Effects of melatonin on oxidative stress parameters and pathohistological changes in rat skeletal muscle tissue following carbon tetrachloride application

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Animal models demonstrating skeletal muscle (SM) disorders are rarely investigated, although these disorders accompany liver disorders and can occur during prolonged exercise/training. In cases of SM disorders exogenous antioxidants, such as melatonin, could help by generally improving tissues antioxidant capacities. We aimed to analyze the potential of melatonin in preventing biochemical and structural changes in rat biceps muscle (BM) occurring after an acute exposure to carbon tetrachloride (CCl4). Biceps muscles obtained from male Wistar rats belonging to different experimental groups were biochemically (determination of tissue MDA, total antioxidant capacity, GSH, CAT, SOD and GPx activities) and pathologically analyzed. Also, serum levels of potassium, LHD and CK were analyzed in all experimental animals. The obtained results were statically compared with those from vehicle-treated control group. The applied melatonin prevented potassium and intracellular enzyme leakage (CK and LDH) that was induced by CCl4, as well as an increase in tissue MDA. From a panel of determined oxidative stress parameters melatonin was able to statistically significantly prevent changes in total antioxidative capacity and in CAT, SOD and GPx activities induced by CCl4. Microscopic analysis of BM from the animals exposed to CCl4 revealed significant muscle fiber disorganization and massive inflammatory cell infiltration. All these changes were significantly ameliorated in the group that received melatonin prior to CCl4. Changes in serum and tissue biochemical parameters accompanied the observed pathological changes, which demonstrated a significant influence of melatonin in preventing skeletal muscle damage induced by CCl4.

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1. Introduction

Carbon tetrachloride (CCl4) is a synthetic industrial chemical often used in paints, solvents and extinguishers with a long atmospheric half-life that varies from 30 to 100 years (Pope and Rall, 1995). In the experimental research conducted on laboratory animals, this chlorine containing compound is used for the induction of different tissue (liver, kidneys, brain, muscles, lungs and testes) oxidative damage (Radulović et al., 2013). The mechanism underlying CCl4 toxicity involves the generation of trichloromethyl free radicals via a biotransformation by cytochrome P450, that are further transformed into trichloromethyl peroxide radicals which could cause a damage to different cell structures (Vural et al., 2017; Radulović et al., 2013). As the result of CCl4 inhalation, ingestion, skin absorption or intraperitoneal administration, the generated free radicals induce mainly necrotic cell and tissue damage leading to the intracellular enzyme leakage into the blood stream (Radulović et al., 2013). However, up to know other tissue damaging effects, extrahepatic tissue, of CCl4 are completely understood.

Among three types of body muscles, skeletal muscles (SM) are the only ones that can contract deliberately allowing locomotion and body posture. Such function can be achieved due to SM specific...
antibacterial and physiological organization (Thews et al., 1999). Under certain conditions (diseases) human body SM mass and function can be significantly altered. This frequently arises as a consequence of protein degradation, since SM represent an important source of amino acids needed for protein synthesis (Wolfe et al., 2006). Such a decrease in SM weight and function is emphasized in several liver disorders such as cirrhosis or fulminant hepatic failure (Warren et al., 2007). Additionally, under physiological conditions, muscle damage can occur during prolonged exercise/training as a result of an imbalance between generated reactive oxygen species (ROS) and tissue antioxidant capabilities (Bentley et al., 2015). It is worth mentioning that SM are able to successfully recover their structure and function within few weeks following mechanical/chemical injury (Kozakowska et al., 2015).

Antioxidants are known to prevent different tissue injury induced by CCl₄ application (Popović et al., 2016; Radulović et al., 2013), however only there are a limited number of papers evaluating effects of different substances on SM tissue damage caused by acutely administered CCl₄ (Hwang et al., 2016). Melatonin (MLT) is an indolamine that has been isolated from the bovine pineal tissue for the first time, although nowadays it is considered to be synthesized by numerous, if not all, cells and organs (Mehanna et al., 2017). Besides its hormone related activity in human organism, MLT possesses strong antioxidant potential and is able to maintain tissue redox homeostasis (Mehanna et al., 2017). The potential of MLT in preventing SM tissue injury by different physical/chemical agents is poorly studied and up to now only several publications are dealing with these problems (Mehanna et al., 2017). However, there are no publications dealing with the effects of MLT on acute SM damage induced by CCl₄.

Thus, the goal of the present study was to detect and describe, for the first time, the biochemical and histopathological changes occurring in SM of rats treated with MLT and acutely exposed to CCl₄.

2. Materials and methods

2.1. Chemicals, reagents and instruments

All chemicals and reagents used in this experiment were purchased from eider Sigma (St. Louis, MO, USA) or AbCam (Cambridge, UK). All chemicals were of analytical grade and all reagents were prepared prior to the experiment. Melatonin was dissolved in absolute ethanol (<0.1%, v/v) and further diluted, daily, in a sterile saline solution (0.9% NaCl) prior to the administration at a dose of 50 mg/kg. Spectrophotometric analyses were performed using V-1800 Shimadzu spectrophotometer and/or microplate reader (Multiscan Ascent; Labsystems, Helsinki, Finland).

2.2. Animals and housing

Male Wistar rats, weighting 250–300 g, housed in groups of 6, were obtained from the Vivarium of the Institute of Biomedical Research, Faculty of Medicine, University of Niš, Serbia. The animals were maintained under standard laboratory conditions: temperature 22 ± 2 °C and humidity 60%, with food and water available ad libitum. All experimental procedures with the animals were conducted in compliance with the declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU) and were also approved by the local Ethics Committee.

2.3. Muscle tissue damage induction

Before the experiment, all animals were divided into four groups of 6 rats each: the control group (group I) where the animals were intraperitoneally (i.p.) administered with vehicle (olive oil) in the dose of 10 ml/kg and the second control group that received MLT (50 mg/kg, applied by an i.p. injection) only (group II). Experimental groups with CCl₄-treated animals were i.p. administered with CCl₄ (1 ml/kg) (group III), known to cause significant liver damage (Radulović et al., 2013), while the last group (group IV) received MLT (i.p.) in a dose of 50 mg/kg 1 h before CCl₄ (Ohta et al., 2000). All animals were sacrificed by an overdose of ketamine 24 h after the last treatment.

Skeletal muscle tissue samples collected, using scissors and tweezers, for histological analyses included left biceps muscle (BM) specimen. Blood samples, withdrawn by a cardiac puncture, and right BM were used for the evaluation of biochemical parameters and were kept at −80 °C until analyses.

2.4. Blood biochemical measurements

The blood samples were centrifuged at 2500 rpm at 4 °C for 15 min to obtain sera in which lactate dehydrogenase (LDH), creatine kinase (CK) and potassium (K) levels were assayed using Olympus AU680 Chemistry-Immuno Analyzer.

2.5. Tissue biochemical measurements

Collected BM tissue specimens were homogenized in an ice-cold distilled water (10%, w/v) and centrifuged afterward (12,000 rpm, 10 min, 4 °C) in order to obtain a clear supernatant which was further used for the determination of tissue oxidative damage. Homogenate protein content, calculated based on the albumin standard curve, was determined using Lowry’s method (Lowry et al., 1951).

2.5.1. Determination of malondialdehyde (MDA) concentrations

Malondialdehyde in muscle tissue homogenates was determined using a spectrophotometric method that is based on the reaction between thiobarbituric acid and MDA under increased heat (Popović et al., 2016). The absorbance of the reaction mixture was recorded at 532 nm and the concentration was expressed as nmol per mg of protein.

2.5.2. Determination of tissue antioxidant capacity (TAC)

Tissue antioxidant capacity (TAC), expressed as Trolox equivalents (mM), was determined using a colorimetric assay kit (Abcam65329) in a reaction based on Cu²⁺ conversion to Cu⁺ ions. The reaction end-product was measured at 570 nm.

2.5.3. Determination of catalase (CAT) activity

Catalase (CAT) activity was measured spectrophotometrically, after a reaction with H₂O₂ as a substrate and ammonium molybdate, at 405 nm (Göth et al., 1991) and the results are presented as U/g of proteins.

2.5.4. Determination of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was assessed in skeletal muscle tissues homogenates using a colorimetric assay kit (Abcam653454). The assay utilized water-soluble tetrazolium salt (WST-1) as a reagent, which in the presence of superoxide anion results in decrease of WST formation that can be detected at 450 nm. Therefore, SOD activity was calculated on the basis of the percent inhibition of WST-1 reduction, which in turn reflected the percent inhibition of the superoxide anions compared to the control group.

2.5.5. Determination of xanthine oxidase (XO) activity

Xanthine oxidase (XO) activity in BM homogenates was determined based on the amounts of uric acid produced in a reaction...
after xanthine (0.6 mM) addition (Sokolović et al., 2015). The activity of XO was expressed as U/mg tissue protein.

2.5.6. Determination of glutathione peroxidase (GPx) activity

Glutathione peroxidases (GPx) activity was determined spectrophotometrically following previously described method (Popović et al., 2016), based on the reaction between the remaining reduced glutathione (GSH), after the reaction with H2O2, and DTNB reagent [5,5-dithiobis(2-nitrobenzoic acid)]. The activity of GPx was expressed in U per mg of proteins.

2.5.7. Determination of reduced glutathione (GSH) concentration

Tissue GSH concentration was estimated using a standard method previously described by Ellman (1959), where after short incubation with DTNB reagent the absorbance of the reaction mixture was measured at 412 nm. The amount of GSH was determined based on the standard curve and expressed as μmol of GSH/mg of proteins.

2.6. Histopathological observations

The BM tissue specimens separated for histopathological examination were fixed in buffered formaldehyde solution (10%, w/v). The fixed tissues were then dehydrated with ethanol solutions of differing concentration (50–100%, v/v), embedded in paraffin, cut into 4–5 μm thick sections and stained with hematoxylin and eosin (HE). The extent of CCl4-induced muscle tissue damage was evaluated on HE stained tissue sections at >40 magnification lens. From each animal BM sample was taken for pathohistological analysis and at least 20 high power fields were analyzed per sample. Disorganization of muscle fibers, inflammatory infiltrate and edema of the interstitial fluid were scored on HE-stained muscle tissue specimens as absent (0), mildly (1), moderately (2) or severely (3) present (Rizo-Roca et al., 2015).

2.7. Statistical analysis

Data presented as mean ± SD were compared using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test for multiple comparisons (GraphPad Prism, ver. 5.03; San Diego, CA). Probability values (p) less than 0.05 were considered to be statistically significant.

3. Results

Serum levels of K+, LDH and CK were statistically significantly higher in CCl4-treated animals compared to the group I (Fig. 1A–C). Pre-treatment with MLT statistically significantly prevented an increase in K+, LDH and CK levels induced by CCl4, however the differences in serum levels of these three parameters were in the group IV still significantly higher compared to the control group (Fig. 1A–C).

The data presented in Table 1 reflect BM tissue biochemical parameters found in control (group I and II) and CCl4-treated groups (group III and IV) of animals. Muscle tissue obtained from the rats exposed to CCl4 only (group III) had statistically significantly larger amounts of MDA (p > 0.001) compared to the tissue of control group animals (Table 1). The amounts of MDA in animals that received MLT prior to CCl4 were the same (statistically speaking) as in the control groups (Table 1). The activity of XO was statistically significantly increased in the group of rats that received CCl4, while in animals treated with MLT and CCl4 such significant increase was suppressed (Table 1). Animals exposed to CCl4 only (group III) had statistically significantly lower TAC and SOD, CAT and GPx activities in BM compared to the group I (Table 1). Applied MLT was able to statistically significantly prevent CCl4-induced decrease in BM tissue TAC, SOD and CAT activity, but not the GPx activity (Table 1). The amount of GSH in BM tissue was statistically significantly decreased, compared to the group I, in both groups that received CCl4, regardless whether the MLT was applied (Table 1). Even though MLT was able to significantly diminish harmful effects of CCl4 in BM, it did not completely abolish all changes since the significant difference compared to the control group existed for the majority of investigated parameters (Table 1).

The BM specimens obtained from healthy control group and the one treated with MLT consisted mainly of uniform polygonal myofibers with peripherally positioned nuclei, intact sarcolemma and sarcoplasm (Fig. 2A and B; Table 2), free of any pathological changes (pathological score was 0). On the other hand, BM of animals treated only with CCl4 had abnormal fiber morphology with

![Fig. 1. Serum levels of K+ (A), LDH (B) and CK (C) obtained from experimental groups of rats. Data are presented as mean ± SD (n = 6). *p < 0.001 vs. group I; #p < 0.001, ##p < 0.001 vs. CCl4-treated rats.](image)
edematous (swollen) rounded muscle fibers and with a splitting of the sarcoplasm (Fig. 2C and Table 2; score 2 for changes in muscle fibers), while several samples even had areas of necrosis (or cell melting). The interstitial compartment was moderately distended on majority of the samples from the CCl4 group (score 2), while muscle fibers from the control group were tightly packed together. Inflammatory cell infiltrate, comprised of either neutrophils, lymphocytes or macrophages, was present both around blood vessels and between muscle fibers in the SM from animals treated with CCl4 (Table 2 and Fig. 2D). Muscle tissue specimens from the group

| Tracked parameter                  | Vehicle control (group I) | MLT control (group II) | CCl4 treated (group III) | MLT + CCl4 treated (group IV) |
|-----------------------------------|---------------------------|------------------------|--------------------------|-------------------------------|
| MDA (μmol/mg of proteins)         | 7.3 ± 1.6                 | 6.9 ± 1.3              | 17.5 ± 3.7               | 9.2 ± 0.3*                   |
| XO (U/g of proteins)              | 3.5 ± 0.3                 | 3.4 ± 0.1              | 21.5 ± 1.8               | 13.7 ± 1.2*                  |
| SOD (% of decrease in activity)   | 0 ± 18                    | 15 ± 4                 | 96.8 ± 15.3              | 52.3 ± 8.4*                  |
| CAT (U/g of proteins)             | 137.9 ± 7.4               | 127.4 ± 9.3            | 105.8 ± 5.4              | 52.3 ± 8.4*                  |
| TAC (mM)                          | 54.1 ± 1.4                | 56.2 ± 2.3             | 31.3 ± 4.6               | 44.7 ± 2.9*                  |
| GPx (U/g of proteins)             | 24.6 ± 0.8                | 23.6 ± 0.2             | 22.8 ± 0.6               | 22.9 ± 0.4                   |
| GSH (nmol/mg of proteins)         | 31.8 ± 3.5                | 28.8 ± 1.6             | 9.5 ± 6.5                | 11.7 ± 6.1*                  |

The results are given as mean ± SD (n = 6).

* p < 0.001.
** p < 0.05 vs. group I.
# p < 0.001 vs. CCl4-treated rats (group III).

Fig. 2. Microscopic appearance of skeletal muscle tissue obtained from the control and CCl4-treated group. Normal morphology of muscle fiber was observed in the control group (A (×200) and B (×400)), while in the CCl4-treated group, muscle fibers appeared edematous or necrotic/melting (C, ×200), with abundant inflammatory cell infiltrate present between muscle fibers (D, ×200); in the group of animals treated with melatonin and CCl4 only mild muscle fiber damage and interstitial distension, without inflammatory infiltrate, was found (E and F, ×200).
of animals treated with MLT and CCl₄ possessed the same changes seen in animals treated with CCl₄ only, however their intensity was significantly milder (Table 2 and Fig. 2E and F). The edema was only present in limited number of muscle fibers (score 1) and the distension of the interstitial compartment (score 0.75) was not so evident (Fig. 2E), also inflammatory cell infiltrate was comprised on fewer inflammatory cells (Fig. 2F), while there were no areas with necrotic SM cells. Evidently, pathological scores estimated for fiber degeneration, inflammation and interstitial compartment (Table 2) of the muscles obtained from all four experimental groups were in accordance with the described changes. The total score in group III (treated with CCl₄) was 5.75, while in group that received MLT prior to CCl₄ the total score was 2.25 (Table 2).

### 4. Discussion

The muscle studied, BM, represent important hind-limb muscles that enable animal movement. Biceps femoris muscle is the largest muscle in the hind limb and has multiple functions that involve thigh abduction, hip extension and knee flexion (Armstrong and Phelps, 1984). Its importance and significance for extremities proper functioning prompted us to evaluate the effects of MLT treatment when BM are damaged by an acute CCl₄ administration. The administration of CCl₄ leads to trichloromethyl free radicals generation by cytochrome P450 and the process is mainly happening in the liver (significant cytochrome P450 expression), as well as other tissues expressing these enzymes, including SM (Smith et al., 2000).

An increase in potassium ion, LDH and CK serum levels indicate an statistically significant muscle damage occurring after acute CCl₄ administration (Fig. 1A–C). Such claims are supported by the findings that show that the mentioned serum biochemical parameters are in good correlation with skeletal muscle state (Brancaccio et al., 2017). The applied MLT was able to significantly prevent an increase in serum potassium ion, LDH and CK levels (Fig. 1A–C), which can be attributed to MLT ability to scavenge free radicals, since the free radicals are directly responsible for tissue damage caused by CCl₄ (group III). Furthermore, MLT is known to modulate the reduction in MDA levels by MLT most probably reflected the free radicals scavenging activity (estimated throughout MDA concentration) (Table 1). This activity can directly be attributed to MLT ability to scavenge free radicals, since the free radicals are directly responsible for the MDA formation (Kocic et al., 2017; Popovic et al., 2016). Thus, the reduction in MDA levels by MLT most probably reflected the SM cell membrane function, making it more stable, which mirrored on the serum potassium levels and serum LDH and CK activities (Fig. 1). In the present model of CCl₄-induced tissue damage MLT is suggested to prevent tissue injury by scavenging cytochrome P450 generated CCl₄O₂ (Nišković et al., 2018).

Besides liver cytochrome P450 generated free radicals in this model, muscle tissue XO is also contributing to ROS induced muscle tissue damage by generating large amounts of ROS as well (Steinbacher and Eckl, 2015). Thus, after CCl₄ application both systemic and locally generated ROS significantly affect skeletal muscle tissue enzymatic and non-enzymatic capacities (Table 1). The applied MLT was able to statistically significantly reduce CCl₄-induced muscle tissue XO increase and these effects might be attributed to MLT direct inhibitory effects on XO (Kocic et al., 2017; Teixeira et al., 2003). The activity of XO is significantly increased by the generated ROS, thus MLT ability to reach to all tissue/cell compartments and to scavenge ROS (Mohamed, 2015) could be one of the mechanism involved in preventing XO increase as well.

All myofibrils possess their own distinct defense enzymatic and non-enzymatic mechanisms that could cope with excessive ROS (Conchar, 2005). Estimated TAC in BM of animals treated with MLT prior to CCl₄ showed a significant improvement compared to animals that did not receive MLT (group III). Such finding can be potentially the consequence of MLT ability to increase/preserve skeletal muscle tissue antioxidative capacities, enzymatic and non-enzymatic ones. It is proven that muscle tissue TAC significantly correlates with citrate synthase activity and myoglobin content (Masuda et al., 2003), that probably remained unchanged, or slightly decreased, within the skeletal muscle cells of animals treated with MLT and CCl₄. Such assumptions are being made based on the MDA amounts in group III and IV (Table 1), where the MLT (group IV) was able to prevent cell/organelle membrane lipids damage caused by CCl₄ (group III). Furthermore, MLT is known to cause the activation of enzymatic antioxidants (SOD, CAT and GPx) in different rat tissue (Rodriguez et al., 2004) and one can expect of such to occur in skeletal muscles as well.

The activity of SOD, cytosolic and mitochondrial enzyme, in skeletal muscle cells is essential for both cell ROS regulation and myogenic fiber contraction (Kozakowska et al., 2015). Another antioxidative enzyme, CAT can be found in muscle cell peroxisomes and is involved in H₂O₂ breakdown (Kozakowska et al., 2015). Also, it is well established that highly oxidative muscle fibers have higher CAT and SOD activities than other muscle fibers (Kozakowska et al., 2015). Additionally, in some other animal models mimicking SM cell damage (Alzheimer’s disease) it is suggested that CAT activity is significantly decreased, correlating with changes found in same SM (Monteiro-Cardoso et al., 2015). The results of the present study demonstrated that MLT was able to statistically significantly prevent a dramatic decrease in these two enzyme activities (Table 1). These results could be explained by MLT potential to cause an increase in tissue antioxidant enzyme

| Table 2 | Pathological scores obtained BM from different experimental animal groups. |
|---------|-----------------------------|
| Tracked parameter | Vehicle control (group I) | MLT control (group II) | CCl₄ treated (group III) | MLT + CCl₄ treated (group IV) |
| Muscle fiber | 0 | 0 | 2 | 1 |
| Infiltration | 0 | 0 | 1.75 | 0.5 |
| Interstitial compartment | 0 | 0 | 2 | 0.75 |

The intensity of changes were scored as follows: absent (0), mildly (1), moderately (2) or severely (3) present.
activity (Rodriguez et al., 2004). However, the results of a recent study showed that MLT (10 mg/kg) was not able to reduce neither inflammation or tissue damage resulting from exhaustive exercise in rats (Beck et al., 2015). These results showed that serum SOD activity and GSH amounts, remain unaffected by the MLT treatment (Beck et al., 2015).

In CCl4-induced tissue damage a decrease in cellular GSH content represents a logical occurrence, since this nonprotein thiol is involved in xenobiotics detoxification (Mohamed, 2015) including free radicals generated from CCl4. Although MLT was able to prevent CCl4-induced MDA formation and cellular enzymes leakage it was unable to preserve/increase cellular GSH (Table 1). Also, the decrease in GPx activity was found in muscles of rats exposed to CCl4 regardless of the MLT treatment (Table 1). The activity of GPx, found in cytosol and mitochondria, is of great importance for eliminating H2O2 by transferring electrons from GSH, thereby converting it into two water molecules (Rodriguez et al., 2004). These findings are somewhat unexpected since it is known that MLT is able to increase antioxidant enzyme activity by increasing their production (mRNA expression) and activity (Rodriguez et al., 2004). Partial explanation for such findings might lie in the fact that skeletal muscle tissue antioxidant enzymes (SOD, CAT and GPx) activities are 4-5 time less than the activities of the same enzymes in the liver (Kwon et al., 2014). Thus, the changes caused by CCl4 and/or ameliorated by MLT might be difficult to track in skeletal muscle tissue.

Pathological scores designated for skeletal muscle structures provided detailed information concerning the extent of damage produced by CCl4 application and the ability of MLT to abolish this damage (Table 2). The results of the pathological and biochemical studies are in agreement with a general notion that by preventing an excessive increase in ROS, myofibril disruption could be prevented as well (Clanton and Klawitter, 1999). In addition, since within each muscle different myofibril have different antioxidant defense capacity (Gonchar, 2005) it is not strange that some of the fibers in rats treated with CCl4 were necrotic (Fig. 2). Such dramatic muscle damage, necrosis, was not observed in animals treated with MLT, which suggests that MLT was able to increase all skeletal muscle cells ability to cope with an increase in ROS production. Besides necrosis a significant increase in ROS, seen after CCl4 application, can induce cell apoptosis (Yu et al., 2005), which is characterized by an increase in pro-apoptotic protein (Bax), TGF-β and Smad-2 expression (Al-Rasheed et al., 2016). Previous studies evaluated the potential of MLT in preventing anti-Fas antibodies induced cell damage, where the MLT almost fully prevented an increase in Bax expression (Kocic et al., 2017). The effects of MLT in preventing myofibre damage induced by CCl4 were not solely observed under the microscope (Fig. 2), but also were supported by the changes in serum CK levels (Fig. 1). The toxic damage of CCl4 and its radicals leads to direct damage of myofibers, which in turn lead to the release of highly sensitive MM isoform of CK from the SM cells (Brancaccio et al., 2010). Comparing the pathological finings with the amounts of MDA one can say that the greater the lipid peroxidation, the higher the muscle fiber degeneration (Tables 1 and 2), which is logical if one has in mind that a majority of MDA arises from damaged cell membrane lipids (Popović et al., 2016).

5. Conclusion

The results demonstrated that the skeletal muscle damage caused by CCl4 are partially diminished by a single 50 mg/kg dose of melatonin. These findings are the first one pointing to acute effects of melatonin in CCl4-induced rat skeletal muscle damage model estimated by tissue/serum biochemical parameters and pathological analysis. The treatment with melatonin prior to CCl4 statistically significantly prevented skeletal muscle tissue damage i.e. decreased serum levels of muscle cell damage markers, prevented MDA formation, preserved/increased TAC, SOD and CAT activity and partially preserved muscle tissue morphology.

Conflict of interest

Authors declare no conflict of interest.

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