Lutein and Zeaxanthin Isomers Induces Mitochondrial Biogenesis and Improves Endurance Capacity in Muscle Cells

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Abstract

Lutein (3R, 3R, 6'R-β, ε-carroten-3,3′-diol) and zeaxanthin (3R,3'R-β,β-caroten-3,3′-diol) isomers from marigold flower extract are oxygenated carotenoids. In this study, we evaluated the beneficial effects of Lutein and Zeaxanthin isomers (L/Zi) (100 µg/ml) on enhancing the capacity of skeletal muscle using murine C2C12 myoblasts. To examine effect of L/Zi on skeletal muscle function, we investigated the effects of L/Zi on mitochondrial function including mitochondrial mass, mitochondrial oxygen consumption rate, IGF-1 and markers of muscle stress and damage in fully differentiated C2C12 myoblasts. We found that L/Zi treatment increased mitochondrial function by improving mitochondrial mass and mitochondrial oxygen consumption rate in skeletal muscle cells. L/Zi increased the IGF-1 concentration and reduced the concentration of cortisol and activities of the enzymes lactate dehydrogenase and creatine kinase, the indicators of fatigue and exercise induced stress. These results suggest lutein may be ergogenic functional food or dietary supplement.

Keywords: Macular Carotenoids; Mitochondrial Biogenesis; Mitochondrial Mass; Inflammation

Introduction

Lutein (3R,3R,6'R-β,ε-carroten-3,3′-diol) and zeaxanthin (3R,3'R-β,β-caroten-3,3′-diol) isomers are oxygenated carotenoids that are abundantly found in extract of Marigold flowers [1]. Lutein and zeaxanthin, often termed xanthophylls, are well-known to be protective against age-related vision loss or disorders like macular degeneration and cataracts whose major risk factor is oxidative stress [2]. These carotenoids are capable of antioxidant function by its effective lipid oxidation prevention by quenching free radicals [3]. Lutein and zeaxanthin isomers (L/Zi) supplementation has been reported to modulate genes involved oxidative stress and inflammation which protects from retinal damage induced by high fat diet [4]. Plasma lutein decreases oxidized LDL suggesting its potent antioxidant and anti-inflammatory effects that may protect against development of atherosclerosis and promotes cardiovascular health [5,6]. Some novel studies suggest some possible role of lutein and zeaxanthin on enhancing exercise performance [7,8]. Recent studies have demonstrated a positive association between serum levels of the carotenoids lutein and zeaxanthin and physical activity [9].

The influence of plant based food supplementation on improving the exercise performance has been a focus of our research team during the past several years. Antioxidant phytochemical supplements are used by physically active individuals as ergogenic functional foods for improving physiological or metabolic responses that may enhance exercise performance [10]. High intensity exercise training leads to excessive levels of reactive oxygen species, pro-inflammatory cytokines and excess concentration of cortisol which may hamper sports performance, induce fatigue, and delay recovery [11]. Therefore, developing antioxidant phytochemicals to prevent exercise-induced oxidative stress along with improving adaptation responses to endurance training is an attractive strategy for physically active individu-

als. The purpose of the present study was to assess in vitro effects of Lutein and Zeaxanthin isomers (L/Zi) on mitochondrial function, exercise-induced hormones - cortisol and IGF-1, antioxidant activity and markers of muscle damage using C2C12 murine myoblasts. Lutein and Zeaxanthin isomers (L/Zi) (Lutemax 2020™) extracted from Marigold flowers (*Tagetes erecta* L) was provided by OmniActive Health Technologies Ltd. (Pune, India). Caffeine, a popular exercise performance-enhancing supplement is used as positive control in the study [12].

**Materials and Methods**

**Cell culture**

C2C12 mouse myocytes and NCI-H295R human adrenal cortex-derived cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). C2C12 myoblasts was cultured at 37°C in humidified 5% CO₂ incubator in a growth medium containing high glucose DMEM supplemented with 10% heat inactivated fetal bovine serum (Corning, NY, USA), 1% L-glutamine (Corning) and 1% penicillin-streptomycin solution (Corning). For each assay, C2C12 cells were seeded in 96 or 24 well treated plates and incubated for 24h prior to switching to differentiation medium, which was DMEM supplement with 1% horse serum, for 5 days allowing myocyte differentiation. NCI-H295R cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Lonza, NJ, USA) supplemented with 2.5% Nu-Serum IV (Corning), and 1% ITS universal culture (Corning) supplement premix containing insulin, 6.25 µg/ml; transferrin, 6.25 µg/ml; selenium, 6.25 ng/ml; 1.25 mg/ml bovine serum albumin and 5.35 µg/ml linoleic acid and 1% penicillin-streptomycin (Corning). For cortisol assay, 2 × 10⁴ cells were plated in each well of a 24 well-plate and incubated for 24h before adding treatment.

**Cell viability assay**

Cytotoxicity assay was carried out using an AlamarBlue cell viability assay (Thermofisher, Waltham, USA). AlamarBlue is a cell-permeable non-fluorescent dye, which can be converted into red fluorescence on reductive reactions within live cells. C2C12 cells were seeded in 96-well black treated plates at a density of 1 × 10⁴ cells/mL and induced to differentiate. L/Zi was dissolved in dimethyl sulfoxide (DMSO) according to manufacturer’s instruction. Cell viability was assessed 24 h after incubation with L/Zi at the final concentrations of 1, 10, and 100 µg/ml or the drug vehicle (DMSO). Following the treatment, added AlamarBlue reagent as 10% of the sample volume followed by a 3 hours incubation at 37°C / 5% CO₂ incubator and then fluorescence was measured (excitation wavelength 570 nm and emission wavelength at 590 nm) using Biotek Synergy Hi Hybrid microplate reader (Biotek, VT, USA).

**Mitochondrial density assay**

C2C12 cells were seeded in 96-well black treated plates at a density of 1 × 10⁴ cells/mL, induced to differentiate, and supplemented with L/Zi (100 µg/ml), caffeine (500 µM) as positive control and vehicle DMSO control. After 24h of treatment, media was replaced with NAO probe (Invitrogen, MA, USA) (100 ng/mL) in PBS and incubated for 30 minutes at 37°C in a humidified atmosphere of 5% CO₂/95% air. NAO is a metachromatic dye that specifically binds cardiolipin, an inner mitochondrial membrane lipid regardless of the energetic state of the cell. Probe fluorescence was measured (excitation 380 nm, emission 645 nm) using a fluorescence Biotek Synergy Hi Hybrid microplate reader (Biotek, VT, USA). Fluorescent intensity relative to untreated control was then calculated.

**Oxygen Consumption Rate Assay**

Oxygen consumption was assessed using an oxygen consumption rate assay kit (MitoXpress-Xtra HS method) (Luxcel Biosciences) according to the manufacturer’s instructions. Briefly, C2C12 cells were grown in 96-well plates (2 × 10⁴ cells/well) until confluence and induced to differentiate into myotubes and treated L/Zi (100 µg/ml), caffeine (500 µM) as positive control and vehicle DMSO control. After 24h of treatment, C2C12 myotubes were treated with 10 µl of MitoXpress-Xtra (a phosphorescent oxygen-sensitive probe whose signal increases over time when O₂ is depleted in the solution) immediately before measuring the fluorescence intensity (excitation/emission, 380 nm/650 nm at 37°C, 0.5-minutes interval) using a time-resolved fluorescence plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, Biotek). Measurement was performed under a sealed environment (by overlaying each well with HS mineral oil), in which

the exchange of $O_2$ was limited. Oxygen-consuming glucose oxidase was used as a reference.

**Antioxidant Capacity**

C2C12 cells were grown in 24-well plates ($3 \times 10^4$ cells/well), induced to differentiate into myotubes and treated with L/Zi (100 µg/ml), resveratrol (100 µM) as positive control and vehicle DMSO control. After 24h of treatment, the total antioxidant capacity was determined in C2C12 homogenates by commercial kit from Sigma Co. (St. Louis, MO, USA) following the manufacturer’s instructions. Briefly, the antioxidant assay is based on the formation of a ferryl myoglobin radical from myoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) to produce a radical cation ABTS⁺, a soluble green color chromogen that can be determined at 405 nm. In the presence of antioxidants, the radical cation is suppressed to an extent dependent on the activity of the antioxidant and the color intensity is decreased proportionally. Trolox, a water-soluble vitamin E analogue, serves as a standard or a control antioxidant.

**Lactate dehydrogenase and Creatine Kinase activity**

Muscle injury can was assessed by determining the release of the cytosolic enzyme lactate dehydrogenase (LDH) and creatine kinase (CK) activity. Briefly, myoblast cultures ($1 \times 10^4$ cells) in a 24 well plate were incubated with L/Zi (100 µg/ml), and vehicle DMSO control for 24h. After the pre-treatment, cells were exposed 2, 4-dinitrophenyl (DNP), a mitochondrial uncoupling agent for an hour to induce injury. Following treatments, supernatant of cells was harvested and used for determination of LDH and CK activity using a commercial kit (Roche, QC, Canada).

**IGF-1 Elisa assay**

C2C12 cells were grown in 96-well plates ($2 \times 10^4$ cells/well) until confluence and induced to differentiate into myotubes and supplemented with L/Zi (100 µg/ml), caffeine (500 µM) as positive controls and vehicle DMSO control and incubated for 24h. To evaluate the effect of treatments on IGF-1 concentration, the mouse IGF-1 enzyme-linked immunosorbent assay (ELISA) kit (Sigma Aldrich) was utilized. The kit provides a quantitative measurement of mouse IGF-1 concentrations in cell culture supernatants by employing an antibody specific coated 96-well plate. Standards and samples were added to the coated plate and IGF-1 present in the sample bound to the immobilized antibody. After removing any unbound antibody, a biotin-conjugated antibody specific for IGF-1 was added to the wells. Then horseradish peroxidase (HRP)-conjugated streptavidin was added to the wells. The wells were washed again, followed by addition of a colorimetric substrate solution. Color developed in proportion to the amount of bound IGF-1. Then the color intensity was read at absorbance 450 nm using a microplate reader. Blank-corrected unknown sample protein concentrations were then extrapolated from a known standard curve.

**Cortisol Elisa assay**

H295R were grown in 24-well plates ($5 \times 10^4$ cells/well), induced to differentiate into myotubes. and treated with L/Zi (100 µg/ml), caffeine (500 µM) as positive control and vehicle DMSO control. After 24h of treatment, DetectX cortisol enzyme immunoassay kit (Arbour assays, Ann Arbor, MI) was used to exam cortisol concentrations in the cell culture medium following manufacturer’s instructions. 50 µL of samples or standards were pipette into wells in the plate, followed by 25 µL of DetectX cortisol conjugate and 25 µL DetectX cortisol antibody. The plate was incubated at room temperature for 1 hour, washed 4 times with wash buffer. 100 µL of TMB substrate was added to each well, incubated at room temperature for 30 minutes. 50 µL of the stop solution was added to each well. Optical density generated from each well was read in a plate reader at 450 nm; cortisol concentrations in cell culture medium with treatments were calculated based on absorbance.
**Statistical analysis**

In all experiments, each sample was assayed in triplicate wells, in at least three independent experiments. The results were expressed as the mean ± SD of three separate experiments and analyzed using student t test and ANOVA. Values of p < 0.05 were considered to be statistically significant differences.

**Results**

**Lutein and Zeaxanthin isomers have no effect on C2C12 cell viability**

As shown in the figure 1, the cell viability of myotubes not shown no significant difference compared to DMSO control when exposed to various concentrations of L/Zi. The results indicated that L/Zi was not toxic to myotubes at concentrations of 1, 10, 100 µg/ml and L/Zi concentration of 100 µg/ml was used for further analysis in this study.

**Figure 1:** Effects of lutein and zeaxanthin isomers (L/Zi) on cell viability in C2C12 cell after 24h incubation at a concentration of 1, 10 and 100 µg/ml. Data are presented as mean ± SD.

**Lutein and Zeaxanthin isomers increases mitochondrial mass and oxygen consumption in C2C12 cells**

To investigate the effect of L/Zi on mitochondrial density, we treated C2C12 myotubular cells with or without 100 µg/ml L/Zi for 24h. After 24h, the cells were stained with NAO, a mitochondrial specific fluorescent dye which is considered to be a surrogate marker of mitochondrial content. As shown in figure 2, assay revealed significantly (p > 0.001) greater NAO fluorescent intensity in L/Zi treated cells than in control cells. There was 22% increase in mitochondrial mass in L/Zi treated cells compared to DMSO control cells. To further establish the effect of lutein and zeaxanthin on mitochondrial biogenesis, we analyzed cellular respiration. Cellular respiration assay evaluated whether functional changes accompanied the apparent increase in mitochondrial mass. Using the mitoxpress kit, we measured

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mitochondrial oxygen consumption assay in intact cells. L/Zi treated cells had a significantly ($p > 0.05$) higher oxygen consumption rate than control cells (Figure 3). The data suggest that L/Zi treatment results in increased oxidative capacity. The pattern of mitochondrial density and oxygen consumption displayed with L/Zi supplementation were similar to the effect to positive control caffeine.

**Figure 2:** Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) and caffeine (500 µM) (positive control) treatment increased mitochondrial mass in C2C12 cells after 24h incubation. Data are presented as mean ± SD. *$P < 0.05$ significantly different to control.

**Figure 3:** Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) and caffeine (500 µM) (positive control) treatment increased mitochondrial oxygen consumption rate in C2C12 cells after 24h incubation. Data are presented as mean ± SD. *$P < 0.05$ significantly different to control.

Lutein and Zeaxanthin isomers enhances antioxidant potential in C2C12 cells

In order to maintain mitochondrial function, reactive oxygen species reduction is important. Therefore, we assessed the effects of L/Zi treatment on antioxidant systems in C2C12 myotubes. The capacity of the antioxidant potential in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analogue. As shown in figure 4, L/Zi and positive control resveratrol treatment dramatically increased antioxidant potential in C2C12 cells compared to control cells.

![Figure 4: Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) and resveratrol (100 µM) (positive control) treatment increased antioxidant potential in C2C12 cells after 24h incubation. Data are presented as mean ± SD. *P < 0.05 significantly different to control.]

Lutein and Zeaxanthin isomers improves IGF-1 production in C2C12 cells

IGF-I exerts acute anabolic actions on protein and carbohydrate metabolism by increasing the cellular uptake of amino acids in cells. In order to determine the effect of L/Zi on cellular hypertrophy, IGF-1 secretion in C2C12 cells was used as marker. L/Zi or caffeine treatment induced significant (p < 0.05) increase in the secretions of IGF-I in C2C12 cells compared to control cells (Figure 5).

Lutein and Zeaxanthin isomers lowers the lactate dehydrogenase and creatine kinase in C2C12 cells

LDH and CK activities are used as indicator of the occurrence of muscle cell damage or disturbance. Muscle injury was induced in pre-treated cells using DNP. Compared to DNP control, CK and LDH activity levels in the cells that treated with L/Zi for 24h was significantly (p < 0.05) decreased by 36 and 85% respectively (Figure 6 and 7).

![Figure 5](image5.png)

**Figure 5:** Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) and caffeine (500 µM) (positive control) treatment increased IGF-1 concentration in C2C12 cells after 24h incubation. Data are presented as mean ± SD. *P < 0.05 significantly different to control.

![Figure 6](image6.png)

**Figure 6:** Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) treatment decreased creatine kinase (CK) activity in C2C12 cells exposed to DNP after 24h of pre-treatment. Data are presented as mean ± SD. $ P < 0.05 significantly different to control, *P < 0.05 significantly different to DNP control.
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H295R cells are a transformed human adrenal cell line which secretes all the steroid intermediates including cortisol of the steroidogenesis pathway, and has been found useful for studying steroidogenesis [13]. As shown in figure 8, L/Zi and caffeine showed a non-significant decreasing trend with respect to control cells in the secretion of catabolic hormone cortisol in H295R cells.

**Figure 7: Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) treatment decreased lactate dehydrogenase (LDH) activity in C2C12 cells exposed to DNP after 24h of pre-treatment. Data are presented as mean ± SD. $ P < 0.05$ significantly different to control, $* P < 0.05$ significantly different to DNP control.

**Figure 8: Lutein and zeaxanthin isomers (L/Zi) (100 µg/ml) and caffeine (500 µM) (positive control) treatment increased cortisol concentration in C2C12 cells after 24h incubation. Data are presented as mean ± SD. $* P < 0.05$ significantly different to control.
Discussion

In this study, we observed lutein and zeaxanthin isomers treatment effectively enhance skeletal muscle function in C2C12 cells. C2C12 cells represent a reliable model to study the effect of ergogenic molecules commonly utilized in improving exercise performance [14]. The primary finding of the present study was that L/Zi treatment stimulated mitochondrial function, specifically increasing mitochondrial density and oxygen consumption rate in C2C12 myoblasts. We also observed L/Zi enhances the anabolic insulin like growth factor-1 hormone and tended to decrease the catabolic cortisol in protein metabolism in myotubes. Furthermore, we observed L/Zi treatment increases antioxidant potential and have the ability to protect muscle cells from injury by decreasing muscle enzymes LDH and CK.

The improvement of mitochondrial function by L/Zi was demonstrated by the results of mitochondrial mass which is commonly used in cells to evaluate mitochondrial function. Mitochondrial content is an important quantitative indicator of oxidative capacity and is often used measures of muscle bioenergetic capacity [15]. We also found L/Zi regulation of mitochondrial mass in muscle cells to be associated with the stimulation of oxygen consumption rate. By promoting mitochondrial content, L/Zi increased mitochondrial oxygen consumption rate implying that L/Zi could improve energy metabolism. In addition, L/Zi treatment activates the antioxidant system in the cells which could protect cells against oxidative stress accompanied by increased oxygen consumption. Therefore, we speculated that lutein zeaxanthin isomers activates antioxidant systems and mitochondrial function that might contributed to the protective effects against exercise induced oxidative stress in skeletal muscle cells.

In this study, we found that L/Zi induced insulin-like growth factor -1 (IGF-1) which plays a major role in the regulation of skeletal muscle growth. Similar to these effect, recent studies have revealed that carotenoids induce IGF-1 concentration in the skeletal muscle, leading to AKT activation and muscle hypertrophy [16]. Kitakaze., et al. reported that administration of carotenoids increases mass and induces functional hypertrophy in the animal models by promoting IGF-1-mediated protein synthesis and by reducing ubiquitin-mediated protein degradation [17]. In addition, IGF-1 acts as mitochondrial protector in several experimental models [18] and reported to have several metabolic and anabolic properties related with mitochondrial function. Furthermore, IGF-1 pathway act as an intrinsic mediator of skeletal muscle repair and adaptation, increasing the proliferation potential of satellite cells, promoting their differentiation, enhancing muscle regeneration and eventually determining protein synthesis and increase muscle mass [19]. The levels of cortisol, LDH and CK are known as indicators of accumulated fatigue and exercise induced stress. L/Zi supplementation effectively inhibited these increase of cortisol, LDH and CK in C2C12 cells supporting the antifatigue effect of L/Zi.

The beneficial effect of L/Zi have could be ascribed to its antioxidant potential. L/Zi suppress the free radical formation which showed its potent antioxidant property. The oxygen free radicals production increases with the rise in oxygen consumption during high intensity exercise which in turn leads to reduced strength and increased muscle soreness. Consumption of antioxidants and anti-inflammatory molecules is crucial to counteract these negative side effects. Cheng., et al. showed lutein protects against skeletal muscle injury by down regulating oxidative stress and inflammatory mechanisms [21]. The exogenous antioxidants from diet interact with endogenous antioxidants to form a cooperative antioxidant network, preventing exercise-induced oxidative stress and reducing physical fatigue by scavenging the free radicals and reactive oxygen species [22]. The finding of this study suggests that L/Zi is a nutraceutical supplements which is likely to exert positive effects on muscle strength and facilitate recovery from fatigue and attenuate exhaustive exercise-induced oxidative damage.

Conclusion

In conclusion, our data suggest that the lutein and zeaxanthin isomers exerts beneficial effects on exercise endurance by improving mitochondrial function, anabolic IGF-1, enhancing muscle strength and downregulating factors related to fatigue. All these diverse effects may be related to antioxidant property and amelioration of skeletal muscle injury by lutein and zeaxanthin isomers on skeletal muscle cells. Further animal and human studies are indeed required to prove the efficacy of application of lutein and zeaxanthin isomers as a potential ergogenic aid in physically active individuals.

Bibliography


