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Palladium α-lipoic acid complex formulation enhances activities of Krebs cycle dehydrogenases and respiratory complexes I–IV in the heart of aged rats

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ABSTRACT

Age-related decline in the capacity to withstand stress, such as ischemia and reperfusion, results in congestive heart failure. Though the mechanisms underlying cardiac decay are not clear, age dependent somatic damages to mitochondrial DNA (mtDNA), loss of mitochondrial function, and a resultant increase in oxidative stress in heart muscle cells may be responsible for the increased risk for cardiovascular diseases. The effect of a safe nutritional supplement, POLY-MVA, containing the active ingredient palladium α-lipoic acid complex, was evaluated on the activities of the Krebs cycle enzymes such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase as well as mitochondrial complexes I, II, III, and IV in heart mitochondria of aged male albino rats of Wistar strain. Administration of 0.05 ml/kg of POLY-MVA (which is equivalent to 0.38 mg complexed α-lipoic acid/kg, p.o), once daily for 30 days, was significantly ($p < 0.05$) effective to enhance the Krebs cycle dehydrogenases, and mitochondrial electron transport chain complexes. The unique electronic and redox properties of palladium α-lipoic acid complex appear to be a key to this physiological effectiveness. The results strongly suggest that this formulation might be effective to protect the aging associated risk of cardiovascular and neurodegenerative diseases.

1. Introduction

The heart is one of the organs, which highly depends on oxidative energy generated in mitochondria by oxidative phosphorylation (OXPHOS). Age dependent somatic damages to nuclear and mitochondrial DNA (mtDNA) in heart muscle cells and thickening of arteries may be responsible for the increased risk for cardiovascular diseases (Corral-Debrinski et al., 1992; Kim et al., 2000). Further, anatomic changes during aging in the heart are dominated by left ventricular (LV) hypertrophy due to an increase in LV mass and wall thickness (Lakatta and Levy, 2003). Muscari et al. (1996) reported that the aging heart undergoes significant functional and structural alterations leading to atrophy and a compensatory hypertrophy, followed by myocardial fibrosis. In addition, there is an age-related decline in the capacity to withstand stress, such as ischemia and reperfusion (Lesnefsky et al., 2001). In its most severe form, cardiac decay results in congestive heart failure, one of the leading causes of death in people over the age of 65. Although the mechanisms underlying cardiac decay are not clear, loss of mitochondrial function and a resultant increase in oxidative stress has been proposed to be one of the key factors in myocardial aging (Hagen et al., 2001).

Mitochondria are a major source of reactive oxygen species (ROS) production and oxidative stress during the aging process and therefore are a central model in the age-associated decline in tissue function (Lenaz, 1998; Finkel and Holbrook, 2000; Huang and Manton, 2004). ROS are produced in vivo by electron leakage from electron transport chain (ETC) complexes during normal respiration. In particular, complex I and complex III are the primary sites of ROS production, which leads to decreased electron transportation, OXPHOS, decreased energy production or loss of calcium homeostasis (Lenaz et al., 2002; Liu et al., 2002; Chen et al., 2003; Starkov and Wallace, 2006). Alpha-ketoglutarate dehydrogenase, a key enzyme in the Krebs cycle, has also been implicated in the generation of ROS (Starkov et al., 2004; Tretter and Adam-Vizi, 2004).

Preservation of mitochondrial function is important for maintaining overall health during aging. In order to preserve the genomic and structural integrity of mitochondria and to increase the

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functional life span, diet supplementation with antioxidants, such as vitamins, *N*-acetyl cysteine and DL-α lipoic acid has been suggested (Chow, 1991; Arivazhagan et al., 2001). The small molecule antioxidants such as L-carnitine, ascorbate, α-tocopherols, reduced coenzyme Q_{10}, urate and glutathione, are chain-breaking antioxidants with a capacity to repair oxidizing radicals directly (Buettner, 1993; Haripriya et al., 2004). Alpha-lipoic acid is well known as a powerful biological antioxidant and its therapeutic potential has been explored extensively (Packer et al., 1995).

Palladium complexes seem to exhibit biological action very different from those of toxic platinum complexes. While the main target of platinum-based drugs is DNA, palladium based drugs show preferential targets such as enzymes and lysosomes (Caires, 2007). Covalent palladium α-lipoic acid complex formulation is a safe nutritional supplement. The commercially available supplement, POLY-MVA, is formulated with palladium α-lipoic acid complex. In addition to the active ingredient, palladium α-lipoic acid complex, a proprietary liquid blend contains molybdenum, rhodium, ruthenium, thiamine, riboflavin, cyanocobalamin, *N*-acetyl cysteine and *N*-formyl methionine. Global ischemia experiments with palladium α-lipoic acid formulation demonstrated that it serves as both a highly active free radical scavenger and alternative energy source to the vulnerable hippocampus of the brain (Antonawich et al., 2004). The aim of our study was to evaluate the effect of the complexed palladium α-lipoic acid in a formulation on tricarboxylic acid cycle (Krebs cycle) enzymes and mitochondrial complexes of the ETC in aged rats.

2. Materials and methods

2.1. Chemicals

Rotenone, antimycin-A, 2,6-diclorophenol indophenol sodium salt (DCPIP), decyl ubiquinol, coenzyme A, trisodium isocitrate, nicotinamide adenine dinucleotide (NAD^+) sodium salt, thiamine pyrophosphate, sodium dithionate, α-ketoglutarate, bovine serum albumin (BSA), potassium cyanide, nicotinamide adenine dinucleotide reduced (NADH), oxalo acetate and cytochrome-C were purchased from Sigma Chemical Company, Saint Louis, MO, USA. DL-α-lipoic acid and palladium α-lipoic acid formulation (POLY-MVA) were obtained as a gift from Garnett McKeen Laboratory, Inc., USA and all other chemicals used were of reagent grade.

2.2. Animals

Male albino rats of Wistar strain weighing approximately 350 ± 50 g (age more than 24 months) were considered as old rats used for this study. The experiment was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and by approval of Institutional Animal Ethics Committee, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India.

2.3. Effect of POLY-MVA on the mitochondrial dehydrogenases and respiratory complexes in the heart of aged rats

Animals were divided into different groups such as aged control group, α-lipoic acid group, and palladium α-lipoic acid formulation group, having six rats in each group. The α-lipoic acid used for this study was dissolved in alkaline solution (0.25% NaOH, w/v). The α-lipoic acid group received 5 mg/kg body weight in 2.5 ml/kg of alkaline solution (p.o) and palladium α-lipoic acid formulation group received 0.05 ml/kg of POLY-MVA, administered as diluted solution in a net volume of 2.5 ml/kg (which is equivalent to 0.38 mg complexed α-lipoic acid/kg, p.o). The aged control group was orally administered with 2.5 ml/kg of alkaline solution. On completion of 30 days of α-lipoic acid and palladium α-lipoic acid formulation administration, the animals were sacrificed by cervical decapitation. The heart was excised and kept at −70 °C for mitochondrial enzyme assay.

2.3.1. Preparation of mitochondrial fraction

The mitochondrial pellets were prepared according to the method as described in our previous report (Sudheesh et al., 2009). The isolated mitochondria were suspended in 50 mmol/L phosphate buffer (pH 7.0). The mitochondrial fraction was frozen and thawed 3–5 times to release the enzymes (except complex IV which was extracted with 0.5% Tween 80 in phosphate buffer, v/v). The protein was estimated in the supernatant using the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.3.2. Determination of activities of the Krebs cycle dehydrogenase

The dehydrogenases activities such as activities of isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) were determined at room temperature according to the methods described in Sudheesh et al. (2009) using a double beam spectrophotometer (Systronics-2202 UV–VIS double beam spectrophotometer, Systronics India Ltd., Hyderabad, India). Briefly, ICDH was determined from the rate of reduction of NAD^+ in the presence of trisodium isocitrate at 340 nm (Fatania et al., 1993) and expressed in μmoles of NAD^+ reduced/min/mg protein using the extinction coefficient of NADH (6.3 mM^{-1} cm^{-1}). Similarly, α-KGDH activity was determined from the rate of reduction of NAD^+ in the presence of α-ketoglutarate (potassium salt) (Reed and Mukherjee, 1969). The activity was expressed as μmoles of NAD^+ reduced/min/mg protein using the extinction coefficient of NADH. SDH activity was determined by the method of Nulton-Persson and Szweda (2001) with slight modifications. The activity was determined from the rate of decrease in absorbance at 600 nm after treating the mitochondria with the reaction mixture containing sodium succinate. The extinction coefficient of 2,6-diclorophenol indophenol (DCPIP) (19.1 mM^{-1} cm^{-1}) was used to calculate the activity and expressed in μmoles of DCPIP reduced/min/mg protein. MDH activity was determined by the method of Mehler et al. (1948). The activity was calculated from the rate of oxidation of NADH and expressed in μmoles of NADH oxidized/min/mg protein using the extinction coefficient of NADH.

2.3.3. Determination of the activity of respiratory complexes

Activities of respiratory complexes were determined according to the methods described in Sudheesh et al. (2009). Briefly, the activity of complex I and complex II were estimated by the method of Janssen et al. (2007). The activities were calculated from the linear part of the absorbance–time curve at 600 nm and expressed as μmoles of DCPIP reduced/min/mg protein using the extinction coefficient of DCPIP. Rotenone (1 μmol/L) was used to inhibit the complex I. Complex III activity was determined by the method of Krahenbuhl et al. (1991) with slight modifications. The reaction was started by the addition of decylubiquinol and monitored for 2 min at 550 nm and again after the addition of antimycin-A (1 μmol/L). The activity was calculated from the linear part of absorption–time curve, which was not less than 30 s. The extinction coefficient of ferricytochrome-C (21 mM^{-1} cm^{-1}) was used for the calculation of complex III activity and expressed in μmoles of ferricytochrome-C reduced/min/mg protein. Complex IV activity was determined by the method of Capaldi et al. (1995) with slight modifications. The reaction was started by the addition of enzyme source to solution of ferrocytochrome-C and monitored at 550 nm with an interval of 30 s for 4 min. The activity was expressed as μmoles of ferrocytochrome-C oxidized/min/mg protein using the extinction coefficient of ferricytochrome-C.

2.4. Statistical analysis

All data were represented as mean ± SD. Data were statistically analyzed using one-way analysis of variance (ANOVA) (using the Graph Pad Instat software package). The significant difference between the aged control group and α-lipoic acid or palladium α-lipoic acid formulation administered groups were analyzed by Bonferroni's *t*-test. $p < 0.05$ was considered as significant.

3. Results

Table 1 represents the effects of administration of palladium α-lipoic acid formulation (POLY-MVA), 0.05 ml/kg body weight (which is equivalent to 0.38 mg complexed α-lipoic acid/kg), to aged rats for 30 days on enzymes of tricarboxylic acid cycle (TCA) or Krebs cycle. The administration of palladium α-lipoic acid formulation significantly increased the Krebs cycle enzyme activities as evidenced by the activities of ICDH, α-KGDH, SDH and MDH when compared to the aged control animals. A positive control of DL-α-lipoic acid was also effective to increase the activities of TCA enzymes. For both the palladium α-lipoic acid formulation and α-lipoic acid administered groups, the ICDH activity was ~3.7 fold more than that of the aged control group. Similarly, the fold increase in activity of α-KGDH, SDH, and MDH was ~1.2, 1.6, and 4.4 respectively for the palladium α-lipoic acid formulation administered group compared to ~1.8, 0.9, and 3.9 for the positive control α-lipoic acid treated groups. These values were calculated using the maximum values (mean + SD value) for aged control group and minimum values (mean − SD value) for the two treated groups so that assessments will be modest and the expectations in the enhanced activities are a minimum.

Table 1
Effect of POLY-MVA treatment on the activity of Krebs cycle enzymes.

Groups	ICDH	α-KGDH	SDH	MDH
Aged control	702.8 ± 133.4	63.0 ± 15.1	42.4 ± 14.2	260.9 ± 26.1
DL-α-lipoic acid (5 mg/kg body weight)	3428.2 ± 348.9***	189.0 ± 50.4***	73.4 ± 21.2**	1386.1 ± 265.5***
POLY-MVA (0.05 ml/kg body weight)#	3483.1 ± 388.9***	145.0 ± 50.6*	98.6 ± 7.4***	1305.7 ± 56.4***

Values are mean ± SD; $n = 6$.
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (one-way ANOVA followed by Bonferroni's test) significantly different from the aged control group.
Units: isocitrate dehydrogenase (ICDH) – μmoles of NAD^+ reduced/min/mg protein; α-ketoglutarate dehydrogenase (α-KGDH) – μmoles of NAD^+ reduced/min/mg protein); succinate dehydrogenase (SDH) – μmoles of DCPIP reduced/min/mg protein; malate dehydrogenase (MDH) – μmoles of NADH oxidized/min/mg protein.
0.05 ml/kg body weight (which is equivalent to 0.38 mg complexed α-lipoic acid/kg).

Table 2
Effect of POLY-MVA treatment on the activity of respiratory complexes I–IV.

Groups	Complex I	Complex II	Complex III	Complex IV
Aged control	23.34 ± 2.12	26.75 ± 2.09	13.57 ± 3.89	30.85 ± 1.31
DL-α-lipoic acid (5 mg/kg body weight)	62.04 ± 11.90**	45.55 ± 28.25ns	21.16 ± 8.36ns	47.36 ± 7.54***
POLY-MVA (0.05 mg/kg body weight)#	58.76 ± 31.11*	83.37 ± 28.46**	21.34 ± 3.31ns	48.13 ± 7.32***

Values are mean ± SD; $n = 6$.
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ significantly (one-way ANOVA followed by Bonferroni's test); nsnon-significantly ($p > 0.05$) different from the aged control.
Units: complex I – μmoles of DCPIP reduced/min/mg protein; complex II – μmoles of DCPIP reduced/min/mg protein; complex III – μmoles of ferricytochrome-C reduced/min/mg protein; complex IV – μmoles of ferrocytochrome-C oxidized/min/mg protein.
0.05 ml/kg body weight (which is equivalent to 0.38 mg complexed α-lipoic acid/kg).

The effect of palladium α-lipoic acid formulation and α-lipoic acid on the respiratory complexes I, II, III, and IV in aged rats is shown in Table 2. The enhanced activities of complexes I, III, and IV were very similar for both the palladium α-lipoic acid formulation and α-lipoic acid administered groups. By using the average values, we find that the activities of complexes I, III, and IV were increased ~2.5, 1.6, and 1.6 fold, respectively in the palladium α-lipoic acid administrated group compared to the aged control group. In the case of complex II, the palladium α-lipoic acid formulation administered group had ~1.8 fold increases in activity compared to the α-lipoic acid administered group. Though the activities of Krebs cycle dehydrogenases and respiratory chain complexes in both treated groups were statistically non-significant from each other, we found that to elicit similar energy enhancing effect, 5 mg/kg of DL-α-lipoic acid, which is 13 times the equivalent dose of complexed α-lipoic acid (0.38 mg complexed α-lipoic acid/kg in the formulation), was needed.

Table 3
Composition of POLY-MVA*.

Palladium α-lipoic acid complex (1:1)	3.72×10^{-2} mol/L
Thiamine	2.17×10^{-3} mol/L
N-acetyl cysteine	1.13×10^{-3} mol/L
Riboflavin	4.62×10^{-4} mol/L
N-formyl methionine	1.46×10^{-4} mol/L
Cyanocobalamin (vitamin B12)	1.37×10^{-4} mol/L
Rhodium	1.34×10^{-4} mol/L
Molybdenum	4.63×10^{-4} mol/L
Ruthenium	1.42×10^{-5} mol/L
Sodium chloride	2.64×10^{-1} mol/L

*Data supplied by Manufacturer of POLY-MVA, El-Gen LLC, 7 Shirley Street, Bohemia, NY 11716-1735, USA.

4. Discussion

Results of this study reveal that palladium α-lipoic acid formulation (POLY-MVA) significantly enhanced the mitochondrial TCA cycle dehydrogenases and ETC complexes. A single dose of palladium α-lipoic acid formulation was selected in this study based on our previous observations using 11.4 and 0.38 mg/kg (equivalent dose of complexed α-lipoic acid in the POLY-MVA) orally once daily for 30 days, which produced maximum activity at 0.38 mg/kg. Aging affects all types of nucleated cells. However, the degree of age-related declines of most of the heart mitochondrial enzymes and membrane functions have been reported (Coleman et al., 1987; Savitha and Pannerselvam, 2006). Therefore, in the present study, we selected only aged rats to evaluate the effect of the palladium α-lipoic acid formulation on age-linked declines in mitochondrial enzyme activity. The details of the POLY-MVA composition, as supplied by the manufacturer are included in Table 3. It is clear that the active ingredient, Palladium α-lipoic acid complex in the formulation is about 17 times higher than any other minor components. Previous ischemia studies have demonstrated that the POLY-MVA vehicle, devoid of palladium α-lipoic acid is not significantly different from saline treatment (Antonawich et al., 2004). The dose and selection of α-lipoic acid as a reference standard was based on study of Arivazhagan et al. (2001) that reported the improved mitochondrial-supported bioenergetics from the treatment of α-lipoic acid. However, the dosage of α-lipoic acid used in the reported studies was at least 100 mg/kg. Since palladium α-lipoic acid is the major component present in the POLY-MVA, α-lipoic acid at a dosage of 5 mg/kg has been chosen as the standard control.

The activities of all the TCA cycle enzymes studied were elevated significantly during the 30 days oral administration of palladium α-lipoic acid formulation. The increased activities of ETC complexes especially that of complex I, II and IV, further support the marked effect of administration of palladium α-lipoic acid formulation. Most of the reducing equivalents enter at complex I of the ETC. Therefore, high levels of reduced coenzymes due to increased activity of TCA dehydrogenases in the palladium α-lipoic acid formulation administered group can be correlated to the enhanced activity of complexes of ETC except complex III, which did not statistically differ from that of aged control rats. A similar effect was found for the α-lipoic acid administered group. However, no statistically significant differences were observed between the α-lipoic acid and palladium α-lipoic acid formulation administered groups except for complex II. Heddi et al. (1996) reported that a reduction in the activities of complex I and IV during aging might be due to a decrease in both mitochondrial transcription and translation levels with aging. However, no significant decline of complex II has been observed during aging since no subunits

of this complex are encoded by mitochondrial DNA (Wallace, 1992).

The exact mechanism by which palladium α-lipoic acid formulation enhances the activity of mitochondrial enzymes is not yet determined. However, since the palladium α-lipoic acid complex serves as a potent redox molecule, it may facilitate the formation of the high-energy intermediates (NADH and $FADH_2$). Furthermore, certain trace components of palladium α-lipoic acid formulation, such as *N*-acetyl cysteine, are known to be good antioxidants (De Vries and De Flora, 1993; Scott et al., 1994). DL-α-lipoic acid is a low molecular mass dithiol antioxidant and is a coenzyme in pyruvate dehydrogenase (PDH) and α-KGDH of mitochondria. A decrease in the level of lipoate has been demonstrated during the process of aging (Lykkesfeldt et al., 1998). However, there is no free α-lipoic acid in palladium α-lipoic acid formulation since it is covalently bound to palladium (Garnett, 1995).

The Oxygen Radical Absorbance Capacity (ORAC) analysis provides a measure of the scavenging capacity of antioxidants against the peroxyl radical, which is one of the most common ROS found in the body. The $ORAC_{hydro}$ (μmoles of trolox equivalent/g) values determined by Brunswick Laboratories, Wareham, MA, USA, and reported by Antonawich and Valane (2007) are 5.65, 1.6, 1.4, 1.12, and 1.0 for POLY-MVA, Vitamin A, α-lipoic acid, Vitamin C, and Vitamin E, respectively. These results demonstrate that POLY-MVA is approximately five times more potent antioxidant than α-lipoic acid. However, the exhibited activity in this study cannot be fully explained by the antioxidant activity of the components of palladium α-lipoic acid formulation. Palladium α-lipoic acid complex's unique electronic and redox properties appear to be the key to its physiological effectiveness (Garnett, 1997, 1998; Krishnan and Garnett, 2006).

Several minor components contained in POLY-MVA are intended to enhance its effectiveness as a dietary supplement. *N*-acetyl cysteine is a known antioxidant with numerous properties (De Vries and De Flora, 1993). Thiamine and lipoic acid are essential cofactors in the conversion of pyruvate to acetyl-CoA or α-ketoglutarate to succinyl-CoA. In vitro free radical scavenging activities of thiamin and thiamine diphosphate were reported recently (Okai et al., 2007). Riboflavin or vitamin B_2 is the central component of the cofactors FAD and FMN and reduced forms of these flavoproteins exhibit a wide range of redox potential and therefore can play a key role in the intermediary metabolism. Apart from the normal functioning of the brain, nervous system, and for the formation of blood, Vitamin B12 is also reported to be an in vitro antioxidant (Okai et al., 2006). *N*-Formyl methionine is used in the initiation of protein synthesis. Molybdenum is an essential trace element for all higher organisms. While transition metals such as rhodium and ruthenium are heavily involved in electron transfer processes (Astruc, 1995), their role in the activities of Krebs cycle and respiratory complexes I–IV are not well established. Hence, the role of these minor components present in the palladium α-lipoic acid formulation in enhancing the age related mitochondrial enzyme activity, if any, cannot be ignored.

The toxicological studies indicated that the LD_{50} of palladium α-lipoic acid formulation exceeded 5000 mg/kg. Moreover, no mutagenic effect of the combination was observed in the Ames test (Bunger et al., 1996). Unlike its relative platinum, studies demonstrate no evidence of any mutagenic property for palladium which is combined with α-lipoic acid. Increased production of ROS with concomitant decreases in antioxidant status, DNA modifications and a progressive decline of over all protein synthesis has been reported to accompany aging (Shigenaga et al., 1994; Richter, 1995). Though aging affects all types of nucleated cells, the degree of age-related changes in cardiomyocytes was found to be high (Coleman et al., 1987). This is most probably due to the lower levels of protective enzymes such as superoxide dismutase and catalase in heart than in any other tissues of the body. Therefore, mitochondria of cardiac myocytes become less efficient with increasing age, resulting in greater damage to DNA and proteins.

Cumulative free radical damage can also leads to significant changes in brain mitochondrial functions with aging (Sastre et al., 2000; Floyd and Hansley, 2002). Of particular interest are the changes in the activity of enzyme complexes of the respiratory chain during the aging process and the role of such changes in the progression of neurodegenerative diseases (Navarro and Boveris, 2004). Any decline in the activity of brain respiratory chain enzyme complexes could have a significant impact on brain function particularly on the etiology of neurodegenerative disorders (Beal, 1998; Trushina and McMurray, 2007). Parkinson's disease, Alzheimer's disease, Huntington's disease and Friedreich's ataxia have been associated with several mitochondrial alterations including impaired oxidative phosphorylation (Banaclocha, 2001). Therefore, the exhibited effects of POLY-MVA on the mitochondria possibly explain the effectiveness against the aging related neurodegenerative disorders also.

Results of this study reveal that palladium α-lipoic acid formulation may be an effective agent to protect the age-linked decline of mitochondrial enzymes and to enhance the energy production of normal cell mitochondria. Since palladium α-lipoic acid formulation is non-toxic to normal cells, the cells can actually benefit from the energy boost. Moreover, treatment with palladium α-lipoic acid formulation may protect the mitochondrial integrity through enhancing the energy production by improving the activities of complexes I and IV. Since we do not have data presently available for α-lipoic acid concentration matching to that in complexed α-lipoic acid in POLY-MVA, strict comparisons are not feasible. However, while analyzing the effects of α-lipoic acid and POLY-MVA treated groups on Krebs cycle enzymes and respiratory complexes, though statistically non-significant from each other, the present results strongly suggest that to get similar enzymatic activities for both Krebs cycle and respiratory complexes I–IV, the concentration needed for the complexed form of α-lipoic acid with palladium in POLY-MVA is about 13 times less than that of pure α-lipoic acid. However, further studies especially the role of minor components of this formulation on the mitochondrial energy status and also the in vivo antioxidant properties of palladium α-lipoic acid complex are needed.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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