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Ellagic acid improves muscle dysfunction in cuprizone-induced demyelinated mice via mitochondrial Sirt3 regulation

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ABSTRACT

Sirt3 enzyme and mitochondrial abnormality can be related to excess fatigue or muscular dysfunction in multiple sclerosis (MS).Ellagic acid (EA) has a mitochondrial protector, iron chelator, antioxidant, and axon regenerator in neurons.In this study the effect of EAon muscle dysfunction, its mitochondria, and Sirt3 enzyme incuprizone-induced model of MSwas examined.

Demyelination was induced by a diet containing 0.2% w/w cuprizone (Cup) for 42 days and EA administered daily (5, 50, and 100 mg/kg P.O) either with or without cuprizone in mice. Behavioral tests were assessed, and muscle tissue markers ofoxidative stress, mitochondrial parameters, mitochondrial respiratory chain activity, the Sirt3 protein level, and *Sirt3* expression were also determined.

Luxol fast blue staining and the behavioral tests were performed toassess the implemented model. In Cup group an increased oxidative stress in their muscle tissues was observed. Also, muscle mitochondria exhibited mitochondria dysfunction, lowered mitochondrial respiratory chain activity, Sirt3 protein level, and *Sirt3* expression.EA prevented most of these anomalous alterations.

Sub-chronicEA co-treatment dose-dependently ameliorated behavioral and muscular impairment in mice that received Cup.EA can effectively protect muscle tissue against cuprizone-induced demeylination via the mitochondrial protection, oxidative stress prevention and Sirt3 overexpression.

1. Introduction

Multiple sclerosis (MS) is an immune-mediated process in which abnormal response of the body's immune system is directed toward CNS.Mitochondria are the most important cellular compartments for ATP production, which also play a vital role in programmed cell death [1].A pathogenic role was suggested for mitochondrial dysfunction in axonal degeneration. Increased ROS production, mtDNA dysfunction [1] and reduced activity of mitochondrial respiratory chain complex were reported in MS [2].Several studies revealed that muscular weakness in MS comes from central nervous system damage [3]; however, environmental factors might also play an important role in inducing muscle dysfunctionin MS [4]. The excess fatigue and muscular dysfunction can be explained by reduced ATP production and mitochondrial abnormality [1].

Sirt3 is an important mitochondrial enzyme with significant role in the mitochondria-related disorders including Alzheimer's disease, cancer, diabetes and aging.Previous studies indicated an over expression of Sirt3 suppressed ROS associated with neuronal death.On rat brain, mitochondrial dysfunction was directly associated with the Sirt3appropriate activity [5].Among sirtuins (sirt1-7), Sirt3 enzyme suggested to be involved in myelination preservation of neurons in the mouse brain [6]. These preliminary findings suggest that Sirt3 enzyme can potentially be a novel target for managing mitochondria associated

Abbreviations: MS, Multiple sclerosis; $C_{14}H6O8$: EA, Ellagic acid; COX-2, Cyclooxygenase 2; Cup, cuprizone; DCFH-DA, 2′,7′-Dichlorofluorescein diacetate; MTT, 3-[4,5dimethylthiazol-2-yl] -2,5- diphenyl tetrazolium bromide; Rh 123, Rhodamine123; LFB, Luxol Fast Blue; TBARS, Thiobarbituric acid reactive substances; TPTZ, 2, 4, 6-tripyridyl-s-triazine; SOD, succinate dehydrogenase activity; DMSO, dimethyl sulfoxide; OD, optical density; Complex I, NADH ubiquinone oxido-reductase; Complex II, succinate dehydrogenase; Complex III, ubiquinol cytochrome c reductase; Complex IV, Cytochrome c oxidase

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diseases including MS.

Ellagic acid ($C_{14}H_6O_8$, EA) is a polyphenolic natural compound found in fruits and nuts such as cocoa, tea, strawberry, walnut, and pomegranate; with a variety of biological effects against inflammation, proliferation, angiogenesis, carcinogenesis, and oxidative stress [7].

Previous studies indicated that EA plays a regulatory role in inflammatory responses screened in various animal models, and the anti-inflammatory properties are possibly due to suppressed NF-kB transcription and nitric oxide synthase [8], Cyclooxygenase 2 (COX-2), interleukin 6 and TNF-α down-regulation [9].EAdisplayed strong neuroprotective property most likly *via* the mitochondrial protection [9].A number of poly phenols other than EA were previously reported to affect members of the sirtuin family, however, theEA effectiveness and its significant role on sirtuins family is yet to be understood.

Previous studies revealed that EA was a mitochondrial protector [10], iron chelator, antioxidant and axon regenerator in neurons [11]. With such properties the improvement of multiple sclerosis symptoms can be expected.The current investigation was designed to evaluate the effect of EA supplementation on muscle complications, mitochondrial functionality, Sirt3 enzyme, and locomotor activity impairment following cuprizone (Cup) administration in C57BL/6 mice.

2. Materials and methods

2.1. Chemicals

Cuprizone, 2′,7′-Dichlorofluorescein diacetate (DCFH-DA),3-[4,5dimethylthiazol-2-yl] -2,5- diphenyl tetrazolium bromide (MTT), Rhodamine123 (Rh 123), Luxol Fast Blue (LFB) stain, and Ellagic acid were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Total RNA Extraction Kit and Easy™ cDNA Synthesis kit were purchased from Parstous Company (Iran).Sirt3 primer was synthesized by Metabion International AG Company (Germany) and Sirt3 assay kit was purchased from Aviva Systems Biology (USA).All materials for mitochondrial respiratory chain activity were purchased from Sigma-Aldrich Co. (St. Louis, MO) or Merck Co.(Whitehouse, NJ). The salts used to mak buffer solutions were of the analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Animals

All experiments were performed on male C57BL/6 mice of 6–9 weeks old. Mice were purchased from Pasteur Institute of Iran. Animals were housed under controlled laboratory conditions,12-h light, 12-h dark cycle, an ambient temperature of 23 °C, and 40% humidity. Tap water and standard food pellets were freely available in the cages at all times. All procedures were approved by the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (1396-01-05-14567; April 2018).

2.3. Experimental setup

C57BL/6 mice(6–9 weeks old)were randomly allocated into six groups (n = 12).Animal were fed with 0.2% (w/w) Cup in ground breeder chow ad libitum for 6 weeks(Torkildsen et al., 2008). Mice were treated as follows: 1) Control (Vehicle treated group), 2) Cup (0.2% (w/w), mix to fed); 3) EA (100 mg/kg/day, gavage); 4) Cup (0.2% w/w) + EA (100 mg/kg/day, gavage); 5) Cup (0.2% w/w) + EA (50 mg/kg/day, gavage); 6) Cup (0.2% w/w) + EA (5 mg/kg/day, gavage). Tap water was used as Cup vehicle. Cup and EA were administered daily (42 consecutive days). The EA [12]and Cup (0.2% w/w mixed to feed) doses was selected based on the previous studies [13,14].At the end of treatments, behavioral tests were performed andthe subjects' brain were collected for histopathological assessment.Also we divided all six groups into two subgroups, six animals per group to determine markers of oxidative stress, and six animals per groups were employed for respiratory-chain complex activity, mitochondrial indices evaluations and Sirt3 assay.Finally, animals were sacrificed (ketamine/xylazine; 100/10 mg/kg, i.p) and their gastrocnemius muscles were collected.Bodyweight and food intake of the mice were recorded every day and they were continuously exposed to Cupduring the 6 weeks of feeding period. Data normalized by the protein concentration was measured, using the Bradford method.

2.4. Luxol fast blue histochemical staining

For histopathological evaluation, brain tissue samples were fixed in a buffered formalin solution (0.4% sodium phosphate monobasic, NaH2PO4, 0.64% sodium phosphate dibasic, Na2HPO4, and 10% formaldehyde in distilled water; pH = 7.4). Finally, paraffin-embedded sections (5 μm) of the brains were prepared and stained with Luxol Fast Blue (LFB) prior to light microscope viewing (× 100). Briefly, the brain tissue was incubated for 12 h in LFB at 56 °C. First, it was washed with 95% ethanol. The tissue was then placed in a lithium carbonate solution for 15 s to allow the white matter to be easily identifiable from the gray matter of the brain, then washed with distilled water and 80% alcohol three times, and then slides were prepared by fresh Xylenol. Finally, the slides were mounted with Entellan®and demyelinationwas determined.Brain tissue slides were blindly analyzed by a pathologist, using a light microscope (Olympus CX21®, Japan).

2.5. Behavioral tests

2.5.1. Gait test

To obtain footprints, the hind paws of the mice were coated with a nontoxic paint. The animals were allowed to walk along a 60-cm-long, 10-cm-wide runway (with 10-cm-high walls) into an enclosed box and a sheet of white paper was placed on the floor of the runway. To characterize the walking pattern of each mouse the average distance between the points of the left and right paws were measured and recorded (in millimeters).

2.5.2. Locomotor activity

Open field behavior was used as an index of animals' locomotor activity in the animal models of neurological as MS. The open field test was conducted for each group before anesthesia and muscle sample collection. The apparatus was made of a white wooden box (freshly cleaned open field box 25 cm L × 25 cm W × 25 cm H). The open field arena was equipped with a webcam [15]; -Megapixel, Gigaware, UK) and all activities were monitored and recorded in a separate room. Animal's behavior was recorded for 15 min and the total number of crossed squares were counted (Total locomotion) [16,17].

2.5.3. Rota-rod test

Following the reported procedure, each mouse underwent five sessions of rotarod performance on a rotarod apparatus. The rotarod speed was 10 rpm with a cut-off point of 300 s. The time, up to which the mice stayed on the rotating rod, was automatically recorded [18].

2.6. Oxidative stress markers

2.6.1. Lipid peroxidation in muscle tissue

Thiobarbituric acid reactive substances (TBARS) were assessed in muscle tissues as an index of lipid peroxidation. The reaction mixture consisted of 0.375%, w/v thiobarbituric acid, 1% w/vphosphoric acid (pH = 2), and 500 μL of tissue homogenate (10% w/v in KCl, 1.15% w/v). The mixture was shaken well and heated in 100 °C water for 45 min. After the incubation period, n-butanol (2 mL) was added and vigorously mixed. Finally, samples were centrifuged at 3000 g for 5 min and the absorbance of developed color in n-butanol phase was measured at 532 nm using an Ultrospec 2000®UV spectrophotometer [16].

2.6.2. Reactive oxygen species (ROS) formation in muscle tissue

Reactive oxygen species (ROS) in muscles was estimated using method described by Gupta et al. with some modifications [19]. Briefly,1:10 w/v of muscle tissues were homogenized on ice-cold 40 mM Tris-HCl buffer (pH = 7.4). 100 μL of the resulted tissue homogenate was mixed with 1 mL of Tris-HCl buffer and 5 μL of 2′, 7′ dichlorofluorescein diacetate (10 μM). The mixture was incubated for 30 min in 37 °C (Gyromax™ incubator shaker). Finally, the fluorescence intensity of the samples was assessed using a FLUOstar Omega® multifunctional microplate reader (λ excitation = 485 nm and λ emission = 525 nm) [20].

2.6.3. Ferric reducing antioxidant power (FRAP) of muscle tissue

FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue colored Fe II-tripyridyltriazine compound from colorless oxidized Fe III form by the action of electron donating antioxidants. Briefly, the working FRAP reagent was prepared by mixing 10 vol of 300 mmol/L acetate buffer(pH 3.6) with 1 volume of 10 mmol/L 2, 4, 6-tripyridyl-s-triazine (TPTZ) in hydrochloric acid (40 mmol/L)and with 1 volume of ferric chloride (20 mmol/L). All solutions were used on the day of preparation. Muscle tissue was homogenized in cooled Tris buffer (0.25 M, containing 0.2 M sucrose and 5 mM DTT, pH 7.4). Then, 50 μL of tissue homogenate and 150 μL of deionized water was added to 1.5 mL of the FRAP reagent. The reaction mixture was incubated at 37 °C for 5 min. Finally, the absorbance of the developed color was measured at 595 nm by an Ultrospec 2000 ® spectrophotometer [20].

2.6.4. HPLC analysis of mitochondria and muscle tissue glutathione content

Reduced (GSH) and oxidized (GSSG) glutathione content in the muscle tissue were determined by HPLC analysis. Briefly, de-proteinized samples by TCA (50% w: v), derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene. Finally, 25 μl of samples were injected into the HPLC system with the following properties: the mobile phases consisted of buffer A (Acetate buffer: Water; 4:1 v/v), buffer B (Methanol: Water; 4:1 v/v), and a gradient method with a steady increase of buffer B to 95% in 40 min.

2.7. Mitochondrial indicates

2.7.1. Muscle mitochondria isolation

Mice's muscle were washed and minced in an ice-cold buffer medium (0.1%Trypsin, 70 mM mannitol, 220 mM sucrose, 2 mM HEPES, 0.5 mM EGTA and 0.1% BSA, pH = 7.4). Minced tissues were transferred into a fresh buffer (5 ml buffer/1g of the muscle) and homogenized. Mitochondria were isolated by differential centrifugation of the homogenate. First, unbroken cells and nuclei were pelleted at 1000 × *g* for 10 min at 4 °C.Second, the supernatant was centrifuged at 10000 × *g* for 10 min at 4 °C to pellet the mitochondria. This step was repeated three times using a fresh buffer medium. Final mitochondrial pellets were suspended in an incubation buffer (70 mM mannitol, 220 mM sucrose, 2 mM HEPES, and 0.5 mM EGTA, pH = 7.4).For mitochondrial ROS, membrane potential and swelling determination, mitochondria suspended in respiration buffer containing 320 mM Sucrose, 10 mM Tris, 20 mM MOPS, 50 μM EGTA, 500 μM MgCl2, 0.1 mM KH2PO4 and 5 mM Sodium succinate, pH = 7.2, MMP assay buffer containing 220 mM Sucrose, 68 mM Mannitol, 10 mM KCl, 5 mM KH2PO4, 2 mM MgCl2, 50 μM EGTA, and 10 mM HEPES, pH = 7.2 and swelling buffer containing 125 mM Sucrose, 65 mM KCl, 10 mM HEPES, pH = 7.2.

2.7.2. Mitochondrial succinate dehydrogenase activity (MTT assay)

The 3-(4, 5-dimethylthiazol-2-yl)-2, the 5-diphenyltetrazolium bromide (MTT) assay was applied as a colorimetric method to determine mitochondrial succinate dehydrogenase activity [21]. Briefly, 0.5 mg protein/ml of mitochondrial suspension was incubated with 0.4% of MTT (37 °C for 30 min). The product of purple formazan crystals was dissolved in 1 mL dimethyl sulfoxide (DMSO) and the optical density (OD) at 570 nm was measured with an EPOCH plate reader (Bio-Tek Instruments, Highland Park, USA) [22].

2.7.3. Mitochondrial membrane potential

Mitochondrial uptake of rhodamine 123 (the cationic fluorescent dye)was used for the estimation of mitochondrial depolarization. Rhodamine 123, accumulates in intact mitochondria by facilitated diffusion. When the mitochondrion is damaged and depolarized, there is no facilitated diffusion and the amount of rhodamine 123 in the supernatant is increased. In the current investigation, 0.5 mg protein/ml of mitochondrial fractions were incubated with rhodamine 123 (10 μM) in the MMP assay buffer for 15 min. Then, samples were centrifuged (15000 × *g*, 1 min, 4 °C) and the fluorescence intensity of the supernatant was monitored using a FLUOstar Omega® multifunctional microplate reader with λ excitation = 485 nm and λ emission = 525 nm [23].

2.7.4. Reactive oxygen species (ROS) in isolated muscle mitochondria

2′, 7′-dichlorofluorescein diacetate (DCFH-DA) was used as a fluorescent probe to assess mitochondrial ROS formation. In brief, isolated muscle mitochondria (0.5 mg protein/ml) were placed in the respiration buffer. Then, 5 μl of DCFH-DA was added (Final concentration, 10 μM) [24] 0.2962) and the mixture was incubated for 15 min in the dark. The fluorescence intensity of samples was measured using a FLUOstar Omega® multifunctional microplate reader with λ excitation = 485 nm and λ emission = 525 nm [25].

2.7.5. Muscle mitochondrial swelling

Analysis of muscle mitochondrial swelling was estimated through changes in light scattering as monitored spectrophotometrically at 540 nm (25 °C). Briefly, isolated mitochondria (0.5 mg protein/ml) were suspended in swelling buffer (125 mM Sucrose, 65 mM KCl, 10 mM HEPES, pH = 7.2) and the absorbance was monitored at 540 nm during 30 min of incubation using an EPOCH plate reader (Bio-Tek® Instruments, Highland Park, USA). A decrease in absorbance is an indication of increased mitochondrial swelling [26].

2.7.6. Muscle tissue and isolated mitochondria ATP content

Muscle ATP activity content was determined by a luciferase-luciferin-based kit (ENLITEN® from Promega). Briefly, muscle tissue and mitochondrial samples (500 μL, 1 mg protein/mL) were mixed in ice 200 μL of TCA 0.3% solution and centrifuged at 15,000 × *g*, 15 min. Then, 100 μL of the supernatant was treated with 100 μL of ATP kit content (in the dark) and luminescence intensity of samples was measured at λ = 560 nm by FLUOstar Omega® multifunctional microplate reader. Samples protein concentrations, for standardization of data, were determined by the Bradford method.

2.7.7. SOD activity

Superoxide dismutase (SOD) activity assay was performed according to Nasdox™ Superoxide Dismutase (SOD) Activity Assay Kit. In brief, SOD activity evaluated based on the inhibition of the pyrogallol autoxidation reaction. pyrogallol is a substance that is oxidized under normal conditions in the presence of air. Having a certain concentration of this substance will determine its half-life of autoxidation.

2.8. Mitochondrial respiratory chain-complex enzyme activity

The mitochondrial suspension was freeze-thawed (3 times) to lyse the cell membranes, and enzymatic activities were measured ELISA reader at 37 °C as described by Ojano-Dirain et al. for Complex V and Bouhours-Nouet et al. for Complexes I–IV, with the exception that we used 20 mg of mitochondrial protein in 1 mL final volume in lieu of 5 mg in 120 μL. Enzyme activities were expressed as units (U)/mg of

mitochondrial protein.

2.8.1. Complex I (NADH ubiquinone oxido-reductase)

Rotenone-sensitive activity of Complex I, in the absence and presence of rotenone was used to measure NADH ubiquinone oxido-reductase activity. Briefly, about 10–20 mg of mitochondrial protein was incubated in 240 µL high-performance liquid chromatography-grade water (at 37 °C for 2 min). Hence, the reaction medium containing 10 mM KH2PO4, pH 7.5, 2 mM KCN, 2 mg/mL antimycin A, 100 mM decylubiquinone, 1 mg/mL bovine serum albumin (BSA), and 5.2 mM MgCl2 was added to incubation solution. The reaction was initiated by the addition of 200 mM NADH and the decrease in the absorbance at 340 nm was monitored 5 min before and after the addition of rotenone (2 mg/mL). The extinction coefficient for quantification of enzyme activity was considered 6.22 mM/cm.

2.8.2. Complex II (succinate dehydrogenase)

Complex II of mitochondrial respiratory chain activity was determined by the reduction of 2,6-dichlorophenolindophenol in the presence of phenazine methosulfate. First, approximately 10–20 mg of mitochondrial protein was added to medium (50 mM KH2PO4, pH 7.5, 16 mM sodium succinate, 1.5 mM KCN and 100 mM phenazine methosulfate) and incubated at 37 °C (3 min). The absorption difference (600 nm) was determined 5 min after addition of 100 mM 2,6-dichlorophenolindophenol. The extinction coefficient for quantification of enzyme activity was considered 21 mM/cm.

2.8.3. Complex III (ubiquinol cytochrome c reductase)

About 10–20 mg of mitochondrial protein was incubated (37 °C for 30s) in the reaction medium (35 mM KH2PO4, pH 7.5, 5 mM MgCl2, 2.5 mg/mL BSA, 1.8 mM KCN, 125 mM oxidized cytochrome c, and 10 mg/mL rotenone). To adding 31.8 mM reduced ubiquinone 2 (ubiquinol 2), the reaction was initiated and the increase in absorbance was assayed for 3 min in the absence and presence of 10 mg/mL antimycin A. Ubiquinol cytochrome c reductase activity was determined at 550 nm, using an extinction coefficient of 19.2 mM/cm.

2.8.4. Complex IV (cytochrome c oxidase)

Oxidation of reduced cytochrome *c* and *a* decrease in absorbance at 550 nm was assessed as an index of the activity of complex IV respiratory chain (cytochrome c oxidase) in isolated muscle mitochondria according to a previously reported procedure (). Mitochondrial protein (about 10–20 µg) was incubated in 10 mM of KH2PO4 (pH 7.0) at 37 °C for 3 min. Afterward, Reduced cytochrome c (96% reduced by using dithionite sodium) was mixed with samples and the decrease in absorbance was measured by mitochondrial activity in lung tissue following the oxidation of cytochrome c for 90 s. The cytochrome c oxidase activity was measured by using an extinction coefficient of 21 mM/cm.

2.9. Sirt3 enzyme

2.9.1. Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from freshly frozen muscle tissue of mice stored in -80 °C via RNA extraction kit according to the manufacturer's instruction.

We applied Total RNA Extraction Kit and Easy™ cDNA Synthesis kit (Parstous) to synthesize RNA and cDNA, respectively.Sirt3 primers were designed and synthesized with Metabion International AG Company (Germany) forward primer: 5′- TGCCAGCTTGTCTGAAGCA-3′, and reverse primer: 5′- GTCCACCAGCCTTTCCACA-3′.GAPDH served as an internal control with forward primer: 5′-AGGTCGGTGTG AACGGATTTG-3′and reverse primer: 5′-TGTAGACCATGTAGTTGAGG TCA-3′. For qRT-PCR, cDNA was amplified with SYBER Green Real time-PCR kit (Thermo Scientific)by Step one plus Real Time System (Applied Biosystem, Step one plus (ABI), USA). QRT-PCR cycling conditions were 95 °C for 10 s, 45 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. The comparative Ct for quantitative gene expression of *Sirt3* and GAPDHwas analyzed by Step one software v2.1.The relative alteration of *Sirt3* gene expression was calculated with the 2(–ΔΔCt) equation.

2.9.2. Sirt3 assay

The Sirt3 Assay was based on standard sandwich enzyme-linked immune-sorbent assay technology. An antibody specific for Sirt3 has been pre-coated onto a 96-wellplates. Standard or test samples were added to the wells and incubated. A biotinylated detector added, incubated and followed by washing. The avidin-peroxidase conjugate is then added, incubated and unbound conjugates washed away. An enzymatic reaction is produced through the addition of TMB substrate which is catalyzed by HRP. A blue color changes to yellow after adding acidic stop solution. Absorbanceat 450 nm is quantitatively proportional to the amount of sample Sirt3.

2.10. Statistical analysis

Graph Pad Prism 6 software (Graph Pad Software Inc., San Diego, CA, USA) was used for the statistical analysis.$P < 0.05$ was considered to be statistically significant.Data are presented as the Mean ± SEM. Data comparison was performed by the one-way analysis of variance [27] with Dunnett post-test.

3. Results

3.1. Behavioral tests

Elevation in behavioral tests showed reduction in the locomotor activity and motor coordination of Cup group (Fig. 1). The average distance between the points of the left ($P < 0.01$) and right ($P < 0.001$)paws were significantly lower in Cup group [28]; 1. C and D). Furthermore, different dosage of EAimprovedlocomotor activity, motor coordination and gait tests (Fig. 1).

3.2. Histopathological changes

LFB histochemical staining was also performed to detect Cuprizone-induced demyelination in the mice brain (see Supplemental Material, SF1). Diffuse white matter injury was a dominant feature that was mainly detected in corpus callosum (C.C). In Cup group, sever demyelination was presented in C.C (LFB × 100).

3.3. Oxidative stress markers

The markers of oxidative stress were affected by Cup. It was found that MDA levels($P < 0.001$) and ROS formation ($P < 0.01$) had significantly increased in Cup animals. Also, lipid peroxidation levels were significantly($P < 0.001$) reduced at EA 100 mg/kg dosage in comparison with the control group. The data is shown in Fig. 2 A and B, demonstrating that muscleMDA levels and ROS formation improved were significantly reduced in Cup + EA groups in comparison with the Cup group.

As shown in Fig. 2. C and D, muscle antioxidant capacity and glutathione content were markedly depleted after Cup administration in comparison with the control group. EA(at a dose of 100 mg/kg) significantly($P \leq 0.001$) improved antioxidant capacity as compared to the control group. Additionally,Co-treatment of Cup with EA(all dosages)were able to significantly increase antioxidant capacity and GSH/GSSG ratio.

3.4. Mitochondrial indicates

The role of mitochondria function in the cuprizone-induced model

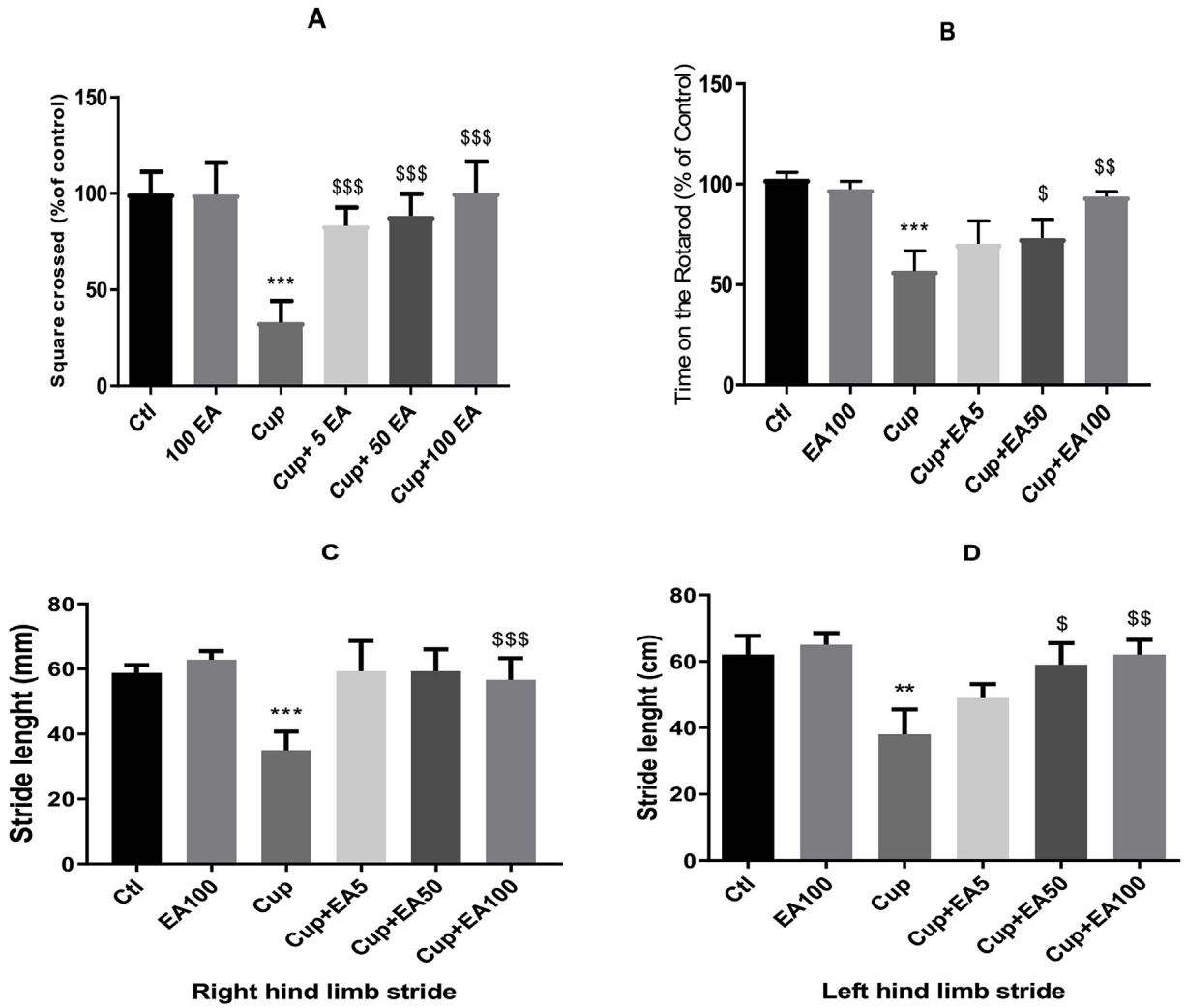

Fig. 1. Behavioral tests: Behavioural tests (Locomotor activity; Fig A, Motor coordination; Fig B, and gait stride (Fig C and D) in the mice of control and treatment groups. Values are Mean ± SEM (n = 12). (One-way ANOVA followed by Dunnett post-test). Ctl: Control, Cup: Cuprizone, EA: Ellagic acid. ** Significant difference in comparison with control group ($P < 0.01$). *** Significant difference in comparison with control group ($P < 0.001$). $ Significant difference in comparison with Cup group ($P < 0.05$). $$ Significant difference in comparison with Cup group ($P < 0.01$). $$$ Significant difference in comparison with Cup group ($P < 0.001$).

of demyelinationwas evaluated. It was found that mitochondrial dehydrogenases activity was significantly ($P < 0.01$) reducedin Cup animals (Fig. 3A). On the other hand, significant mitochondrial depolarization and swelling were evident in Cup group (Fig. 3. B and D). Mitochondrial levels of ROS were also higher in Cup animals in comparison with the control mice (Fig. 3. C). It was also found that ATP content was significantly ($P < 0.01$) lower in the muscle tissue from Cup group (Fig. 3F). Additionally, EA (at doses of 5, 50, and 100 mg/kg)can probably repress these imbalances induced by the Cup (Fig. 3 A-F).As can be seen in Fig. 3G, in comparison with the control group, the muscle mitochondria GSH/GSSG ratio was significantly reduced in Cup groups ($p < 0.01$), with no significant differences in 100 mg/kg EA group. The muscle mitochondria GSH/GSSG ratio in Cup + EA 5, 50, and 100 mg/kg ($p < 0.001$) groups was significantly higher than what was observed in Cup group.As it can be seen in Fig. 2E, SOD activity was significantly reduced in Cup group ($p < 0.01$) compared to the control group; however, no significant difference was observed in EA 100 mg/kg groups. Moreover, SOD activity was notably increased in three Cup + EA groups ($p < 0.05$, $p < 0.01$, $P < 0.01$ respectively).

3.5. Mitochondrial respiratory chain-complex enzyme activity

In another part of this investigation, respiratory chain-complex activities were evaluated on muscle tissue (Fig. 4; A-D). Cup inhibited the respiratory chain-complex activities in mice as revealed by decreases complex I, II, III and IV activities.Moreover, a significant increase in the succinate dehydrogenase, ubiquinol cytochrome c reductase, and Cytochrome c oxidase activities of muscle tissue were detected in the Cup + EA 50 and 100 mg/kg groups in comparison with the Cup group. However; complex III activity was significantly ($P < 0.01$) higher in the mitochondria isolated from Cup + EA 5 mg/kg group.In complex III activity no significant difference was found among EA groups as compared with the Cup animals.

3.6. Sirt3 enzyme

As shown in Fig. 5A and B, Sirt3 level and *Sirt3*expression in the muscle were significantly ($P \leq 0.05$)reduced by following the induction of MS by Cup. Data showed that pretreatment withEA was significantly effective to counteract the reduction of the Sirt3 level and *Sirt3*expression in the muscle of Cup group.

4. Discussion

This study was performed with the aim to explore the mechanisms involved in the protective effect of EA on muscle tissue injury and

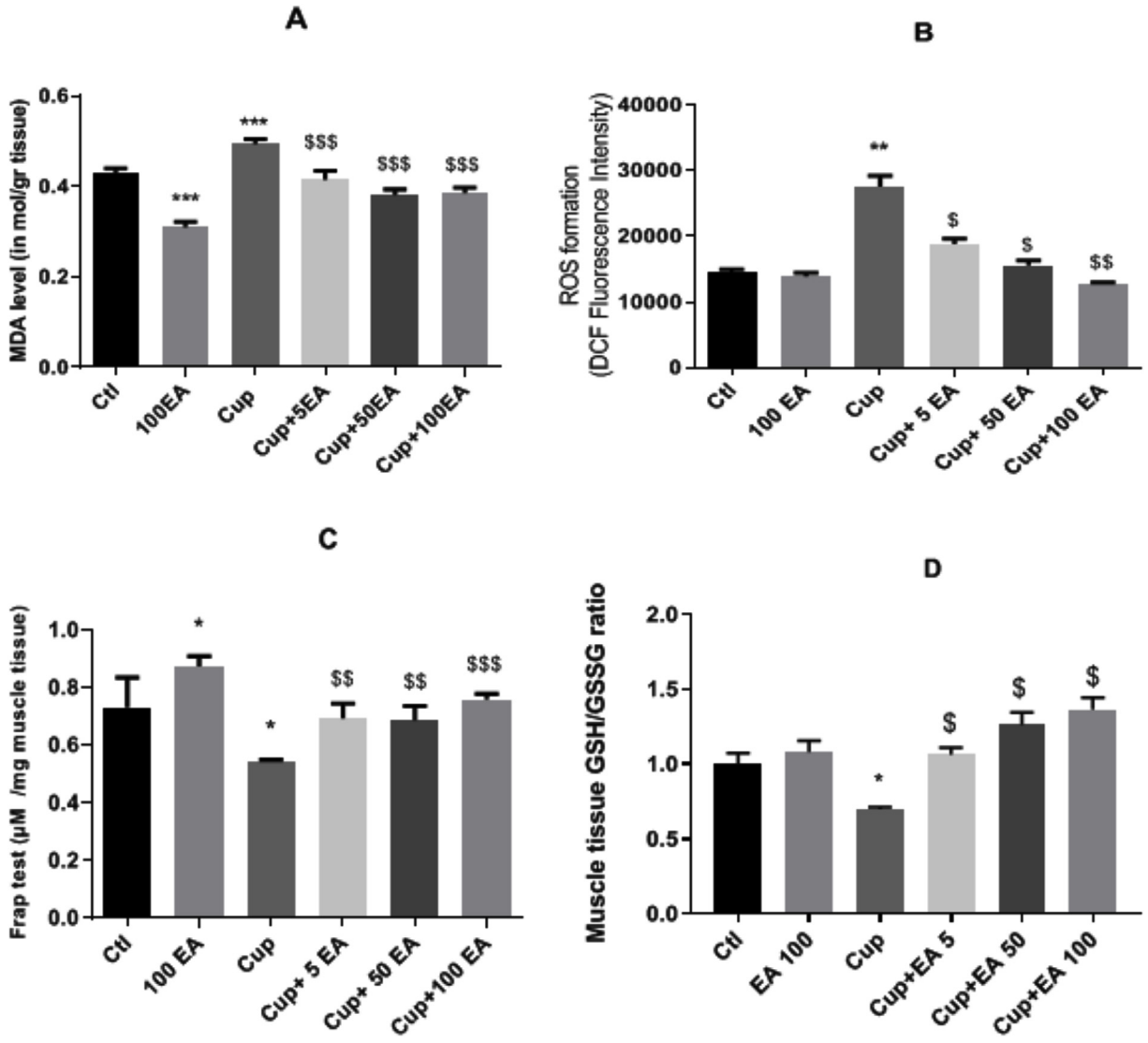

Fig. 2. Markers of oxidative stress (Lipid peroxidation; Fig. A, Reactive oxygen species (ROS) content; Fig. B, antioxidant capacity; Fig. C, GSH/GSSG ratio, Fig. D) in the muscle tissue mice in the control and treatment groups (A–D). Values are Mean ± SEM (n = 6). (One-way ANOVA followed by Dunnett post-test). Ctl: Control, Cup: Cuprizone, EA: Ellagic acid. * Significant difference in comparison with the control group ($P < 0.05$). ** Significant difference in comparison with the control group ($P < 0.01$). *** Significant difference in comparison with the control group ($P < 0.001$). $ Significant difference in comparison with Cup group ($P < 0.05$). $$ Significant difference in comparison with Cup group ($P < 0.01$). $$$ Significant difference in comparison with Cup group ($P < 0.001$).

locomotor dysfunction induced by Cup treatment. The findings revealed that EAsupplementation improved the performance of cuprizone-treated mice.This was done by improving muscle mitochondrial functionality, *Sirt3* expression andSirt3 content, and decreasing tissue oxidative stress markers seem to play a significant role in the EA mechanism of protection in the muscles of animals with MS.

Our findings on behavioral tests in Cuprizone-induced demyelinated male C57BL/6 mice were consistent with the previous reports. In this regard, it was shown that a diet containing 0.2% w/w Cup for 5–6 weeks can lead to demyelination [13,29] and brain mitochondrial dysfunction in male C57BL/6 mice(Faizi et al., 2016). Previous studies showed that behavioral dysfunction occurred parallel to the demyelination in the mice brain [30,31].Our data confirmed the above mentioned results and at the same time showed a significant muscle dysfunction along with brain damage.Regimens containing other concentration of Cup in different periods have also impaired neuronal function that leads to severe status spongiosus [32], epileptic seizures, hippocampal damage [33], epilepsy, and schizophrenia [13].

The toxic effects of Cup suggested to be mediated *via* copper chelating in the C.C [14].Our histopathological evaluations revealed sever demyelination regions in the same area of the brain(see Supplemental Material, SF1).Furthermore, we detected damage and mitochondrial dysfunction in the muscle tissue of all animals that received Cup, which were in agreement with the results obtained from behavioral tests including open field, rotarod test, and gait test. All of these alterations are suggestive of increased oxidative stress in the muscle tissue following Cup. In our study, sub chronic co-admininstartion of EA, dose-dependently improved locomotor activity, gait test and motor coordination in mice. Consistent with our findings, but for shorter duration and by means of higher doses, previous reports documented the beneficial effect of EA in various models of neurodegeneration [7,9,11].

Evidence suggest that ROS over production and decreased antioxidant capability are correlated to the severity of muscular dysfunction. Also, lower ratio of GSH/GSSG, SOD activity, and antioxidant capacity in Cup animals confirms the occurrence of oxidative stress in the muscle tissues.EA in our study attenuated oxidative stress and improved antioxidant defense system.EA alone reduced muscle MDA content in mice compared to control group, which might contribute to its polyphenolic nature. In addition, EA lowered mitochondrial ROS level and muscle impairment, although its net effect on muscle tissue

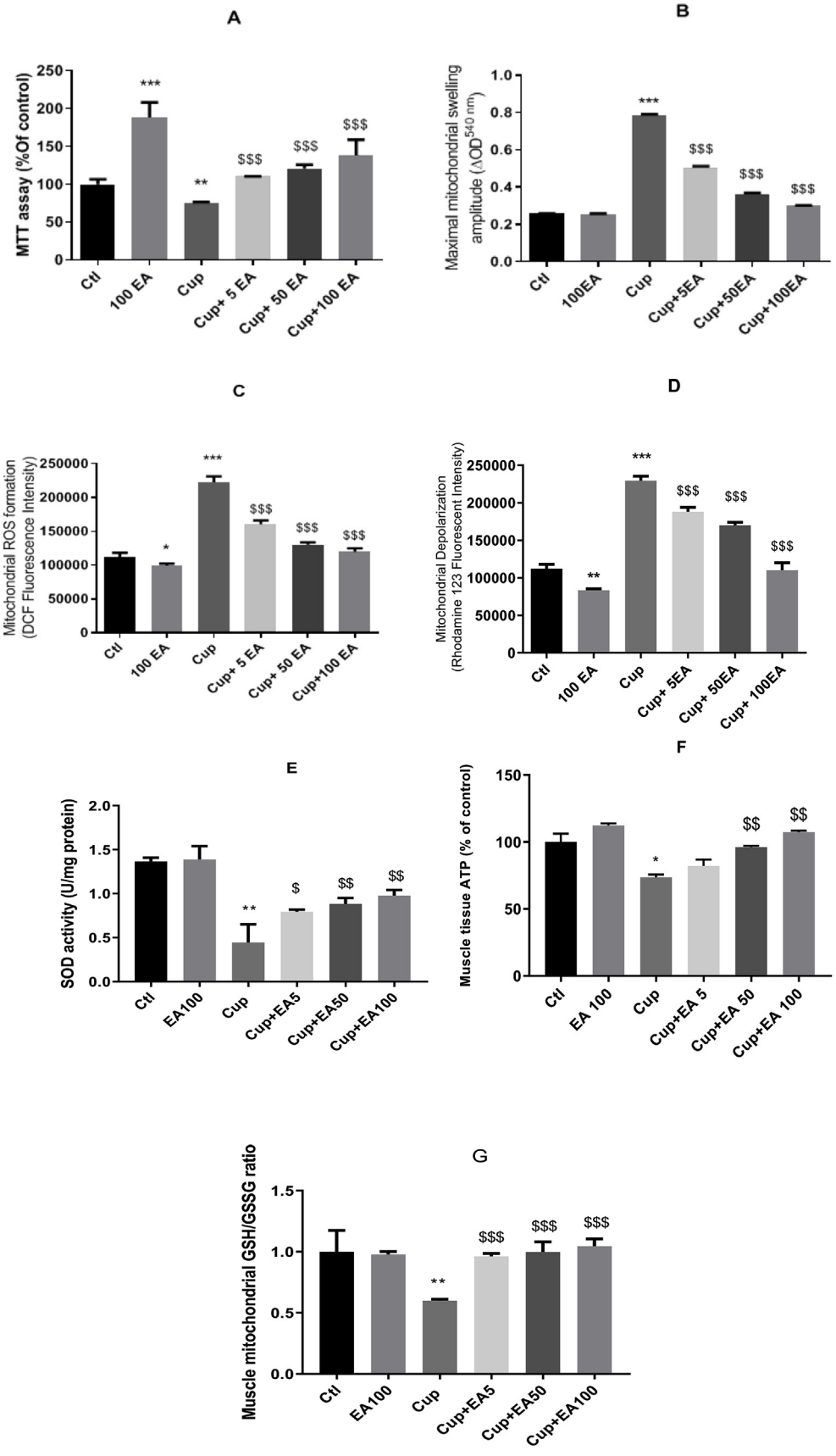

Fig. 3. Mitochondrial indices of functionality (MTT assay; Fig. A, swelling; Fig. B, Mitochondrial Reactive oxygen species (ROS) content; Fig. C, Collapse of the membrane potential; Fig. D, SOD activity; Fig. E, ATP content; Fig. F, Muscle mitochondrial GSH/GSSG ratio; Fig. G)in the muscle tissue mice in control and treatment groups. Values are Mean ± SEM (n = 6). (One-way ANOVA followed by Dunnett post-test). Ctl: Control, Cup: Cuprizone, EA: Ellagic acid. * Significant difference in comparison with control untreated group ($P < 0.05$. ** Significant difference in comparison with control untreated group ($P < 0.01$). *** Significant difference in comparison with control group ($P < 0.001$). $$$ Significant difference in comparison with Cup group ($P < 0.001$).

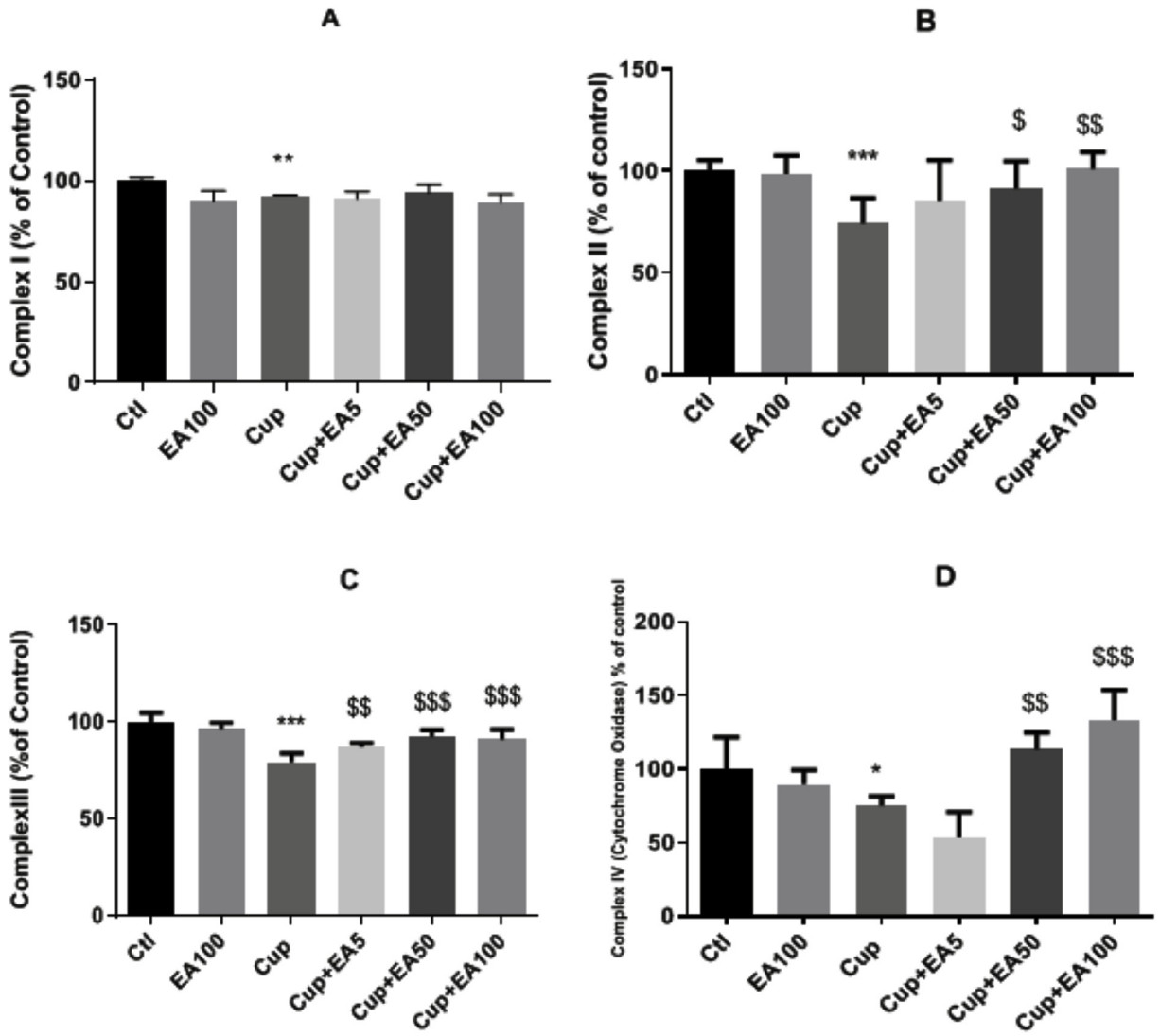

Fig. 4. Respiratory chain-complex activities (Complex I-IV)in the muscle tissue mice in control and treatment groups (A–D). Values are Mean ± SEM (n = 6). (One-way ANOVA followed by Dunnett post-test).Complex I = NADH ubiquinone oxido-reductase; Complex II = succinate dehydrogenase; Complex III = ubiquinol cytochrome c reductase; Complex IV: cytochrome c oxidase. Ctl: Control, Cup: Cuprizone, EA: Ellagic acid. * Significant difference in comparison with control untreated group ($P < 0.05$. ** Significant difference in comparison with control untreated group ($P < 0.01$). *** Significant difference in comparison with control group ($P < 0.001$). $$$ Significant difference in comparison with Cup group ($P < 0.001$).

might be indicative of protection due to other sources for ROS production other than mitochondria such as peroxisomes and endoplasmic reticulum [34,35].

Mitochondria is an important organelle involved in neurodegenerative diseases [1].Inthe neurodegenerative conditions like Alzheimer's disease [36], Parkinson's disease [37] and Huntington's disease [25], noticeable mitochondrial dysfunction might occur.

The results showed that dehydrogenase activity in the muscle was reducedin Cup group and EA pretreatment dose-dependently reversed this undesirable alteration. In line with these results, EAreported to play a defensive role against mitochondrial ROS production and oxidative stress induced by arsenic in isolated rat liver mitochondria [38].

We also measured some mitochondrial dysfunction-related markers including ROS production, mitochondria membrane potential, swelling, muscle mitochondrial GSH/GSSG ratio, and ATP content in muscle tissue. In this regard, EAmight be capable ineffectively reducing the level of mitochondrial ROS production and swelling.In this way, preventing the MMPcollapse and dehydrogenase inactivity might be as a result of EAco-treatment by protecting muscle tissues against oxidative damage.

Interference with mitochondrial function coulddisrupt energy metabolism and ATP production.Mitochondrial dysfunction has a pathogenic role in axonal degeneration and it is considered as a neuropathological consequence in neurodegenerative diseases like MS [39]. This pathology is also associated with activated oligodendrocytes [13].Cup chronically induced oxidative stress, ER stress, and oligodendrocyte apoptosis that led to neuronal damage and demyelination [13].Also, a significant role was given to mitochondria in Cuprizone-induced demyelination [39].In the muscles of patients with MS, oligodendrocyte apoptosis occurs along with muscle weakness and atrophy [40], Muscle weakness and fatigue might be due to reduced ATP production and mitochondrial dysfunction [1].

In line with previous investigations, we found that muscle tissue mitochondria activity was drastically reducedin Cup group. However, there is no evidence of cuprizone-induced mitochondrial dysfunction in the muscle tissue. As a result, Cup treatment leads to inhibition of ATP production and impaired muscle mitochondrial respiratory chain; hence, expect complex I activity, whereEA can possibly repress these imbalances. Mitochondrial dysfunction in the brain following Cup exposure was associated with behavioral impairment [39]. Previous

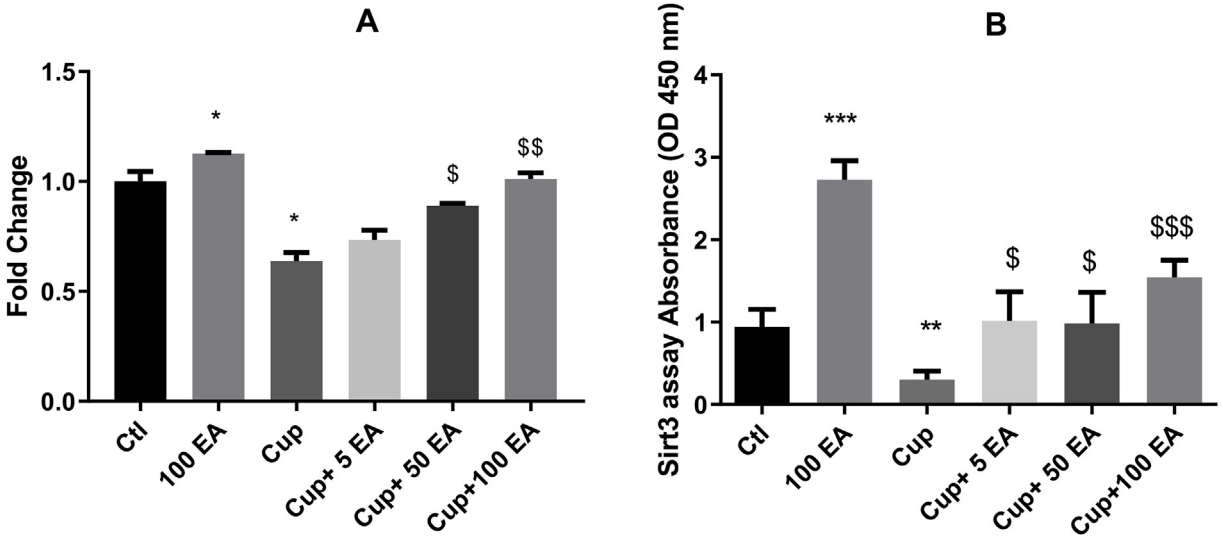

Fig. 5. Real-time quantitative PCR displayed the levels of sirt3 expression and Sirt3 level in the muscle tissue from mice in the control and treated groups. Values are Mean ± SEM (n = 6). (One-way ANOVA followed by Dunnett post-test). Ctl: Control, Cup: Cuprizone, EA: Ellagic acid. * Significant difference in comparison with control untreated group ($P < 0.05$). Ellagic acid. ** Significant difference in comparison with control untreated group ($P < 0.01$). *** Significant difference in comparison with control group ($P < 0.001$). $ Significant difference in comparison with Cup group ($P < 0.05$). $$$ Significant difference in comparison with Cup group ($P < 0.001$).

report indicated that EA increased mature oligodendrocytes population, and decreased apoptosis compared with the Cuprizone-induced demyelinatedmice [41]. Also, an earlier report showed that treatment with short term EA (80 mg/kg) significantly decreased neuro inflammation and the population of activated macrophages, but not reactive astrocytes compared with those in Cuprizone-induced demyelinatedmice [9].

The data obtained from the current study suggest that mitochondrial activity dysfunction, mitochondrial respiratory chain dysfunction, and energy crisis as a primary mechanism of muscle injury and behavioral dysfunction in demyelination-associated brain dysfunction. Moreover, mitochondrial respiratory chain-complex activities is related to progressive tissue demyelination [42].

The sirtuin family of proteins consists of seven members in mammals (SirT1-T7). Sirtuin family is involved in cancer, diabetes, heart disease [43], Alzheimer's disease, and aging [5].Sirt3 is located within the matrix of mitochondria, and its activity is directly related to the mitochondrial normal function. Sirt3 directly mediates adaptive neuronal responses to bioenergetic disturbances and oxidative stress [44]. A study indicated that sirt3 levels in the brain of Alzheimer's model of rats were reduced [45], indicating that it might have a role in neurodegenerative diseases. Our results showed that *Sirt3* expression and Sirt3 content were reduced in Cuprizone-induced demyelination group and that the treatment with EA significantly returnedSirt3 to its normal values. EA alone increased*Sirt3* expression and Sirt3 content, presumably due to improved mitochondrial function as it appears in complex II and ROS results. EA stimulate complex II and tricarboxylic acid (TCA) cycle and also the electron transport chain for ATP production [46].As a result, Sirt3 modification might be a subcellular mechanism involved in demyelination and remyelination processes.

According to data, reduced activity of mitochondria was present in the muscle of Cup group, and EA 50 and 100 mg/kg restored Sirt3 enzyme and mitochondrial activity. Muscle damage parameters including MDA level, ROS production, GSH/GSSG ratio, and antioxidant capacity werereversed by EA treatment. Earlier reports revealed that EA has a neuroprotective effect against Alzheimer's disease, [47]) Parkinson disease [48] and also multiple sclerosis [9]. After administration of EA (50 mg/kg for 10 days) in a rat model of Parkinson disease induced by 6-hydroxydopamine (6-OHDA), the glutathione and superoxide dismutase activities improved and also the levels of MDA in the rat's brain significantly declined [48].

The pharmacoknetic study in mice showed that EA given orally is absorbed almost within 2 h [49]. In urine, bile and blood detected free EA and EA conjugates with sulphate ester, glucuronide, and glutathione [50]. Pharmacodynamic studies of EA have demonstrated that most of phase I and phase II metabolism of EA occurred in the GI tract and the liver. Different studies indicate that food condition such as pH, constituents, and processing can have a crucial impact on EA absorption in the proximal part of the GI tract. At 24 h, the majority of EA and its metabolites are excreted in urine and faeces, with 19% and 22% excreted in faeces and urine, respectively. Although, Paradox results of absorption and metabolism of EA in rat were reported, EA is extensively metabolised by the intestinal microflora to urolithins A, B, C, and D in animals and humans [51]. Hence, earlier studies reported the EA has the ability for plasma protein binding and it is capable to pass through the blood-brain barrier at small quantities [24]; and EA was detected in the rat's brain after its administration. This agent might be related to the neuroprotection associated with the intake of certain fruits and foods [52,53].Our data suggest that sub chronic EA co-administration had a direct repairing effect on muscle mitochondrial function in the Cup induced demyelination. Other mechanisms related to the mitochondria needs further investigation.Until now, there is no effective therapy of EA that can stop or at least slow down the sign of MS disease in the clinical situation. Although; most of in vitro and in vivo studies showed protective effect of EA on neurodegeneration diseases such as Alzheimer diseases [54]and MS [9,41]. Moreover, studies discovered some of polyphenols in the same category of EA such as quercetin, luteolin, curcumin, resveratrol, and epigallocatechin-3-gallate (EGCG) have positive effect in MS disease in clinical trial studies [55]. Regarding to in vivo and in vitro studies of EA on neurodegenerative disease we hope to focus on evaluated effect of EA on MS disease in clinical trial in future projects. Hence, some of medicine that use in treatment of neurodegeneration disease, they have protective effect on mitochondria and due to this property could have treatment effect. Then according to data and protective effect of EA on mitochondria, might it will be success treatment to least slow down the sign of MS disease.

In brief, sub chronic co-treatment with EA, a natural phenolic compound, improved Cuprizone-induced demyelination in mice in a way that dose-dependently recovered muscular dysfunction, motor

incoordination, and locomotor inactivity, which was probably *via* the mitochondrial protection, activity of mitochondrial respiratory chain, Sirt3 modification and oxidative stress.

Ethical standards

Compliance with ethical standards.

Declaration of competing interest

The authors declare that there are no conflicts of interest in this research project.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116954.

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