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Research Article

Evaluating the Effect of Vitamin C on Myocardial Angiogenesis Under Oxidative Stress Induced by Exhaustive Exercise in Rat

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ABSTRACT

Background: The main purpose of the present study was to assess the effects of exhaustive swimming with the consumption of a vitamin C supplement on indices of myocardial oxidative stress and gene expression related to angiogenesis.

Methods: Wistar rats were randomly divided into six groups of normal (C), 100 and 200 mg/kg of vitamin C, (VC100 and VC200), exercise with 100 and 200 mg/kg of vitamin C (Ex+VC100 and Ex+VC200) and exercise without treatment (Ex). Finally, the serum activity of serum creatine phosphokinase (CK) and lactate dehydrogenase (LDH) and heart tissue oxidant/antioxidant parameters, besides gene expression of Vascular endothelial growth factor-B (VEGF-B), angiopoietin 1 (ANGPT-1) and matrix metalloproteinases 2 (MMP-2) was measured.

Results: Significant increase in LDH level was seen in group Ex which was remarkably attenuated in group Ex+VC200 ($p<0.001$). The tissue oxidative stress was observed in group Ex where daily intake of vitamin C could remarkably regulate this property ($p<0.01$). Vitamin C could ameliorate significant upper gene expression of VEGF-B and MMP-2 remarkably ($p<0.05$).

Conclusion: Oxidative condition in myocardial besides over expression of MMP-2, could be concluded as a detrimental condition resulting from exhaustive swimming that continued by the proteolytic release of CK and LDH from the muscle. Upper gene expression of VEGF-B and MMP-2 besides no changes of ANGPT-1 can be concluded as an early stage of angiogenesis. All these events were somehow attenuated by vitamin C which confirmed its beneficial effects as an antioxidant and the role of oxidation properties in the regulation of angiogenesis.

Introduction

According to previous beliefs, physical activity has beneficial roles in the prevention of several cardiovascular diseases, such as coronary heart disease.[1] Recent studies have focused on the effects of prolonged exercise on vascular system of healthy individuals and its deleterious effects.[2,3] These adverse effects of exhaustive exercise on the heart were also documented in animal models of rat which lead to elevated troponin T and creatine kinase as the most sensitive and specific indicators of myocardial damage.[4]

Oxidative damage results from the imbalance between low antioxidant defense capacity and high generation of reactive oxygen or nitrogen species. A large number of studies have reported the oxidative effects of unaccustomed intensity and duration of exercise on generation of these reactive radicals and their impact on heart and muscle.[5,6] the susceptibility of these organs is due to their difference in mitochondrial biogenesis and the occurrence of oxidant induced degeneration.[7] Angiogenesis is the important process of the formation of new blood vessel from the existing vasculature in response to proangiogenic factors. These include vascular endothelial growth factor-B (VEGF-B) and angiopoietin 1 (ANGPT-1), which improve cellular perfusion and stimulate vascular regrowth within the infarction area.[8] To address the oxidative stress as the consequence of myocardial infarction, scientists have used a variety of models, such as diabetes mellitus and those who had coronary artery suture to define ischemia-reperfusion injury.[9-11] in acute myocardial damage, delay angiogenic response due to oxidative stress has been reported.[12] In the chronic phase, NADH oxidase (NOX) has also been defined producing ROS and activating down-stream signals by receptors of pro-angiogenic growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF).[13]

The matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which have stimulatory effects of angiogenesis in boosting cancer cell growth.[14] Gelatinase-A (MMP-2) has been reported to be released under VEGF-B stimulation of human dermal

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microvascular endothelial cells,[15] human dental pulp cells[16] and has involved in coronary artery wall formation.[17] Vitamin C is a water-soluble antioxidant molecule, by anti-arrhythmic and anti-atherosclerotic effect, it quenches reactive oxygen species (ROS) and inhibits ROS-mediated nitric oxide (NO) activation.[18,19]
Due to the sensitivity of heart cells to oxidative damages and the effects of exhaustive exercise on oxidative stress, we decided to evaluate oxidative stress related to exhaustive swimming, gene expression of VEGF-B, ANGPT-1, and MMP-2 and evaluate the effect of vitamin C, as a potent antioxidant agent on oxidative stress and gene expression.

Materials and Methods

Chemicals

Vitamin C were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). The dose of 100 and 200 mg/kg of vitamin C in 500µl normal saline was administrated orally (by gavage) per day during the experiment.

Experimental design

Thirty six male Wistar rats weighing about 270-300g were kept in standard condition throughout acclimatization and experiment period. These include appropriate temperature (25 ± 2 ° C), standard hygienic housing (33 × 23×12 cm3), light (12-h light/dark cycles) and adequate diet by free access to commercial rat chow diet and tap water *ad libitum*. This was approved by the local ethics committee of our faculty state, and national medical board, in accordance with the ethics standards of "Principles of Laboratory Animal Care". Grouping of animal was done randomly after one week of acclimatization. The groups include no treatment (Group C; n=6), control which received 100mg/kg of vitamin C (Group VC100; n=6), control group which received 200mg/kg of vitamin C (Group VC200; n=6), exhausted swimming group without supplementary treatment (Group Ex; n=6), exhausted swimming group which received 100mg/kg of vitamin C (Group Ex+VC100; n=6), and exhausted swimming group which received 200mg/kg of vitamin C (Group Ex+VC200; n=6).

Exercise training

Before the start of swimming course for animals, one week swim was designed to accommodate and adapt them to their environment. So the rats began to swim for 30 min in the first session, 40 min for the next session and 60 min for the last session. Training program, consist of ten weeks, five days a week, an hour per session. In each week the train duration was increased for half an hour, so that it rises to three hours per session on the fifth week, and stayed constant until the end of the experiment.[20] The swimming pool designed to be 100 × 60 × 60 cm size and 30 °C to 35 °C heats.

Sample collection

At the end of the experiment, all the rats were sacrificed under anesthesia with chloroform. The blood was drawn by the aspiration of abdominal aorta and centrifuged at 3000×g in 10 minutes to collect sera samples. The samples were then freeze immediately at −20 °C. Heart muscle was dissected, washed rapidly with cold PBS, immediately imprisoned in liquid nitrogen and stored at -75 °C for gene expression and biochemical analysis. All experiments were performed in accordance with the procedures approved by the Animal Care Center-Hamadan University of Medical Sciences.

Analysis of serum biochemical parameters

Serum Creatine Phosphokinase (CK) and Lactate Dehydrogenase (LDH) were measured in serum by routine commercial kits (Parsazmun, Iran).

Antioxidant analysis

The supernatant was prepared by washing 10 mg of heart tissue in cold PBS and homogenized in lysate buffer contained 1% Triton X-100, 500 mM, Tris/HCl, pH 7.6, 200 mM NaCl, and 10 mM $CaCl_2$, protease inhibitor cocktail [Sigma-Aldrich Co. Ltd., Dorset, UK] and centrifuged for 5 min at 4°C and 14000×g. After quantification of protein content by bicinchoninic acid (BCA) method, supernatant was used for the evaluation of the following anti-oxidant assays. The Nitrate/nitrite content and glutathione peroxidase activity (GPX) were measured using a colorimetric assay kit (ZellBio GmbH, Ulm-Germany) according to manufacturer's instructions. The abcam assay kit (Cambridge, MA) was used to analyze the activity of SOD (ab65354). Malondialdehyde (MDA), as a biomarker of oxidative damage of polyunsaturated fatty acids and byproduct of lipid peroxidation, thiobarbituric acid reactive substances (TBARS**)** were measured according to the Yagi method (1984) using 1.1.3.3-tetraethoxypropane as the standard and expressed as pmol/mg protein.[21] Total antioxidant capacity (TAC) was assayed by reducing ferric ion (Fe^{3+}) complex found in the 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) to the ferrous ion (Fe^{2+}) and expressed as nmol/mg protein.[22]

Quantitative Real-Time PCR (q-PCR)

The stages of q-PCR, include RNA extraction, qualification and quantification, complementary DNA (c-DNA) synthesis and real time amplification. For these, TRizol (Thermo Fisher Scientific, USA) was used for RNA extraction. The quality and quantity of RNA were then determined by nanodrop One^c UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) and agarose 2% (Sigma-Aldrich Co. Ltd., Dorset, UK) in TBE (Tris/Borate/EDTA) buffer. Reversed c-DNA was then created by the cDNA synthesis kit (Thermo Fisher Scientific, USA). The gene of VEGF-B, ANGPT1 and MMP-2 was amplified using SYBR *premix* Ampliqon (Odense M, Denmark), by Real-Time PCR on a Roche LightCycler® 96 System (Roche Diagnostics Corporation, Indianapolis-USA) in comparison with hypoxanthine phosphoribosyltransferase 1 (HPRT1) as a housekeeping gene. Gene expression was calculated via

$2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen.[23] The forward and reverse primer sequences used were listed as; HPRT1 forward: 5′- CCTCCTCAGACCGCTTTTCC -3′, and reverse: 5′- CACTAATCACGACGCTGGGA-3′; VEGF-B forward: 5′- GCAACACCAAGTCCGAATG -3′, and reverse: 5′- CTTCACAGCACTCTCCTTTCT-3′. ANGPT-1 forward: 5'-ACAAAGGACGCTGATAACGAC -3' and reverse: 5'-AGTAGTGCCACTTTATCCCAT-3' and MMP-2 forward: 5'- CCCCTATCTACACCTACACCA-3' and reverse: 5'- GCGATGCCATCAAAGACAATG-3'.

Statistical analysis

The mean ± standard deviation was used to express all values presented in the table and figures. The statistical significance was measured using One-way analysis of variance (ANOVA) followed by post hoc Tukey test. SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA) and Graph Pad Prism version 6.0 (Graph Pad Software, San Diego, CA, USA) were used to analyzed the result. $p<0.05$ was considered statistically significant.

Results

Effect of vitamin C and exhaustive exercise on serum level of heart biomarkers

As shown in Figure 1, significant increase in serum CK and LDH was observed in group Ex ($p<0.001$). While 200 mg/kg dose of vitamin C could relief LDH level remarkably ($p<0.001$), the administration of any dose of vitamin C couldn't ameliorate CK level.

Effect of vitamin C and exhaustive exercise on myocardial stress marker

Heart tissue oxidant and antioxidant parameters include: TAC, MDA, GPX, NO, and SOD which are summarized in Table 1. Ten weeks exhaustive swimming strongly caused oxidative stress and significantly increased MDA and NO level ($p<0.001$ for all). It also decreased the level of TAC besides the activity of GPX and SOD ($p<0.001$ for all). The alteration of TAC, MDA, GPX, and SOD ameliorated remarkably by vitamin C in both doses ($p<0.001$ for all). The NO level decreased only by a high dose of vitamin C ($p<0.001$). Higher doses of vitamin C could also increase the TAC level and GPX activity, notably better than lower dose of this agent ($p<0.01$). Between all parameters, only NO and MDA levels could be altered to the normal level of vitamin C.

Effect of vitamin C and exhaustive exercise on gene expression of VEGF-B, ANGPT-1, and MMP-2

According to Figure 2, exhaustive exercise significantly increased gene expression of VEGF-B and MMP-2 but not ANGPT-1 compared with normal levels of group C, VC100 and VC200 ($p<0.001$). While daily oral treatment of high dose of vitamin C could attenuate MMP-2 gene expression ($p=0.003$), both doses of this supplementation could also attenuate VEGF-B gene expression. Interestingly, although the inhibitory effect of vitamin C, in high dose, was notably better than low doses (VEGF-B; $p=0.031$, and MMP-2; $p=0.01$), it couldn't normalize their expression level to the normal level of groups C, CV100, and CV200.

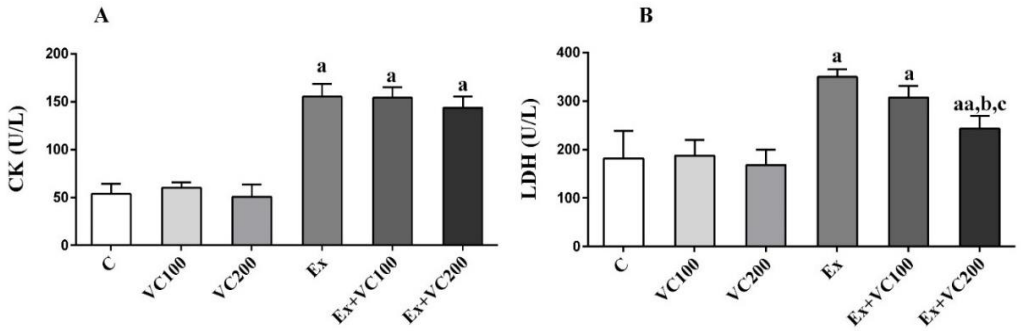

Figure 1. Serum Enzyme activity of creatine kinase (CK, A) and Lactate dehydrogenase (LDH, B) in all groups. Data is represented as mean ± SD. In each row, [a] $p<0.001$ versus group C, [aa] $p<0.05$ versus group C, [b] $p<0.001$ in groups EX+VC100 and EX+VC200 versus group EX, [c] $p<0.05$ in group EX+VC200 versus group EX+VC100 represent significant difference. There is no significant difference in group VC100 and VC200 compared with group C.

Table 1. Hepatic and serum antioxidant level in experimental and control groups.

Parameters	C	VC100	VC200	Ex	Ex+VC100	Ex+VC200
TAC (nmol/mg protein)	280.86 ± 16.62	308.7 ± 19.58	311.74±27.19	105.28±16.47[a]	179.38±14.13[a,b]	232.09±17.15[aa,b,cc]
MDA (pmol/mg protein)	37.14 ± 5.49	36.74± 3.05	26.00±3.54	95.45±13.46[a]	60.73±12.22[a,b]	48.37±6.16[b]
GPX (U/µg protein)	0.61± 0.02	0.62± 0.03	0.62±00.04	0.11±00.01[a]	0.20±00.03[a,b]	0.31±00.01[a,b,c]
SOD (µmol/mg protein)	43.05± 2.24	44.25± 5.22	44.26±02.04	25.14±2.06[a]	35.90±0.87[aa,b]	37.58±0.81[aaa,b]
NO (nmol/mg protein)	1.05± 0.33	0.82± 0.51	0.86±0.15	2.77±0.75[a]	2.01±0.50[aaa]	1.41±0.29[b]

Data is represented as mean ± SD. In each row, [a] $p<0.001$ versus group C, [aa] $p<0.01$ versus group C, [aa] $p<0.01$ versus group C, [aaa] $p<0.05$ versus group C, [b] $p<0.001$ in groups EX+VC100 and EX+VC200 versus group EX, [c] $p<0.001$ in group EX+VC200 versus group EX+VC100 and [cc] $p<0.01$ in group EX+VC200 versus group EX+VC100 represent significant difference. There is no significant difference in group VC100 and VC200 compared with group C.
TAC: total antioxidant capacity, MDA: Malondialdehyde, GPX: Glutathione peroxidase, NO: nitric oxide, SOD, superoxide dismutase.

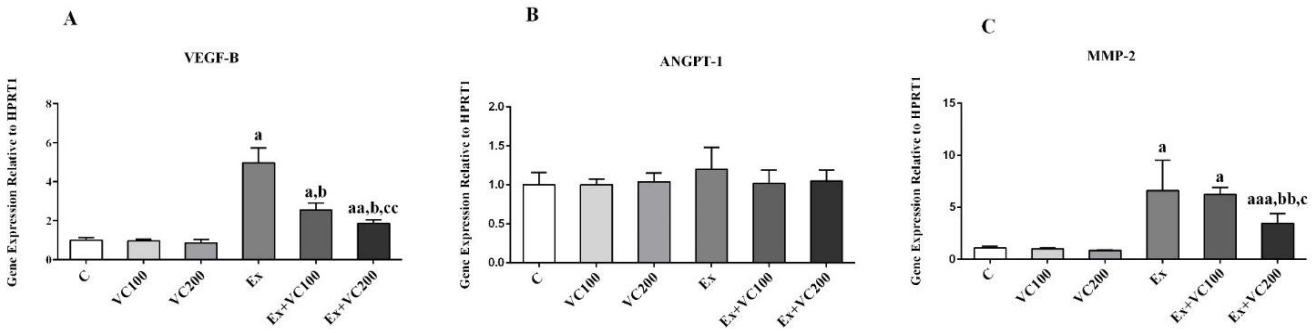

Figure 2. Relative gene expression of VEGF-B (A), ANGPT-1 (B) and MMP-2 (C) in all groups. Data is represented as mean ± SD. In each row, [a] p<0.001 versus group C, [aa] p<0.01 versus group C, [aaa] p<0.05 versus group C, [b] p<0.001 in groups EX+VC100 and EX+VC200 versus group EX, [bb] p<0.01 in groups EX+VC100 and EX+VC200 versus group EX, [c] p<0.01 in group EX+VC200 versus group EX+VC100 and [cc] p<0.05 in group EX+VC200 versus group EX+VC100 represent significant difference. There is no significant difference in group VC100 and VC200 compared with group C.

Discussion

The main purpose of the present study was to explore the oxidative effects of exhaustive exercise on heart muscle, its effect on angiogenesis and to evaluate the relationship between oxidative stresses and angiogenesis. We also used vitamin C by two doses to ameliorate oxidative stress and evaluate the relationship between oxidant, antioxidant parameters, and angiogenesis. Our result showed that vitamin C, especially in high dose, could ameliorate serum biomarkers, balance oxidant, antioxidant parameters of TAC, MDA, GPX, SOD, and NO besides gene expression of VEGF-B, and MMP-2. These muscle degeneration and oxidative biomarkers, like angiogenic genes were changed by exhaustive exercise in a manner which showed the deleterious effects of this activity. Accordingly, excessive fatigue due to the exhaustive swimming leads to drowning, in which the rat remains underwater for more than ten seconds. Organ damage induced by exhaustive exercise resulted in substantial elevation of CK and LDH. So this physical activity induced a varying degree of muscle disturbance manifested by the release of CK, LDH, and aspartate transaminase into the circulation.[24,25] Circulatory creatine kinase was confined to the sarcomere architecture. Surface membrane damage of muscle cell and also modest myocardium disintegration lead to leakage of this enzyme.[26] Lactate dehydrogenase is a NAD (P)-dependent enzyme, which transfers hydride, found in the sarcoplasm of skeletal muscle and cytoplasm of most cells. The level of this enzyme was elevated, especially after fast anaerobic consumption of glucose during muscle contraction.[27] High serum activity of this enzyme points to symptoms of diseases such as acute or chronic muscle cell damaged by pre-oxidative injury through strenuous exercise.[28] The organ injuries due to exhaustive exercise have been reported through the changes of biochemical parameters,[29,30] which is in line with the result of this study. The high level of LDH and CK as a result of cardiac damage induced by corn syrup was previously documented which was significantly attenuated by 200 mg/kg dose of vitamin C.[31] Although in our experiment, vitamin C in higher dose could significantly relief only LDH but not CK, but dose dependent reduction of these serum markers was seen and indicates that higher doses might be able to reduce them significantly. Furthermore, we measured total creatine kinase instead of heart specific creatine kinase (CK-MB) which may justify the ineffectiveness of vitamin C.

The animal trained in the present study was so exhausted that it led to sweeping changes in oxidative factors including lower TAC and higher MDA and NO level besides less GPX and SOD activity. Similar oxidative effects of extreme exercise that induce RBC membrane lipid oxidation were also reported.[32] In exhausted animals, the free radicals produced from the ATP-generating system (which include $O_2^{\bullet-}$ and H_2O_2, respectively) enhanced exercise-related oxidative stress.[33] These free radicals which are dismasted by SOD, an antioxidant defense against hydrogen peroxide could be finally wear off by glutathione peroxidase. Excess production of free radicals besides failure of the antioxidant power could extract a hydrogen atom from the cellular membrane lipids, producing lipid peroxidation end product with longer half-lives and more diffusion power to intracellular and extracellular targets.[34] These conditions can lead to the reduction of antioxidant ability in the body to reduce ferric ion in our experiment which is manifested by lower TAC level. In another mechanism, mild level of nitric oxide has cardio protective effects, but enhanced level during ischemia seems to be detrimental because of the formation of peroxynitrite ($ONOO^-$) by reacting with superoxide ($O^{\bullet-}_2$).[29] It was reported to cause myocardial apoptosis and necrosis in ischemia reperfusion.[35] Higher NO levels after exhaustive exercise in osteoarthritic model of rat were also reported which confirmed our result.[36] Similarly, higher level of NO, MDA besides the lower level of SOD was reported previously as a result of exhaustive exercise and increases in MDA levels in the heart tissue have been reported after exhaustive swimming exercise.[6,37,38] Daily intake of vitamin C in the present study could normalize changes of TAC, MDA, GPX and SOD in both doses, and NO level in higher doses. According to the dual effect of vitamin C in attenuation of oxidative stress[39,40] or as a pro-oxidant compound,[41] our result confirmed the previous hypothesis which mentioned that anti-oxidative effect of

supplementation of vitamin C, has just been observed in situations of high baseline levels of oxidative stress and/or low antioxidant status.[42] According to the vast oxidative changes in the present study, it could be concluded that the beneficial effect of vitamin C was due to high baseline levels of oxidative stress and vitamin C in our study could enhance antioxidant defense system. In the present study, the notable high level gene expression of VEGF-B, and MMP-2 but not ANGPT-1 was observed as a result of ten weeks exhaustive swimming. Exhaustive swimming exercise after maximal exercise tolerance could induce hypoxia and up-regulate gene and protein expression of Hypoxia inducible factor-1 (HIF-1) in various tissues and exactly in skeletal muscle.[43,44] Activation of HIF-1 activates hypoxia responsive element (HRE), which then modulates gene expression of VEGF.[45] Nitric oxide is produced by endothelial nitric oxide synthase (eNOS) in endothelial cell during extreme exercise.[46] Nitric oxide could stimulate VEGF-B in primary steps of extreme activity.[47] High level of NO besides up-regulation of VEGF-B was seen in the present study. In another mechanism, NADH oxidase activation results from ROS and activates pro-angiogenic growth factors such as VEGF-B.[13] High ROS production in the present study via lower SOD activity, besides gene expression of VEGF-B, confirmed the relationship between oxidative stress and VEGF-B expression. Similar to the previous study which indicates the inhibitory effect of vitamin C against VEGF-B,[48] significant lower gene expression of VEGF-B was seen in the present study. Also, slight increase of ANGPT-1 gene expression was seen in trained rats, but it did not reach statistical significance and couldn't change significantly by vitamin C supplementation. While VEGF-B is the most stimulator of angiogenesis, ANGPT-1 supports endothelial cell survival and integrity and is required for the later stages of vascular remodeling.[49] Overexpression of ANGPT-1 dramatically blocks increases in vascular permeability induced by VEGF-B[50] and its enhanced neovessels density in combination with VEGF-B in the corneal implant model.[51] According to this, it could be concluded that the early stages of angiogenesis are ongoing in the present experiment. The notable over expression of MMP-2 was observed by extreme swimming in the present study. The necessity of MMP-2 in angiogenesis and the stimulatory effect of this protein on the release of VEGF-B has been determined.[52,53] the upper expression of VEGF-B might be as a result of MMP-2. On the other hand, it was crystal clear that VEGF, stimulates the release of MMP-2.[15] So it believed that these two compounds stimulate each other and upper gene expression of both was justified in the present study. Dose dependent attenuation of MMP-2 expression was seen by vitamin C treatment in our experiment. In line with this finding regarding the inhibitory effects of vitamin C on MMP-2 expression, other experiments also reported this effect.[54]

Conclusion

In conclusion, MMP-2 was determined as a photolytic and angiogenic compound in extracellular matrix, and overexpression of this, besides the oxidative condition in myocardia could be considered as a detrimental condition that results from exhaustive situation. These conditions were continued by the protolithic release of CK and LDH from the muscle. Upper expression of VEGF-B and its relationship with MMP-2 confirmed the angiogenic events due to oxidative conditions. Vitamin C, due to its anti-oxidant properties could ameliorate oxidative stress, gene expression of MMP-2 and VEGF-B. So these events highlight the deleterious effects of exhaustive extreme exercise on myocardial muscle and the beneficial use of antioxidant agent.

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Conflict of interests

The authors claim that there is no conflict of interest.

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