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Bavachin antioxidant effects on the cardiac myocytes of mice in exhaustive exercise

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Bavachin is the main substance extracted from *Psoralea corylifolia* which is a widely used traditional Chinese medical herb. Studies have shown that bavachin may have a vital antioxidant role in exercise-induced myocardial mitochondrion injury, but the specific antioxidation effect of this herb still remains unexplored. Our study is dedicated to evaluate the antioxidant effect of bavachin in mice with one-off exhaustive swimming, and explore the underlying mechanisms. Bavachin (16 mg·kg^{-1}) was orally administered to mice for 13 days before the forced swimming to exhaustion test. The average swimming time to exhaustion of bavachin-treated mice was prolonged by 56.5% (71.86±28.88 min) compared with that of the control group (45.86 ±12.09 min). Structural analysis showed that myofibrils in the experimental group were lined up in register, the number of mitochondria increased, the membrane structure was clear and kept intact. Malondialdehyde (MDA) contents of the experimental groups significantly decreased ($p < 0.05$) compared with the control group. The activity of H+-K+ ATPase, respiratory chain complex I (RCC I), cytochrome c oxidase (COX) and the levels of superoxide dismutase (SOD) of the experimental groups significantly increased ($p < 0.05$) compared with the control group. Therefore, this study provides a new idea about the protective mechanism of cardiac muscle mitochondria by using bavachin as a natural adjuvant antioxidant to improve the antioxidative levels in mice with one-off exhaustive swimming. These results provide scientific evidence that bavachin has a potential as an antioxidant agent for human beings such as athletes and elderly persons.

Key words: bavachin; one-off exhaustive swimming, mitochondria, energy metabolic enzymes.

INTRODUCTION

Exercise-induced oxidative stress (EIOS) is an imbalance favouring pro-oxidants over antioxidants induced by exhaustive exercise, which may result in the damage of biological components, and has been associated with many pathologic conditions, especially the myocardial mitochondrion injury. The EIOS has been well documented [3,15,23,24]. So far, more than three kinds of mechansms about EIOS have been studied, but researchers still remains divided about what triggers the oxygen free radicals (OFR) booming in EIOS [17]. However, it seems to be obviously summarized that, in the exhaustive exercise, the animal cells produce increased amounts of free radicals [18] and ample reactive oxygen species (ROS), and most OFR are produced by electronic leakage [7] in a series of cytochrome-based enzymes formed in the respiratory chain in the mitochondrial inner membrane, where the mitochondria *in vivo* generate most of the cellular energy supply as adenosine triphosphate (ATP) by ATPase [13]. Under the condition of exhaustive exercise, the OFR cannot be well eliminated by SOD; when the level of OFR exceeds the antioxidant defences, oxidative stress occurs. The surplus OFR disturb the synthesis of ATP in mitochondria and the catalytic activity of H+-K+ ATPase, RCC I and COX. Furthermore, the OFR elevated membrane lipid peroxidation [5] could disturb the protein structure, lead to the structural and functional modification of mitochondria, accelerate the energy metabolism disorder and myocardial cellular damage, and the physiological disturbance of the cardiac muscle. Mitochondrial functional disorders have been implicated in the etiology of several human diseases. In addition, malondialdehyde (MDA) is the main metabolic product of lipid peroxides; the level of MDA reflects the level of OFR and lipid peroxidation, and MDA is commonly used as an indicator of lipid peroxidation [2].

There is a lot of research proving that some natural antioxidants which include many kinds of botanical medicines and medicinal foods, could improve the anti-oxidative level *in vivo*. There are several herbs [9] reported to decrease oxidative stress. Recently, it was indicated that one of the herbs, *psoralea corylifolia*, has more than six pharmacological effects, incuding antioxidant effect [12]. And there are more than 23 kinds of active

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ingredients in *psoralea* [26]. Previous research [19,26] has suggested that bavachin could elevate the activity of SOD in hepatocyte, eliminate OFR, but this has not been studied in EIOS yet.

The objective of the present study is to investigate whether bavachin could attenuate the OFR damage during exhaustive exercise, and explore the underlining antioxidation mechanism. Our study reveals the antioxidant action of bavachin by using exhaustive swimming mice model for the first time. Based on the morphological changes of cardiac myocyte after exhaustive swimming with or without pretreatment of bavachin, our study further verified the antioxidant effect of bavachin.

EXPERIMENTAL

Materials

Plant and reagents

Bavachin was extracted by the Traditional Chinese Medicine Research and Development Laboratories, RongChang Campus, Southwest University (China). The structure of bavachin is shown in Fig 1. Superoxide dismutase (SOD) activity assay kit, malondialdehyde (MDA) assay kit, H+-K+ ATPase activity determination kit and Coomassie Plus (Bradford) assay kit were provided by the Nanjing Jiancheng Bio-engineering Institute (China). TRIZOL®Reagent was purchased from Shanghai Bio-engineering Co., Ltd (China). PrimeScript RT reagent kit with gDNA eraser and SYBR®Premix Ex Taq™ were purchased from Takara Biotechnology Co., Ltd (Japan). 2×Taq PCR master mix kit was purchased from Tiangen Biological Techonology Co., Ltd (China). NADH, cytochrome C and Janus green B were purchased from Sigma (USA).

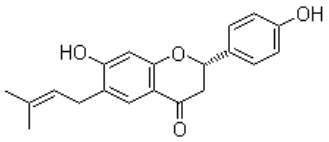


Fig. 1. Molecular formula of bavachin, molecular weight: 324.38, chemical formula $C_{20}H_{20}O_4$, purity of bavachin 97%.

Animals grouping

Male Kunming mice weighing 20-22 g were purchased from the Experimental Animal Center of Sichuan University, used in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research. The study was approved by the ethical committee of Sichuan University. The mice were randomized into the following 5 groups of 10 mice each: control group without any treatment and exercise; untreated group (exhaustive exercise group) which received 16 mg.kg^{-1} body mass of distilled water by gastric perfusion; three treated groups (bavachin-treatment and exercise group) administered with bavachin by gastric perfusion at doses of 24 mg·kg^{-1} body mass, 16mg·kg^{-1} body mass and 12 mg·kg^{-1} body mass, respectively, according to the toxicologic and pharmacologic pretests. In the present study the 16 mg · kg^{-1} body mass group was chosen as the best dose group in the following tests.

Modeling preparation

Swimming was performed in a glass *flume* that was 50 cm long, 40 cm wide and 30 cm high; water temperature was maintained at 32±0.5 °C. Mice in the treated groups were subjected to exhaustive swimming with a load of 5% of their bodyweight attached to the tail of each mouse at 9 a. m., 30 min after giving the drug. The swimming duration until the mouse was exhausted and remained below the water surface for 10 s, without turning over when lying on its back, was recorded. Mice were sacrificed, the heart tissue was removed and divided into two parts: one was used to isolate the mitochondria, the other was used in the study of gene expression.

Myocardial mitochondria isolation and identification

The heart tissue was washed with precooled normal saline three times before it was put into the homogenate medium. The tissue was cut up in the ice bath and homogenized. The heart homogenate was centrifuged for 10 min at 1500 rpm at 4°C, the supernatant was recentrifuged for 10 min at 10,000 rpm at 4 °C, then was discarded. The deposit was resuspended and centrifuged for 10 min at 10,000 rpm at 4 °C. The deposit was resuspended and supplied with 1% Janus green B, the mitochondria particles were dyed in blue-green. Mitochondial protein content was determined with the Coomassie brilliant blue method. The concentration of mitochondial protein was adjusted to be 1.5 mg/ml.

Electron microscope observation

The heart tissues in each group were sectioned and repaired into blocks (1mm×1mm×1mm) for preparing to make paraffin sections after the exhausting swimming. Those sections were fixed and dehydrated according to the methods [27,20,22], then the operator used epoxy resin 821 as the entrapment agent to imbed tissue using UC7 RT ultramicrotome, uranyl acetate and citromalic acid lead double stainings and TECNAI 10 electron

microscope to observe the changes in cellular morphology and ultrastructure.

Measurement of enzyme indexes

The activity of SOD, H+-K+ATPase of mitochondia and the MDA level was measured according to the respective kit instruction.

The activity of COX was measured according to Sciamanna [18] in a 3 ml reaction mixture containing 0.1 ml of 1.5 mg/ml heart mitochondria, 1.5 ml of 0.1 mM phosphate buffer, pH 7.0, and 1.3 ml of distilled water. 0.1 ml of 0.07% reduced cytochrome c was added after 5 min at 36 °C in the water bath. A550 (absorbance at 550 nm) was recorded every 30 s. The activity of COX was represented by the decreasing rate of A550, and it was estimated using the formula: COX (n mol • min^{-1} • mg^{-1}) = 54.6 × the change of A 550/min.

According to the method described by Veitch *et al.* [27], the activity of RCC I was measured as given below. Briefly, 0.03 ml of mitochondrial suspension was added to 0.2M phosphate buffer solution, pH 7.4, 0.3 ml of 10 mM potassium ferricyanide, the volume was adjusted to 2.97 ml. Then, 30 µl of 0.1M NADH (prepared with 10 mM Tris) was added as the initiator. The absorbance was monitored at 420 nm. Activity was measured spectrophotometrically by determining the decrease in absorbance at 420 nm. A molar extinction coefficient of E420 =1.03 mmol•L^{-1}/cm was used in the calculations. Activity was given in µmol of substrate consumed per min per mg of mitochondrial protein.

Measurement of ATPase 6 gene expression level

Total RNA extraction was performed using a TRIzol reagent kit. RNA integrity was verified electrophoretically and spectrophotometrically by OD 260/OD 280 nm, absorption ratio > 1. 95. Total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit with gDNA eraser (Takara) according to the manufacturer's instructions. Real-time PCR was used to determine mRNA levels using a SYBR Premix Ex TaqTM II kit (Takara). Real-time PCR was performed in a Mini Opticon Real PCR Detector (BIO-RAD, USA). β-actin was used as internal control. The PCR primers were designed as follows: the forward primers of β-actin and ATPase 6 are 5'-TCACCCACACTGTGCCCATCTATGA-3'and 5'-ACACCAAAAGGACGAACCTG-3', respectively. The reverse primers of β-actin and ATPase 6 are 5'-CATCGGAACCGCTCATTGCCGATAG-3' and 5'-CGGTGAGAAGTGGGCTAAAG-3', respectively. The PCR products were a 295 bp fragment of β-actin and a 207 bp fragment of ATPase 6. PCR cycles included 94 °C for 30 s, followed by 39 cycles consisting of 95 °C for 5 s, 59 °C for 30 s and 65 °C for 5 s. Measure the fluorescence signal generated during each amplification cycle. The standard curve, the dissolution curve, amplification curve and the cycle threshold (Ct) value were analysed by the Bio-Rad CFX Manager software. Semiquantification of ATPase 6 was calculated using the $2-\Delta\Delta CT$ method [16].

Statistical analysis

The statistical analyses were performed using the SPSS for Windows version 20.0. All results were expressed as means ± standard error. Statistical comparisons of mean values were analyzed by one-way ANOVA. $P<0.05$ was considered as the significant level of difference.

RESULTS AND DISCUSSION

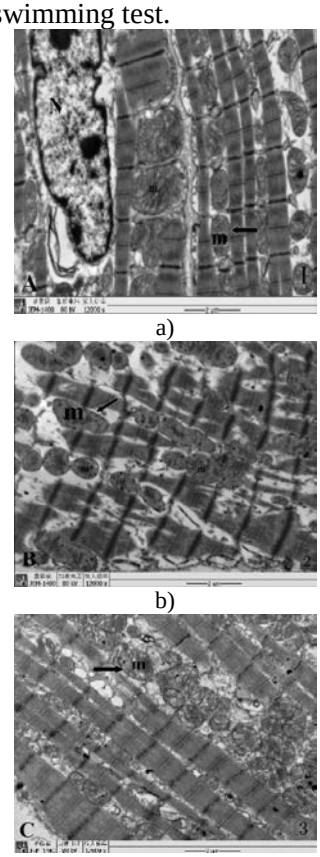
The direct appearance of the anti-fatigue ability of bavachin is the elevated tolerance to exhaustive exercise. Weight-loaded exhaustive swimming duration was used to evaluate the tolerance. The mean duration of exhaustive swimming of the untreated group and mice receiving bavachin in the treated group was 45.86 ± 12.09 and 71.86 ± 28.88 min, respectively. Bavachin given at a dose of 16 mg kg^{-1} significantly lengthened the swimming duration ($p<0.01$). The result of one-off exhaustive swimming time of mice ($\bar{x}$ ± s, n=10) are shown in Table 1.

Table 1. One-off exhaustive swimming time of mice ($\bar{x}$± s, n=10). Data of swimming time are mean ± standard deviation (SD). The control group used as the vacuity contrast.

Groups	Concentration (mg·kg^{-1})	Swimming time (min)
Control group	—	—
Untreated group	—	45.86±12.09
Treated groups	24	75.21±22.16
	16	71.86±28.88
	12	56.22±16.32

Structure alteration is the base of the function alteration, the ultrastructure undergoes the earliest variation. As shown in Fig 2, cardiac muscles were damaged after exhaustive exercise in each group. In the muscle tissue of exhaustive exercised mice, mitochondria showed characteristic vacuolation and varying sizes, cristae disappeared or were deformed, myomeres were incomplete, myofibrils were disarrayed and disasociated when compared with those of the control group. By contrast, bavachin administration in the 16 mg·kg^{-1} dose

group correlated with lesser cardiac myocytes damage. There is no obvious pathology change of myofibrils in the treated groups, but some mitochondria in the treated groups seemed vacuolated and swollen. The obvious pathological changes observed in the untreated group (exhaustive group) revealed that exhaustive exercise may damage the structure of mitochondria and myofibrils, while bavachin can protect against damage in cardiac muscles effectively during the exhaustive swimming test.



a)

b)

c)

□HV □ mag □ — 2 μm —
□80 kV □ 12000 × JEM -1400

Fig. 2. Ultrastructure of mice myocardial cells. A: blank group. B: untreated group (one-off exhaustive swimming group). C: treated group (bavachin group) (A-C, magn., 12000×). M, mitochondrion; N, nucleus; C, chromatin; Scale bar, 2 μm; HV, 80kV.

Compared with those of the control group (A), the myocardial mitochondrial cristae in the untreated group (B) were not clear, vacuolization appeared and mitochondrial morphological multiple myofibrils (M) were incomplete and appeared dissociated. In the 16mg·kg^{-1} bavachin treated group (B), the myofibrils were complete, and the sarcomeres were well arranged, but vacuolization of the mitochondria appeared and the size was irregular.

As shown in Table 2, cardiac myocytes of mice in saline control group had higher MDA levels but lower SOD levels than those of the control group, while MDA and SOD levels of mice in the treated group (16 mg·kg^{-1}) showed significant minimal changes.

MDA and SOD in the control group were obviously higher than those in the control group, which signifies that the one-off exhaustive swimming caused EIOS in mice. Bavachin significantly minimized the concentration variation of MDA and SOD which indicated that bavachin can protect the cardiac mitochondria from the EIOS damage.

Tab 2. Effect on the content of mice myocardial mitochondria MDA and the activity of SOD in exhaustive mice ($\bar{x}$ ±s, n=10).

Groups	MDA (n mol·mgprot^{-1})	SOD (U·mgprot^{-1})
Control group	0.97±0.14	43.51±3.71
Untreated group	2.28±0.390**	33.05±2.63**
Treated group	1.47±0.421$^{\Delta}$	36.06±2.10$^{\Delta}$

Note: Data are mean ± SD. * represents the significant difference compared with the control group (P<0.05), ** represents the extremely significant difference compared with the control group (P<0.01), ΔΔ represents the very significant difference compared with the untreated group (P<0.01), Δ represents the significant differences compared with the untreated group (P <0.05). This note is also applied in fig 3.

As shown in Fig 3, the activity of H+-K+ ATPase, RCC I and COX decreased in the control group. Bavachin (16 mg·kg^{-1}) could protect the cell from exhaustive exercise induced oxidative damage by increasing the activity of H+-K+ATPase, respiratory chain complex and COX.

For the first time this study revealed that bavachin increased the expression of ATPase 6 in cardiac myocytes by RT-PCR. As shown in Figure 6, cardiac myocytes of mice in the untreated group (exhaustive exercise group) had lower ATPase 6 mRNA expression than the control group. The bavachin treatment could significantly up-regulate the expression of ATPase 6 mRNA.

Exhaustive exercise can produce increased ROS which disturbs the oxidant/antioxidant balance, thus elicits oxidative damage. Oxidative stress always followed cardiac damage [8] and other diseases. So there is a necessary and sufficient reason to protect the cardiac muscle from the oxidative damage by using a new antioxidant. This study provides a new idea about using bavachin as a potential natural adjuvant antioxidant to improve the antioxidative levels and anti-fatigue in mice.

In this study, the oxidative stress in mice was caused by one-off exhausted swimming in order to assess the exercise ability and anti-fatigue at the same time. The results shown in Table 1 suggested that after intragastric administration of bavachin,

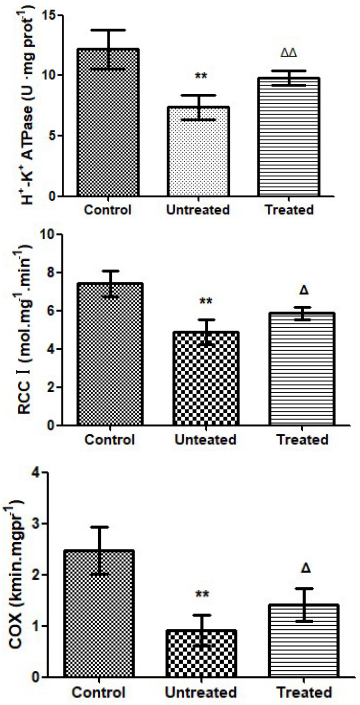


Fig. 3. Effect on the activity of H+-K+ATPase, RCC I and COX in myocardial mitochondria in mice (n=10), (COX, cytochrome c oxidase.)

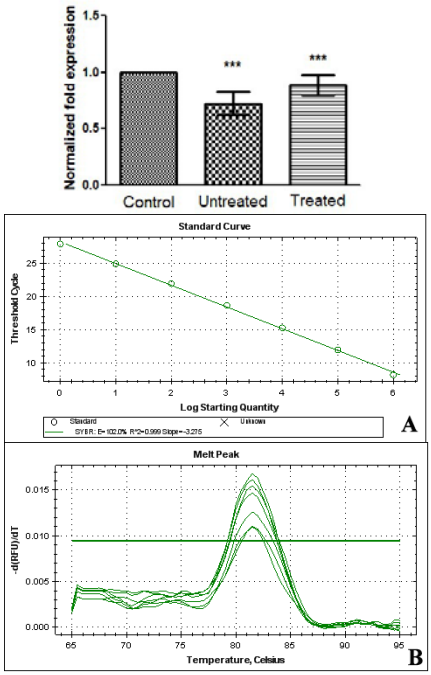


Fig. 4. ATPase6 gene expression in the mice myocardial tissue and standard curve and dissociation curve of fluorescence quantified PCR. A: Standard curve of ATPase subunit 6 gene. B: Dissociation curve of ATPase subunit 6 gene. Data are mean ± SD. $^{***}P<0.01$, treated (n=6) and untreated (n=6) *vs.* control (n=6). Untreated: ATPase6 gene expression after one-off exhaustive training treated without bavachin; and treated: ATPase6 gene expression after one-off exhaustive swimming treated with bavachin.

the time of exhausted swimming was lengthened, and bavachin improved the anti-fatigue ability in mice.

After observing the myocardial mitochondrial ultrastructural organization in one-off exhausted swimming mice by electron microscope, the myocardial mitochondrial cristae were not clear, vacuolization appeared, mitochondrial morphology was abnormal, the size was irregular, multiple myofibrils were broken and dissociation appeared. This indicated that exhausted exercise could damage the structure of mitochondria and myofibrils, and these results were similar to those in ref. [6]. In the bavachin group, the myofibrils were complete and the sarcomeres were well arranged, but vacuolization appeared in the mitochondria and the size was irregular, the degree of injury was lower than that in the one-off exhausted swimming group. This observation revealed that, after giving bavachin, the structure of mitochondria concerned with aerobic metabolism was quickly recovered, in order to accelerate the body recovery.

Mitochondria are the main site where energy and ROS are released [4]. O_2 as the terminal electron acceptor in the respiration chain generates a superoxide anion (O_2•-) after obtaining an electron from complex I, II or III. In the normal exercise, O2•- can be detoxicated by the mitochondrial oxidase SOD. Oxidative stress only occurs while excess ROS cannot be fully cleaned up by antioxidant system. Mitochondrial dysfunction and secondary increase in ROS induce cell death and apoptosis [11]. One important manifestation of muscle fatigue is ATP decrease, energy metabolism abnormality and ROS increase, which can initiate a chain reaction and damage biomacromolecules, then repeatedly damage mitochondria. Muscle fatigue and damaged mitochondria can form an aggravating vicious circle. The results exhibited that bavachin reduced malondialdehyde (MDA) and significantly increased superoxide dismutase levels in myocardial mitochondria.

During exhausted exercise, the body needs to consume massive energy. The energy metabolism gets fast and multiple ROS are produced. If the ROS are not cleared in time, they could attack the cell membrane; lipid peroxides might produce more ROS, attack its double bond, and cause a chain reaction of ROS, causing ion and energy metabolism disturbance. SOD was the primary antioxidative enzyme in the free radicals release clearance mechanism and oxidation-antioxidation mechanism.

There are two main parts of mitochondria participating in the energy transformation process,

one is ATPase and the other is respiration chain. Research shows that the energy of cell comes from the process of oxidative phosphorylation. Several enzymes are involved in this process. H+-K+ ATPase is on the intima of mitochondria which is the key enzyme in ATP synthesis. Enzymatic activity of H+-K+ ATPase was measured in this research. Enzymatic activity of H+-K+ ATPase decreased leading to a decrease in ATP, imbalance of oxidative phosphorylation, dysfunction of energy synthesis and influence on the physiological process. The results show that the enzymatic activity of H+-K+ ATPase in the exhausting exercise group decreased. After gastric perfusion of bavachin for two weeks, the enzymatic activity of H+-K+ ATPase in mice myocardium increased.

Mitochondrial complex I is the biggest enzyme complex in the initiating terminal respiration chain. It is also the sensitive site in ROS damage [10]. The change of complex I is a representative mark for the mitochondrial oxidative phosphorylation. Exhausting exercise can lead to superoxide formation and occurrence of cell death, which can decrease the amount of mitochondria, the mitochondrial membrane potential energy (ATP), the activity of respiratory chain complexes I and IV. The results show that the activity of respiratory chain complexes I in the untreated group obviously decreased; by gastric perfusion of bavachin flavonoid, the enzymatic activity of respiratory chain complexes I increased. So the operator inferred that bavachin provides anti-oxidation protection by respiratory chain complexes I.

COX marks the process of oxidative phosphorylation in the mitochondrial complex, and it is the only cytochrome that can pass electrons to the oxygen molecule. It is the junction between respiratory metabolism and oxygen. COX and succinate dehydrogenase are the key enzyme among mitochondrial respiratory enzymes, the decrease of enzymatic activity of COX and succinate dehydrogenase can affect the process of mitochondrial oxidative phosphorylation. The results show that the enzymatic activity of COX decreases in the exhausting exercise group, which can cause an inhibition of the function in the oxidative phosphorylation electron transport chain. ATP decreases while ROS increases, it can cause energy metabolic disturbance too [1, 25]. By gastric perfusion of bavachin flavonoid, the enzymatic activity of COX increases, so we can concluded that bavachin provides anti-oxidation protection by enzymatic activity of COX.

Compared with other relevant studies, this study ignored some secondary factors, such as ischaemia, oedema, NOS and etc., but it still demonstrates the antioxidant effect of bavachin in a constructive way.

CONCLUSION

In this experiment, the data demonstrate that bavachin could prolong the time of exhaustive swimming, increase the activites of H+-K+ ATPase, COX and RCC I, decrease the oxidative stress reaction in cells, and prevent the myocardial cells from oxidative damage. Through real-time fluorescence quantitative PCR (RTFQ-PCR) it was found that the exhaustive swimming could down-regulate the expression of ATPase 6 mRNA, but after giving bavachin, the expression of ATPase subunit 6 in mice myocardial increased, which suggested that bavachin might up-regulate the expression of ATPase subunit 6.

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REFERENCES

1. A. Sarniak, J. Lipińska, K. Tytman, S. Lipińska, , *Postepy Hig. Med. Dosw.*, **70**, 1150 (2016).
2. A.A.M. Adly, *Res. J. Immunol*., **3**(2), 129 (2010).
3. R.W. Dal Negro, M. Visconti, *Pulmonary Pharmacology & Therapeutics*, **41**, 48 (2016).
4. D.W. Busija, I. Rutkai, S. Dutta, P.V. Katakam, *Compr. Physiol.*, **6**(3), 1529 (2016).
5. E. Altinoz, T. Ozmen, Z. Oner, H. Elbe, M.E. Erdemli, H.G. Bag, *Bratisl. Med. J.*, **117**(7), 381 (2016).
6. W. Wu, S. Chang, Q. Wu, Zh. Xu, P. Wanf, Y. Li, P. Yu, G. Gao, Zh. Shi, X. Duan, Y.-Zh. Chang, *Cell Death & Disease*, **7**(11), e2475 (2016).
7. C.M. Deaton, D.J. Marlin, *Clin. Tech. Equine. Pract.* **2**(3), 278 (2003).
8. S. Zainalabidin, SNFSN Shahidin, S.B. Budin, *Sains Malaysiana*, **45**(2), 207 (2016).
9. I. Paur, M.H. Carlsen, B.L. Halvorsen, R. Blomhoff, *J. Industrial & Engineering Chemistry Fundamentals*, **25**(2), 244 (2011).
10. V. Martínez-Cisuelo, J. Gómez, I. García-Junceda, A. Naudí, R. Cabré, N. Mota-Martorell, M. López-Torres, M. González-Sánchez, R. Pamplona, G. Barja, *Exp. Gerontol.*, **83**, 130 (2016).
11. G. Barrera, F. Gentile, S. Pizzimenti, R.A. Canuto, M. Daga, A. Arcaro, G.P. Cetrangolo, A. Lepore, C. Ferretti, C. Dianzani, G. Muzio, *Antioxidants*, **5** (1), 7 (2016).
12. C.J. Sang, R. Tulasi, S.R. Koyyalamudi, *Cancers*, **8** (3), 33 (2016).
13. H. Tsutsui, S. Kinugawa, S. Matsushima, *Am. J. Physiol. Heart Circ. Physiol.,* **310** (6), 2181 (2011).

14. Y.S. Keum, K.K. Park, J.M. Lee, K.-S. Chun, J. H. Park, S. K. Lee, H. Kwon, Y.-J. Surh, *Cancer Lett.*, **150** (1), 41 (2000).
15. E.N. Chirico, C. Faës, P. Connes, E. Canet-Soulas, C. Martin, V. Pialoux, *Sports Med.*, **46** (5), 629 (2016).
16. M. Yamagishi, *Mol. Breeding*, **36** (1), 3 (2016).
17. N.B.J. Vollaard, J.P. Shearman, C.E. Cooper, *Sports Med.*, **35**(12)**,** 1045 (2005).
18. N. Okudan, S. Revan, S.S. Balci, M. Belviranli, H. Pepe, H. Gökbel, *Bratisl. Lek. Listy*, **113** (7), 393 (2012).
19. Y.L. Qiu, J. Liu, *Chin. J. Vet. Sci*, **46** (3), 55 (2010).
20. E.F. Rosa, R.F. Ribeiro, F.M. Pereira. E. Freymüller, J. Aboulafia, V.L. Nouailhetas, *J. Appl. Physiol.*, **107** (5), 1532 (2009).
21. J. Sastre, M. Asensi, E. Gascó, F.V. Pallardó, J.A. Ferrero, T. Furukawa, J. Viña, *J. Am. J. physiol*, **263** (5 Pt 2), R992 (1992).
22. M.A. Sciamanna, J. Zinkel, A.Y. Fabi, C.P. Lee, *Biochimica et Biophysica Acta*, **1134** (3), 223 (1992).
23. S.K. Powers, Z. Radak, L.J. Li, *J. Physiol.*, **594** (18), 15 (2016).
24. M. Wiecek, M. Maciejczyk, J. Szymura, S. Wiecha, M. Kantorowicz, Z. Szygula, *J. Sports Med. Phys. Fitness*, **57** (7-8), 942 (2016).
25. S. Rahman, J.A. Mayr. Disorders of Oxidative Phosphorylation, Inborn Metabolic Diseases. Springer, Berlin Heidelberg, (2016).
26. H. Haraguchi, J. Inoue, Y. Tamura, K. Mizutan, *Phytotherapy Res. Ptr.*, **16** (6), 539 (2002).
27. K. Veitch, A. Hombroeckx, D. Caucheteux, H. Pouleur, L. Hue*Biochem. J.*, **281** (3), 709 (1992).