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EFFECT OF β-CAROTENE AGAINST COLCHICINE-INDUCED MYOTOXICITY IN MALE RATS

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ABSTRACT
Myopathy affects physical disability and interrupts the health system, hence intrudes life quality of humans. Colchicine is one of several drugs that has many side effects and can induce myotoxicity. Antioxidants should be available in sufficient concentration to keep the balance with oxidative stress and avoid tissue damage. Beta carotene (β-carotene) is an important member of carotenoids which has a powerful oxidative scavenger capacity. There is a great attention to more developed strategies for the use of β-carotene to avoid cardiac myopathy. This study aimed to evaluate the role of β-carotene (10 mg/ kg b.wt/day, orally for 2 weeks) against myotoxicity induced by colchicine (0.05 mg/ kg b.wt/day, intraperitoneanally for 2 weeks) in adult male albino rats. The results showed that β-carotene had improved myopathy markers either by co-administration or post-administration with colchicine. Also, β-carotene restored body and muscle weight gains, rearranged autophagy signals (LC3-II and LAMP-2) and collagen-1. Moreover β-carotene attenuated ROS and NO with increase of SOD levels and improvement of CK and LDH activities. Hence, β-carotene offers an exciting possibility of using it as effective antioxidant against muscle toxicity and atrophy induced by colchicine.

KEYWORDS: β-carotene, myotoxicity, autophagy, antioxidant, colchicine.

INTRODUCTION
Myopathy is characterized by pain, weakness and atrophy which affects physical disability and interrupts the body systems consequently intrudes the quality of life of individuals.[1] Sometimes, drugs used for therapeutic intrusions either alone or in combination may cause myotoxicity, causing variable degrees of symptoms from mild discomfort to permanent damage and disability.[2] Examples for such drugs are colchicine, chloroquine, statins and others.[1] Colchicine is used mainly in the treatment of gout and also valuable in inflammatory diseases as familial Mediterranean fever (FMF).[3] However, colchicine-induced myopathy has been remarkably designated in the literature.[4] Antioxidants are vital for life, and the expectations of antioxidants as health promoting agents were very high.[5,6] Hence, antioxidants should be available in sufficient concentrations to avoid tissue damage.[7] The carotenoids are abundant in nature and are responsible for pigmentation in animals and plants.[8] In recent years most attention has been focused on this group of pigments to understand their function especially as antioxidants.[9] Beta carotene (β-carotene) is an important member of carotenoids that is available in fruits and vegetables.[8] β-carotene is the precursor of vitamin A (Retinol) and can powerfully scavenge free radicals in living animal systems.[10,11] Moreover, its consumption can reduce the risk of various disorders, like cardiac disease and cancer.[12,13] Besides, there is a great attention for the usage of β-carotene in avoidance and managing of cardiac myopathy.[14] Otherwise, this study aimed to evaluate the role of β-carotene against colchicine-induced myotoxicity in male rats.

MATERIALS and METHODS
Experimental Animals
Male Wister rats (150 g average body weights) were kept for one week under conventional conditions before the experiment. Rats were subjected to 12:12 light/dark cycle and allowed to free food and water. The ethical protocols for the experimental animal treatment were followed according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol recommended by Ain Shams University.

Colchicine
Colchicine (50 mg tablets), El Nasr Pharmaceutical Chemical Co., Egypt. Colchicine was injected intra-peritoneally to rats at a dose of 0.05 mg/kg in normal saline for 2 weeks.[15,16]

β-carotene

β-carotene ≥97.0% (UV), Sigma-Aldrich (USA). β-carotene was orally supplemented to rats at a dose of 10 mg/kg/day dissolved in olive oil for 2 weeks.[12]

EXPERIMENTAL DESIGN

Rats were divided into 7 equal groups (6 rats each) according to the following protocol: The 1st is the **normal control** group that was left with no treatment. The 2nd is the **saline** group that was injected intra-peritoneal with colchicine vehicle (0.5 ml saline/day) for 2 weeks. The 3rd is the **olive oil** group that orally received β-carotene vehicle (0.5 ml/day olive oil) for 2 weeks. The 4th is **β-carotene** group that received oral dose of β-carotene (10 mg/kg/day in 0.5 ml olive oil) for 2 weeks. The 5th is the **colchicine** group was injected with intra-peritoneal dose of colchicine (0.05 mg/kg/day in 0.5 ml saline) for 2 weeks. The 6th is **colchicine+β-carotene** group that administered colchicine with β-carotene as the same previous doses and routes for 2 weeks. The 7th is the **colchicine/β-carotene** group that was injected with colchicine for 2 weeks followed by β-carotene for another 2 weeks. At the end of the experimental period, rats were weighted and sacrificed under ether anaesthesia. For the biochemical studies, blood samples were withdrawn by heart acupuncture to separate sera. Muscle tissues (tibialis anterior skeletal muscle) were dissected washed with saline, weighted and homogenated in phosphate buffer solution at PH 7.4 for the biochemical analysis.

EXPERIMENTAL ANALYSIS

PCR analysis: Microtubule-associated protein-1 light chain-II (MAP1LC3-II), lysosomal associated membrane proteins-2 (LAMP-2) and collagen-1 gene expressions in muscle tissue were measured using PCR, polymerase chain reaction. Briefly, tibialis anterior muscle tissues of all animals were used for RNA extraction using Qiagen tissue extraction kit (Qiagen, USA). Total RNA was used for cDNA conversion by using high capacity cDNA reverse transcription kit (Fermentas, USA) according to manufacturer's instructions.[17] Reference β-actin gene was used and SYBR Green was used for double stranded DNA synthesis. The amplifications were completed using 40 cycles of denaturation for 15 seconds at 95 C^o and extension at 60 C^o for 60 seconds. QRT-PCR amplification and analysis were caused using an Applied Bio system with software version 3.1 (StepOne™, USA).[18] The primers were used as the following:

β-actin: F: 5′-TGTTGTCCCTGTATGCCTCT-3′
R: 3′-TAATGTCACGCACGATTTCC-5′.[19]
LC3-II: F: 5′-ACCCTCCCTGCATGCAGCTGTCC-3′,
R:5′- ACCAGGGACATGACGACGTACACAACC-3′.[20]
LAMP-2: F: 5′-TGGCTCAGCTTTCATTGTTTC-3′
R:5′-CATATAAGAACTTCCCAGAGGAGCAT-3′.[20]
Collagen-1: F: 5′-GAG CGG AGA GTA CTG GAT CG-3′
R: 5′-GAT TGG GAT GGA GGG AGT TT-3′.[21]

Reactive oxygen species (ROS): Muscle ROS was measured according to manufacturers' protocols of rat ELISA kit (MyBioSource, Inc. CA, USA), No. MBS775259. Nitric oxide (**NO)**: Muscle NO was measured using NO assay kit, Biodiagnostic, NO. 2533.[22] **Superoxide dismutase (SOD)**: Muscle SOD was measured using SOD assay kit, Biodiagnostic, No. SD 2521.[23] **Creatine kinase (CK)**: Serum CK was measured using BioMed assay kit.[24] Lactate dehydrogenase (**LDH)**: Serum LDH was measured using BioMed assay kit.[25]

STATISTICAL ANALYSIS

Results of the present study were expressed as mean±S.E. of the mean. SPSS (the Statistical Package for the Social Sciences) program, version 16.0 was used to compare the significance between each two groups. The differences were considered significant when $P<0.05$.

RESULTS

As shown in (Table 1) there were no different changes between normal control and vehicles or β-carotene groups (*p>0.05*). On the other hand the administration of colchicine for 2 weeks produced significant changes in different experimental parameters comparing to saline group (*p<0.05)*; where colchicine caused loss in both body and muscle weight gain (-7.4%, b.wt and -12%, m.wt), while colchicine increased autophagy signals represented by high expression of muscular LC3-II and LAMP-2 genes (138.6%, and 89.4%) respectively. Also, gene expression of collagen-1 increased with 273.9%. Concerning to oxidative stress, as shown in (Table 2), results obtained that colchicine elevated muscular ROS and NO contents (172.8% and 158.7%) respectively, while SOD activities decreased with (-62.5%). Moreover, serum CK and LDH activities were elevated after colchicine treatment (129.3% and 90.8%) comparing to saline group. On other side, as shown in (Table 1) and comparing to colchicine group, treatment with β-carotene caused statistical changes (*p<0.05);* either accompanied with colchicine (**colchicine+β-carotene** group) or after 2 weeks of colchicine treatment (**colchicine/β-carotene** group), where body weight and muscle weight gain were increased (5.2%, b.wt and 13.8%, m.wt.) for **colchicine+β-carotene** group and (6.7%, b.wt and 20.7% m.wt) for **colchicine/β-carotene** group. While LC3-II expression was restored again by β-carotene (-65.2% and -59% lower) for **colchicine+β-carotene** and **colchicine/β-carotene** respectively. Parallel results were obtained for LAMP2 expression with changes of (-39% and -28% lower) respectively for the corresponding groups. Also, collagen-1 was rearranged by β-carotene with changes of (-47.5% and -53.5% lower) respectively. Continuously, β-carotene decreased ROS and NO values comparing to colchicine group (-50%, ROS and -31%, NO) for **colchicine+β-carotene** and (-51%, ROS and -36%, NO) for **colchicine/β-carotene.** On the other hand, β-carotene elevated SOD activities with (92% and 118%) for the corresponding groups, Table 2. Moreover, serum CK and LDH

activities were attenuated after β-carotene treatment comparing to colchicine group (-41%, CK and -35%, LDH lower) for **colchicine+β-carotene** and (-41%, CK and -36%, LDH lower) for **colchicine/β-carotene,** Table 2**.**

Table 1: Effects of β-carotene on body and muscle weights as well as myopathy markers in rats treated with colchicine.

Group	Body weight (g)	Muscle weight (g)	LC3-II Relative exp.	LAMP-2 Relative exp.	Collagen-I Relative exp.
Normal control	150.8±2.06	0.34±0.01	0.97±0.04	1.02±0.03	1.04±0.07
Saline	149.3±1.11	0.33±0.03	0.88±0.04	1.04±0.07	1.11±0.05
	NS	NS	NS	NS	NS
Olive oil	150±1.08	0.34±0.01	0.94±0.05	0.98±0.01	1.03±0.03
	NS	NS	NS	NS	NS
β-carotene	150.3±1.75	0.36±0.01	1.13±0.12	1.09±0.07	1.01±0.09
	NS	NS	NS	NS	NS
Colchicine	138.3±0.75	0.29±0.004	2.10±0.12	1.97±0.11	4.15±0.29
	a	a	a	a	a
Colchicine + β-carotene	145.5±0.87	0.33±0.02	0.73±0.03	1.20±0.07	2.18±0.14
	b	b	b	b	b
Colchicine/β-carotene	147.5±0.96	0.35±0.02	0.86±0.04	1.41±0.02	1.93±0.08
	b	b	b	b	b

The results are presented as Mean ± SE (n=6/group). N.S= non-significant change comparing to normal control, p>0.05 a: Significant change from saline, p<0.05 b: Significant change from colchicine, p<0.05.

Table 2: Effects of β-carotene on muscle oxidative state and myopathy enzyme markers of rats treated with colchicine.

Group	ROS pg/g	NO U/g	SOD U/g	CK U/L	LDH U/L
Normal control	28.18±0.63	15.95±0.42	10.70±0.14	124.40±1.30	113.95±0.66
Saline	28.88±0.68	16.35±0.30	10.40±0.33	123.21±0.72	115.30±1.49
	NS	NS	NS	NS	NS
Olive oil	27.90±0.72	14.90±0.83	10.80±0.64	128.50±3.56	115.55±1.65
	NS	NS	NS	NS	NS
β-carotene	25.88±0.51	14.23±0.54	10.98±0.33	121.03±2.32	115.01±1.65
	NS	NS	NS	NS	NS
Colchicine	78.80±0.98	42.30±1.07	3.90±0.15	282.50±1.68	220.01±2.43
	a	a	a	a	a
Colchicine + β-carotene	39.23±0.56	29.04±0.86	7.48±0.30	165.90±2.76	142.1±1.69
	b	b	b	b	b
Colchicine / β-carotene	38.93±0.68	27.15±0.39	8.50±0.38	166.04±3.75	140.9±1.24
	b	b	b	b	b

The results are presented as Mean ± SE (n=6/group). N.S= non-significant change comparing to normal control, p>0.05 a: Significant change from saline, p<0.05 b: Significant change from colchicine, p<0.05.

DISCUSSION

This study demonstrated the protective effects of β-carotene against colchicine-induced toxicity in rat muscle. Our data revealed that β-carotene attenuates muscle atrophy, and suppress oxidative stress induced by colchicine. A brief discussion of these issues is as follows:

Results revealed that β-carotene restored muscle mass gain and consequently increased mean body weights which decreased by colchicine. This means that β-carotene played a protective role against muscle atrophy induced by colchicine. Important fact is that autophagy-lysosome system is a vital mechanism required to maintenance of muscle mass and homeostasis in the face of stresses and either reduced or excessive autophagy function leading to specific myopathies.[26,27] Autophagy is an intracellular recycling mechanism, whereby damaged or terminated cellular components are engulfed in a double membrane structure called the autophagosome, which is ultimately delivered to the lysosome for digestion of its contents.[28] And this occurs via several steps; phagophore initiation and expansion, autophagosome formation, and autophagosome fusion with lysosomes.[29] Thus the disturbances in autophagy such as the activation of autophagy signaling pathways while inhibiting the degradation of synthesized autophagosomes, resulting in myotoxicity and muscle atrophy.[29,30] However, LC3-II is a key identifier of

autophagosomes and LAMP-2 is a lysosomal protein indicator.[31]

It was reported that colchicine induced myotoxicity over prevent autophagosome-lysosome fusion, so autophagosome vacuoles were accumulated.[4,28,29] This is confirmed in this study by high expressions of muscle autophagy-related genes, LC3-II and LAMP-2.[29] Enhanced LC3-II and Lamp-2 levels may be caused by the impaired degradation of autophagosomes which mean that LC3-positive autophagosomes failed to localize with LAMP-2.[28]

Moreover, there was crosstalk between oxidative stress and autophagy disturbances caused by colchicine.[32,33] Duan et al reported that ROS disrupted the early steps of the autophagy pathway which lead to accumulation of ubiquitinated protein and mitochondrial dysfunction that activated tyrosine kinase to induce NO.[34]

On other side, in accordance with Tran et al.[35], β-carotene restored autophagy gene expressions which indicated that β-carotene reorganized autophagy process that maintained muscle mass and induced functional hypertrophy.

Thus the main cure of repaired autophagy role of β-carotene may referred to its powerful action as oxidative scavenger and the ability to overcome oxidative stress which induced by colchicine.[36] This is verified in the current results by decreasing levels of muscle ROS and NO and increasing SOD activity after β-carotene treatment.[37,38] It was reported that β-carotene contributes the most towards antioxidant property compared to phenolic compounds.[39] Besides, according to Filomeni et al.[36], autophagy could be included in the antioxidant and DNA repair systems for help clearing the cells of all irreversibly oxidized biomolecules (proteins, DNA and lipids). Consequently, overexpression of antioxidant superoxide dismutase triggers successful autophagy function.[40]

More to the point, it is well established that muscle atrophy is the consequence of a loss in muscle protein.[10,41,42] Oxidative stress obtained in this study may limit protein synthesis while antioxidant stimulated it. A novel study suggested that a combination of β-carotene, astaxanthin and resveratrol, even in small amounts, stimulated protein synthesis during the muscle hypertrophic process.[10] Continuously, the regulatory autophagy response of β-carotene perhaps caused by promoting insulin-like growth factor1-mediated protein synthesis and by reducing ubiquitin-mediated protein degradation.[43] Therefore, these observations suggest that β-carotene can act as antioxidant effectively competes with colchicine oxidative actions against muscle atrophy.

Type I collagen is normally associated with tissue stiffness, while disarranged structure can compromise the mechanical properties of muscle and affect its behavior.[44] However, the increase in synthesis of fibrillar collagens may induce myofibrosis which impairs muscle function.[10] In this study, type I collagen increased in muscle tissue after colchicine administration while it was rearranged with β-carotene treatment.[7] Upon the fact that ROS production induce fibroblast differentiation into myofibroblasts and increased collagen fibers.[45] Thus, these results may indicate that β-carotene has preventive effects on collagen-mediated fibrosis through mediating ROS production.[46,47]

As it is known, CK and LDH enzymes are markers to show energy status and metabolism especially at the stress condition of muscle.[48,49], and remain the first line biomarkers of muscle injury.[3,50] An increase in LDH and CK levels mean leakage of these enzymes into the serum due to increase in membrane permeability of muscle caused by ROS-lipid peroxidation.[3,51] β-carotene abled to prevent elevation of serum LDH and CK levels, which can be attributed to its membrane stabilizing effects and/or free radical scavenging activity.[52,53] However, better maintenance of LDH and CK indicated better metabolism and membrane health with the administration of β-carotene.[42]

CONCLUSION AND RECOMMENDATION

Nonetheless, many of these effects can be attributed to the action of β-carotene as a scavenger for free radicals and oxidants. Since, β-carotene offers an exciting possibility of using it as antioxidant impact against muscle toxicity and atrophy induced by colchicine. Therefore, a healthy lifestyle including a diet rich in carotenoids such as β-carotene is the best prevention strategy against muscle toxicity.

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