 0.38$; $p = .003$), and processing speed (r = 0.35; $p = .007$). Change in serum lutein was significantly correlated to change in verbal memory ($r = 0.41$; $p < .001$), composite memory ($r = 0.31$; $p = .009$), and sustained attention ($r = 0.28$; $p = .036$). Change in serum zeaxanthin isomers was significantly correlated with change in verbal memory ($r = 0.33$; $p = .017$). Lastly, change in AOC was significantly associated with verbal memory ($r = 0.34$; $p = .021$), composite memory ($r = 0.29$; $p = .03$), and sustained attention (r = 0.35; $p = .016$).

4. Discussion

The results of this study suggest that 6 months of supplementation with MXans (at levels of at least 13 mg/day), significantly reduces IL-1β, leads to significant increases BDNF, MPOD, serum lutein/zeaxanthin, AOC, and significantly enhances several parameters of cognitive performance. Many of these changes were found to correlate with each

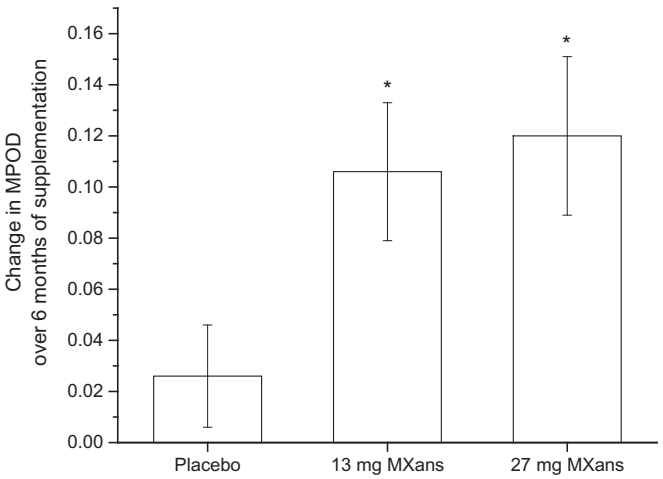

Fig. 1. Change in MPOD over the 6-month study period, for placebo and treatment groups. Asterisk denotes statistical significance versus placebo, $p < .05$. MXan supplement groups did not differ significantly.

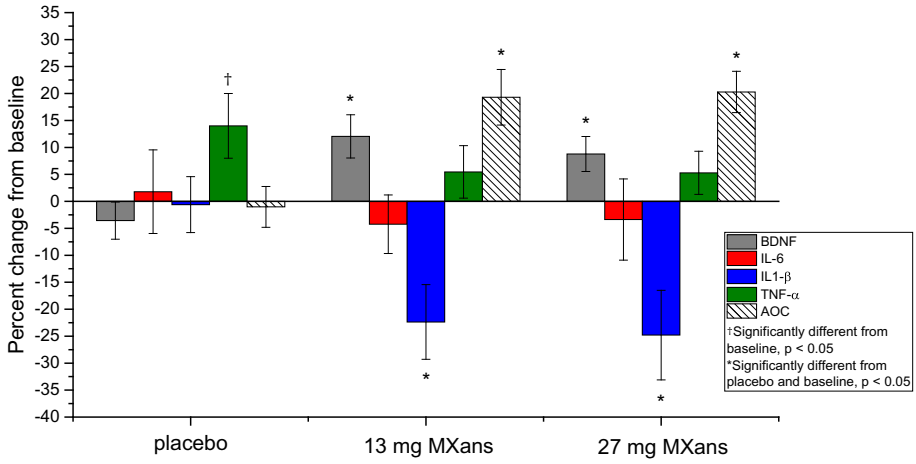

Fig. 2. Percent change from baseline in serum BDNF, pro-inflammatory cytokines and antioxidant capacity over the 6-month study period. See legend for symbol key and designation of statistical significance.

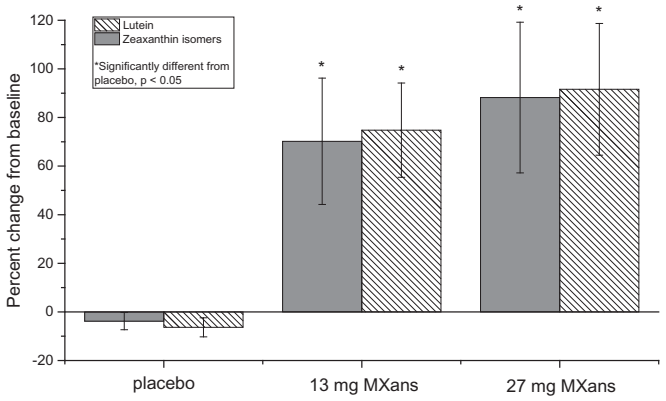

Fig. 3. Percent change from baseline in serum lutein and zeaxanthin isomers over the 6-month study period. See legend for symbol key and designation of statistical significance.

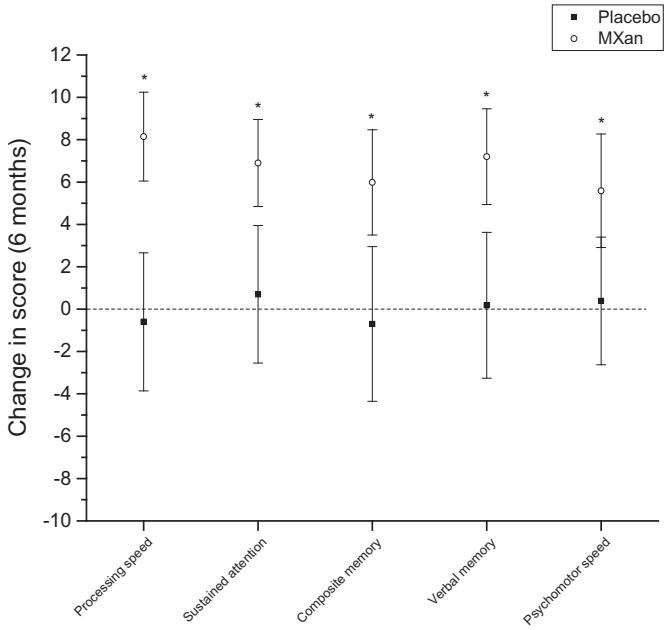

Fig. 4. Change in scores at 6 months in placebo vs. combined supplement groups (MXan), for five parameters of cognitive performance. Data points are means +/− standard deviations. Asterisks indicate statistical significance ($p < .05$).

other, and the relationships are consistent with previous animal work on the matter (e.g., the relationship between inflammation and reduced BDNF; see [13]). Additionally, there were marked differences in findings when comparing baseline with 6-month supplementation data. For example, at baseline (outside of the expected interrelationships between inflammatory parameters), we determined only one significant relationship between measures of antioxidant status (i.e. serum lutein/zeaxanthin, AOC, and MPOD) and other serum parameters (i.e., the correlation between MPOD and BDNF; see Table 2). Moreover, for the cognitive data, only three parameters of cognitive performance were shown to correlate significantly with other measures at baseline: serum lutein/verbal memory; MPOD/reaction time; BDNF/reasoning. By contrast, there were several measures whose changes after the supplementation period correlated significantly (see Table 4). The significant relationships between the change in serum lutein and zeaxanthins, BDNF, and IL-1β over the course of the study lend credence to the idea that regular consumption of MXans can interrupt the inflammatory pathway that leads to the reduction of BDNF. In particular, the strength of the relational changes determined between serum lutein and zeaxanthins/IL-1β is striking. Based on our data, a plausible model for this effect could involve increases in serum lutein and zeaxanthin/AOC, which in turn may reduce signaling for IL-1β and lead to lowered inflammation, which would finally produce increases in BDNF. Although there is no direct, causal link in our data for this idea, the results (coupled with current knowledge on the matter) are suggestive of this process. Moreover, there are several intermediate steps (e.g., inhibition of NFκ-B [71]) in the pathway of inflammation that may be strongly modulated by the MXans, and therefore may serve as mediating factors for the relational changes found in our study. This is especially true for IL-1β, which has been shown to be strongly associated with/modulated by NFκ-B (e.g. [39]). Although the general trend of reduction in the supplement groups (compared to placebo) was seen for both IL-6 and TNF-α, neither achieved statistical significance. This was a little surprising, given previous findings of MXan ability to reduce these pro-inflammatory cytokines in animal models (e.g. [20]) and in human populations (e.g. [15], in coronary artery disease patients). A lack of finding on these points could be explained by a floor effect, which may be present for generally healthy individuals. Previous work (e.g. [15]) has focused on diseased populations (for which pro-inflammatory cytokine levels are decidedly higher). Nevertheless, as noted above, the general pattern of reduction in supplementation groups was seen for both IL-6 and TNF-α (see Fig. 2).

The results of the dietary assessment may provide much explanatory power in terms of the effects of MXan supplementation. Our subjects – who were young, healthy adults – reported consuming a current diet

Table 4
Same as Table 3, but for changes in variables over the 6-month study period.

Measure	BDNF	IL-6	IL-1β	TNF-α	AOC	Lutein	Zeaxanthin isomers	MPOD
BDNF	1	–	–	–	–	–	–	–
IL-6	−0.094	1	–	–	–	–	–	–
IL-1β	−0.47*	0.26*	1	–	–	–	–	–
TNF-α	0.11	−0.138	−0.084	1	–	–	–	–
AOC	0.31*	−0.199	−0.283*	−0.116	1	–	–	–
Lutein	0.38*	−0.188	−0.51*	−0.204	0.79*	1	–	–
Zeaxanthin isomers	0.33*	−0.167	−0.412*	−0.187	0.61*	0.81*	1	
MPOD	0.44*	−0.077	−0.37*	0.121	0.43*	0.62*	0.66*	1

Values are Pearson correlation coefficients, and asterisks denote statistical significance ($p < .05$).

that, on average, was nearly devoid of the foods (e.g. leafy-green vegetables) that contain appreciable amounts of lutein and zeaxanthin. The diet of "young, healthy adults" may therefore not actually be sufficiently healthful. Specifically (based on our results), the level of dietary carotenoids such as lutein are apparently suboptimal. This observation is not new – data from NHANES (2003–2004, and 2011–2014) indicate that American adults consume only about 1–2 mg lutein/day (as cited in [14,34]), which is equivalent to roughly only ¼ cup of raw spinach. If, however, these powerful antioxidants/anti-inflammatory agents are introduced to the diet on a regular basis (as was the case in our study), improvements in terms of oxidative, inflammatory, neurotrophic, and ultimately, cognitive status may occur. To be sure, several of our subjects had average - high MPOD at baseline, which indicates that, at some point in their lives, they consumed foods containing MXans regularly. In other words, accumulation of MXans in the retina as MPOD may have occurred relatively early in life and been maintained and/or remained at a high level. Maintenance of tissue-bound carotenoids is due not only to consistent dietary intake, but also to the fact that physical quenching (which for the MXans accounts for > 99.5% of the quenching) of free radicals is a regenerative process, whereby a carotenoid molecule absorbs the energy of a reactive oxygen species, and then releases that energy as heat [75]. Since the carotenoid remains intact, it can be used over and over again; in the absence of excessive oxidative stress (e.g. brought on by smoking or systemic disease, like diabetes), a tissue-bound carotenoid could theoretically remain viable ad infinitum. Indeed, a relationship between childhood consumption of leafy-green/colored fruits and vegetables and adult MPOD has been reported [59], despite no correlation between current diet and MPOD.

As noted above, only some of the beneficial effects and relationships with MPOD were seen at baseline. This would seem to indicate that increasing the level of systemic, circulating MXans (via regular, consistent supplementation), and not simply increasing tissue concentrations in areas such as the retina or brain, is responsible for many of the beneficial effects seen in our study. By logical extension, the mechanism of action for several of the effects reported in the present study would appear to be the increase in systemic antioxidant/anti-inflammatory capacity in our treatment groups. Based on our results, this line of reasoning would also appear to be applicable to the improvements in cognitive performance found in the supplementation groups (see Fig. 4).

Because our study was conducted on young, healthy individuals, the improvements in cognitive performance are particularly meaningful. One might expect ceiling effects for several cognitive domains, given that young adults tend to be at or near the peak of overall cognitive performance (e.g. [52]). Despite this, students in the supplementation groups improved significantly in several cognitive domains (see Fig. 3). The functional significance of these changes is not small – as can be seen in Fig. 3, improvements ranged from roughly 6–8 points; given that the standard deviation of the cognitive tests is 15 points, an improvement in these domains of roughly 0.5 SD over 6 months is impressive. The only other study (to our knowledge) of lutein/zeaxanthin supplementation and cognitive performance in young, healthy adult participants was conducted by [50]. Like in the present study, they used the CNSVS cognitive assessment. Unlike the present study they used a 12-mg/day lutein + zeaxanthin supplement for a period of 1 year. They found significant improvement versus placebo in one cognitive parameter, visual memory (an increase of 4.5 points over placebo after the 1-year study period). It should be noted that not all of their participants complied with supplementation, and so their results should be interpreted cautiously; this is especially true in light of the fact that several of the effects determined in the present study appeared to be dependent on consistent ingestion of the MXans, and not necessarily deposition in tissues such as the retina or brain.

By obtaining serum parameters such as BDNF, AOC, and inflammatory cytokines, the present study is better suited than previous efforts to address potential mechanisms for effects seen. For example, the significant relational changes between serum parameters/MPOD and cognitive performance give clues as to potential mechanistic pathways involved in facilitating specific improvements. Interestingly, verbal and composite memory were both found to be significantly related to increases in BDNF. Because BDNF is so strongly linked to neuroplasticity (e.g., [11]), the increases in BDNF found in our study may have led to enhancements in areas of the brain that serve memory performance, such as the prefrontal cortex and hippocampus [48]. Improvements in processing speed and psychomotor speed, on the other hand, were significantly related to increases in MPOD. This finding suggests that enhancements in cognitive processing speed are at least partly dependent upon deposition of the MXans in neural tissues. As noted in the Introduction, MPOD is strongly correlated to brain levels of lutein and zeaxanthin [64], and so we assume that the increases seen in MPOD for our subjects corresponded to an increase in brain tissue concentrations. Additionally, a relationship between MPOD and speed of visual processing has been well established ([29,60], [76]). Consequently, what we have found in terms of MPOD and processing speed for cognition may be related to (or perhaps mediated by) increased visual processing speed.

As was noted in the Results section, our obtained serum values (BDNF, TNF-α, IL-6, and IL-1β) were reasonably comparable to those generated from healthy participants in previous investigations. Much is known about values for these parameters in diseased populations (e.g. [4]) – inflammatory parameters are often found to be ten times (or more) as high as controls, and BDNF is generally found to be substantially lower, often at half the levels of normal controls (e.g. in diabetes; [67]). The changes determined in our study were much less dramatic than what is typically found in diseased patients, but yet appear to nevertheless be statistically related to beneficial changes in other serum and cognitive parameters. Strictly speaking, however, the functional significance of the relatively small changes in inflammatory and neurotrophic parameters in the present study is not known, and requires further study.

Lastly, although our results were generated from a sample of young, healthy adults, they may hold promise for older populations, including those at risk for developing cognitive impairment, or perhaps even

those currently with cognitive impairment. Oxidative damage and inflammation are hallmarks of age-related cognitive decline (e.g., Alzheimer's disease [AD]; [72]); interestingly, a recent preliminary investigation of MXan/fish oil supplementation in AD patients indicated that 18 months of supplementation significantly reduced caregiver-observed disease progression [45]. Additionally, perhaps those with systemic, pro-inflammatory disease such as diabetes may benefit from MXan supplementation. It is known, for example, that diabetics have significantly lower MPOD than normal controls [18,54]. Moreover, previous data show that significantly low retinal BDNF levels precipitate diabetic retinopathy in rat models of diabetes, and that administration of BDNF therapy rescues retinal neurons from degeneration [56]. If a simple dietary modification can produce meaningful effects such as those in the present study, the public health benefits would be significant. Our MPOD results indicate that a reasonable level (13 mg/day) of macular carotenoid supplementation can produce a meaningful retinal response; in fact, the 13- and 27 mg/day responses were very similar in our study. However, it should be noted that the similarity in serum and ocular responses between the two groups may be explained by a couple of factors, namely exceptionally robust response in serum and retina for 4 of the members of the 13 mg group, and a relatively weak response for 4 of the members in the 27 mg group. The similarity in response could disappear with larger sample sizes or longer study periods; if this is the case, an effect of a higher dose may be revealed. In terms of MXan supplementation level there is no doubt a point of diminishing returns, but our study is not well equipped to address this point.

5. Conclusion

Based on the results of this study, we conclude that consistent ingestion of MXans significantly increases serum lutein and zeaxanthins, MPOD, BDNF, and AOC, and decreases IL-1β. Additionally, several aspects of cognitive performance were shown to improve. Relationships between changes in serum parameters/MPOD and cognitive performance measures suggest that those parameters of cognitive performance that involve complex processing (e.g., memory) are enhanced by increases in BDNF (perhaps via increased AOC as noted above), whereas parameters that involve speed of processing appear to be influenced by neural deposition of the MXans (i.e. MPOD). Given that cognition is a collection of several different abilities, these differences lend insight into the neural and biochemical mechanisms underlying different parameters of cognitive performance. The connection between the nervous system, the immune system, cognitive performance, and diet represents an exciting new area of research. Planned future studies include further evaluation of the relationship between diet and neuroplasticity, determination of the effects of nutritional intervention with carotenoids in early childhood on the developing brain, and investigation of potential effects of MXan supplementation in diabetes.

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