Effect of POLY-MVA, a palladium α-lipoic acid complex formulation against declined mitochondrial antioxidant status in the myocardium of aged rats

N.P. Sudheesh\textsuperscript{a}, T.A. Ajith\textsuperscript{b}, K.K. Janardhanan\textsuperscript{a}, C.V. Krishnan\textsuperscript{c,d,∗}

\textsuperscript{a} Department of Microbiology, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala 680 555, India
\textsuperscript{b} Department of Biochemistry, Amala Institute of Medical Sciences, Amala Nagar, Thrissur, Kerala 680 555, India
\textsuperscript{c} Garnett McKee Laboratory, Inc., Bohemia, NY 11716-1735, USA
\textsuperscript{d} Department of Chemistry, University at Stony Brook, NY 11794-3400, USA

**Abstract**

Palladium α-lipoic acid formulation – ‘POLY-MVA’ is found to enhance the activities of Krebs cycle dehydrogenases and respiratory complexes in the heart of aged rat. In this study, we aimed to evaluate the effect of POLY-MVA on the activities of antioxidant status in the heart mitochondria of aged rat. We determined the activities of manganese–superoxide dismutase (Mn SOD), catalase (CAT), glutathione peroxidase (GPx), and level of reduced GSH and lipid peroxidation in the heart mitochondria of aged rats, after administering POLY-MVA (0.05 ml/kg; equivalent to 0.38 mg complexed α-lipoic acid/kg) orally once daily for 30 days. DL-α-lipoic acid (0.38 mg/kg, p.o) treated for 30 days was kept as the positive control. We found that the antioxidant in the aged control was declined significantly than the young control. The formulation significantly (\(p < 0.05\)) enhanced the activity of CAT and GPx compared to the aged control. The level of GSH was also significantly improved and the level of lipid peroxidation was decreased significantly (\(p < 0.05\)) by POLY-MVA. The results indicate that POLY-MVA is effective to protect the age-linked decline of myocardial mitochondrial antioxidant status. The findings suggest the use of this formulation against myocardial aging.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Aging has been considered as a major risk factor for human diseases. It is characterized by progressive decline in biochemical and physiological functions of various tissues and organs in an individual. Reactive oxygen species (ROS) which include free radicals (hydroxyl radical, HO\(_2\), and superoxide anion radical, O\(_2^−\)), non-radicals (hydrogen peroxide, H\(_2\)O\(_2\), and singlet oxygen \(\text{^1} \text{O}_2\)), short lived lipid peroxidation products (peroxyl radical, RO\(_2\), and alkoxyl radical RO\(_\bullet\)), and long lived secondary products (malondialdehyde and 4-hydroxyalkenals) are involved in the pathophysiology of various human diseases (Conklin, 2002). Mitochondria are one of the major source of ROS production and oxidative stress during the aging process and therefore play a central part in the age-associated decline of myocardial mitochondrial antioxidant status. They exist in an oxidative steady-state, they are more vulnerable to oxidative damage, when compared to the other organelles. The increased free radical production leads to decreased electron transport, and eventually cause increased mitochondrial damage. The resultant mitochondrial dysfunction will lead either to decreased energy production or to loss of calcium homeostasis. Both conditions are associated with cellular death and will eventually result in a general decline of metabolic activities. According to the mitochondrial theory of aging, the increase in oxidative stress resulting from mitochondrial dysfunction is a basic mechanism of mammalian aging (Herman, 1972).

Recent experimental and clinical investigations have demonstrated that oxidative stress is involved in certain cardiovascular disease states, including atherosclerosis and hypertension. The generation of ROS was found to be increased in the progression of chronic heart failure (HF) (Belch et al., 1991; Hill and Singal, 1996). Kinugawa et al. (2000) found that increased activity of myocardial matrix metalloproteinase (MMP) induced by ROS as one of the mechanisms of left ventricular (LV) remodeling and failure. Following myocardial infarction, oxidative stress mediated mitochondrial DNA damage and dysfunction has been observed (Ide et al., 2001). The degree of age-related changes in cardiomyocytes was found to be high (Coleman et al., 1987). The aging heart undergoes significant functional and structural alterations leading to atrophy...
and a compensatory hypertrophy, followed by myocardial fibrosis (Muscar i et al., 1996). Loss of mitochondrial function and an increase in oxidative stress have been proposed to be one of the key factors in myocardial aging (Hagen et al., 2001). In addition, there is an age-related decline in the capacity to withstand stress like ischemia or reperfusion (Lesn esky et al., 2001). In its most severe form, cardiac decay that may result in congestive HF, is observed during old age. Many experimental studies have demonstrated the importance of diet supplemented with antioxidants, such as vitamins, N-acetyl cysteine, and DL-α-lipoic acid to protect the mitochondria against respiration-linked oxidative stress, to preserve the genomic and structural integrity of these energy producing organelles, and to increase the functional life span (Chow, 1991; Lass et al., 1999; Huertas et al., 1999; Hagen et al., 1999). Antioxidants are found to be effective to prevent the ROS-induced MMP activation and the resulting LV dysfunction and failure in mice (Kinugawa et al., 2000).

POLY-MVA, a commercially available supplement, is formulated with palladium α-lipoic acid complex. In addition to the active ingredient, palladium α-lipoic acid complex, this proprietary liquid blend contains trace amount of molybdenum, rhodium, ruthenium, thiamine, riboflavin, cyanocobalamin, N-acetyl cysteine and N-formyl methionine (Table 1). This covalent palladium ingredient, palladium α-lipoic acid complex formulation is a safe nutritional supplement. Global ischemia experiments with palladium α-lipoic acid complex formulation demonstrated that it serves both as a highly active free radical scavenger and an alternative source of energy to the vulnerable hippocampus of the brain (Antonawich et al., 2004). Our previous experiment with palladium α-lipoic acid formulation found that it can enhance the activities of Krebs cycle dehydrogenases and respiratory complexes in the heart tissue homogenate was prepared in 50 mmol/L phosphate buffer (pH 7.0) containing 0.25 mol/L (w/v) sucrose. Homogenate was centrifuged initially at 3000g for 10 min and the supernatant was subjected to 11,000g for 10 min at 4°C in a cooling centrifuge (Remi C-24 BL; 8 × 25 mL). The mitochondrial pellets were washed twice with phosphate buffer to remove the sucrose and finally suspended in phosphate buffer.

The mitochondrial suspension was frozen and thawed 3–4 times to release the enzymes. The supernatant (contains approximately 3 mg/ml protein) was used for the determination of activities of manganese–superoxide dismutase (Mn SOD), catalase (CAT), and glutathione peroxidase (GPx) and level of reduced glutathione (GSH) and lipid peroxidation. All the estimations were carried out at room temperature using a double beam spectrophotometer (Systronics India Ltd., Hyderabad, India).

Mn SOD activity was determined from the ability of the mitochondrial sample to scavenge the superoxide anion generated from the photo-illumination of riboflavin according to the method of Mc Cord and Fridovich (1969). Mitochondrial CAT activity was determined from the rate of decomposition of H2O2 (Beers and Sizer, 1952). Activity of the CAT was calculated using the molar extinction coefficient of H2O2 (43.6 M−1 cm−1) and expressed in mmoles of H2O2 decomposed/min/mg protein (U/mg protein). Activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H2O2 and NaN3 (Hafe man et al., 1974). One unit of GPx activity was defined as decrease in log GSH by 0.001/min with respect to the non-enzymatic reaction and is expressed in U/mg protein. Reduced GSH was determined according to the method of Moron et al. (1979) based on the formation of a yellow colored complex with Ellman’s reagent. The concentration was calculated from the standard graph of GSH and is expressed in nmol/mg protein. The level of lipid peroxidation was measured as thiobarbituric acid reacting substance (TBARS) and is expressed as equivalents of malondialdehyde (MDA), using 1:13.3–tetramethoxypropane as standard (Ohkawa et al., 1979). Protein content in the tissue was determined using Folin’s phenol reagent (Lowry et al., 1951) and compared to bovine serum albumin (BSA) standard.

2.3.2. Effect of POLY-MVA on the antioxidant status of heart mitochondria

Animals were divided into four groups of six animals each. Group 1: Young control; Group 2: aged control administered with distilled water; Group 3: administered with POLY-MVA (0.05 ml/kg) (equivalent to 0.38 mg/kg complexed DL–α-lipoic acid); Group 4: administered with DL-α-lipoic acid (0.38 mg/kg, administered as 0.05 ml/kg of 7.68 mg/ml of DL–α-lipoic acid dissolved in 0.5% NaOH). The dis-tilled water (aged control), POLY-MVA and DL–α-lipoic acid were administered orally once daily for 30 days using oral gavage. Twenty-four hours after the administration, the animals were sacrificed by cervical decapitation. The heart was excised immediately and kept in 70°C for the determination of enzymatic antioxidant activities and non-enzymatic antioxidant level in the mitochondria.

2.3.3. Isolation of mitochondria and determination of antioxidant status

Mitochondria were isolated from the heart homogenate by differential centrifugation according to the method described in Ajith et al. (2009). Briefly, 10% of the heart tissue homogenate was prepared in 50 mmol/L phosphate buffer (pH 7.0) containing 0.25 mol/L (w/v) sucrose. Homogenate was centrifuged initially at 3000g for 10 min and the supernatant was subjected to 11,000g for 10 min at 4°C in a cooling centrifuge (Remi C-24 BL; 8 × 25 mL). The mitochondrial pellets were washed twice with phosphate buffer to remove the sucrose and finally suspended in phosphate buffer.

The mitochondrial suspension was frozen and thawed 3–4 times to release the enzymes. The supernatant (contains approximately 3 mg/ml protein) was used for the determination of activities of manganese–superoxide dismutase (Mn SOD), catalase (CAT), and glutathione peroxidase (GPx) and level of reduced glutathione (GSH) and lipid peroxidation. All the estimations were carried out at room temperature using a double beam spectrophotometer (Systronics India Ltd., Hyderabad, India).

Mn SOD activity was determined from the ability of the mitochondrial sample to scavenge the superoxide anion generated from the photo-illumination of riboflavin according to the method of Mc Cord and Fridovich (1969). Mitochondrial CAT activity was determined from the rate of decomposition of H2O2 (Beers and Sizer, 1952). Activity of the CAT was calculated using the molar extinction coefficient of H2O2 (43.6 M−1 cm−1) and expressed in mmoles of H2O2 decomposed/min/mg protein (U/mg protein). Activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H2O2 and NaN3 (Hafe man et al., 1974). One unit of GPx activity was defined as decrease in log GSH by 0.001/min with respect to the non-enzymatic reaction and is expressed in U/mg protein. Reduced GSH was determined according to the method of Moron et al. (1979) based on the formation of a yellow colored complex with Ellman’s reagent. The concentration was calculated from the standard graph of GSH and is expressed in nmol/mg protein. The level of lipid peroxidation was measured as thiobarbituric acid reacting substance (TBARS) and is expressed as equivalents of malondialdehyde (MDA), using 1:13.3–tetramethoxypropane as standard (Ohkawa et al., 1979). Protein content in the tissue was determined using Folin’s phenol reagent (Lowry et al., 1951) and compared to bovine serum albumin (BSA) standard.

2.4. Statistical analysis

All data were represented as mean ± SD. The mean values were statistically analyzed using one-way analysis of variance (ANOVA) (using the Graph Pad Instat software package). The significant differences between the groups were further analyzed by Bonferroni’s t-test. P-value less than 0.05 was considered as significant.

3. Results

The effects of the palladium α-lipoic acid formulation (POLY-MVA) and DL-α-lipoic acid on the antioxidant status in the heart mitochondria of aged rats are presented in Table 2 and Fig. 1. The activity of Mn SOD, CAT and GPx in the young control was significantly higher than that of the aged control group. There was approximately 1.75-fold increase in the activity of CAT in the young control than the aged control. Similarly, in the young control the level of GSH was approximately 1.01 higher and the level of MDA was approximately 1.76-fold lower than that of the aged
control. The study clearly showed that the administration of POLY-MVA significantly ($p < 0.001$) improved the antioxidant status (except activity of Mn SOD) in the heart mitochondria of aged rats. There was approximately 1.39 and 2.84-fold increase in the activities of CAT and GPx, respectively and approximately 1.04-fold increase in the level of GSH in the POLY-MVA treated group than that of the aged control group. But in the case of Mn-SOD, the POLY-MVA administrated group showed non-significant ($p > 0.05$) effect with respect to the aged control group. However, the mean value was higher than that of aged control. The level of MDA was significantly ($p < 0.001$) reduced in the POLY-MVA treated group than that of aged control group. Similarly, the level of GSH was significantly ($p < 0.05$) increased in the POLY-MVA treated group with respect to the aged control group. The DL-α-lipoic acid used in the study had significant ($p < 0.01$) effect on the antioxidant status in the aged rats. There was approximately 1.70 and 2.22-fold increase for CAT and GPx, respectively in the DL-α-lipoic acid treated group than that of aged control. The increase in the level of GSH in DL-α-lipoic acid treated group was non-significantly ($p > 0.05$) different from that of control group. Also, the level of MDA in the DL-α-lipoic acid treated group was non-significantly ($p > 0.05$) different from that control group.

4. Discussion

Results of the study reveal that palladium α-lipoic acid formulation significantly enhanced the activities of mitochondrial antioxidant enzyme as well as the level of GSH and reduced the MDA level when compared to that of aged control. The selection of the single dose of palladium α-lipoic acid formulation and DL-α-lipoic acid were based on our previous observations (Sudheesh et al., 2009). The active ingredient, palladium α-lipoic acid complex in the formulation is about 17 times higher than other minor components (Table 1). Previous studies in our laboratory with POLY-MVA devoid of palladium α-lipoic acid complex demonstrated that it could not elicit any significant effect on the activity of mitochondrial dehydrogenases or respiratory chain complexes in the heart of aged rat compared to the aged control or to the palladium α-lipoic acid formulation (unpublished data). Similarly, previous studies on ischemia have demonstrated that the effect of POLY-MVA devoid of palladium α-lipoic acid complex did not significantly differ from the saline treatment (Antonawich et al., 2004). Although the components such as N-acetyl cysteine, and thiamine are known antioxidants, their trace amounts in the formula can plausibly be ignored as a group treated with POLY-MVA devoid of palladium α-lipoic acid complex.

The rate of free radical production and scavenging capacity of the antioxidants are essentially constant or balanced under normal homeostasis. The antioxidant enzymes, Mn SOD and GPx are recognized as primary defense against superoxide anion (O$_2^-$) and H$_2$O$_2$ in eukaryotic cell mitochondria. However, presence of a heme-containing CAT in the rat heart mitochondrial matrix was demonstrated by Radi et al. (1991). The mitochondrial O$_2$ is dismutated to H$_2$O$_2$ by MnSOD, and the H$_2$O$_2$ is converted to H$_2$O by GPx-isoform 1 (GPx1). Our findings such as declined activity of Mn SOD, CAT, and GPx in the heart mitochondria also agree with the previous report of Savitha et al. (2005). Though the MnSOD activity was not altered in the POLY-MVA treated group, a significant elevation of the GPx activity could be observed, that could eventually protect the myocardium from oxidative stress.

GSH, a water-soluble tripeptide, is the most abundant non-protein thiol molecule in tissues with predominant defense against ROS. GSH reacts directly with ROS and electrophilic metabolites, protects essential thiol groups from oxidation. It can promote the regeneration of $\infty$-tocopherol, and serves as a substrate for GSH-related enzymes, such as GPx (Townsend et al., 2003). Treatment with POLY-MVA could significantly increase the mitochondrial GSH level with respect to that of the aged control group. Therefore, the enhanced activity of GPx can be ascribed with the high level of GSH in the myocardium.

The possible reason for the declined mitochondrial antioxidant enzyme activity can be explained with the excess generation of ROS during aging. Mn SOD and CAT are prone to age-associated oxidative damage due to $\cdot OH$ generated from Fenton’s reaction (Jouihan et al., 2008). ROS-induced oxidative modification of many enzyme proteins results in structural alteration and their functional inactivation (Sitte et al., 2000). The decreased GPx activity can be attributed to the decreased level of GSH. 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).

Approximately 1–5% of the total oxygen consumption gives rise to potentially cytotoxic ROS such as O$_2^-$ and H$_2$O$_2$. The cellular sources of ROS within the heart include cardiac myocytes, endothelial cells, and neutrophils. Within cardiac myocytes, ROS can also be produced by NADPH oxidase of plasma membrane,

### Table 2

Effect of POLY-MVA treatment on the activities of Mn SOD, CAT and GPx in the heart mitochondria of aged rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mn SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>GPx (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged control</td>
<td>12.23 ± 2.33</td>
<td>4.05 ± 0.82</td>
<td>22.70 ± 4.24</td>
</tr>
<tr>
<td>Young control</td>
<td>16.34 ± 1.17</td>
<td>9.61 ± 1.17</td>
<td>73.24 ± 20.65</td>
</tr>
<tr>
<td>POLY-MVA (0.05 ml/kg body weight)</td>
<td>15.59 ± 5.31</td>
<td>8.26 ± 1.48</td>
<td>168.58 ± 63.74</td>
</tr>
<tr>
<td>DL-α-lipoic acid (0.38 mg/kg)</td>
<td>12.72 ± 5.94</td>
<td>4.81 ± 1.34</td>
<td>64.19 ± 15.50</td>
</tr>
</tbody>
</table>

Values are mean ± SD; $n = 6$.

$p < 0.001$ significantly different from young control (Bonferroni test).

$p > 0.05$ non-significantly different from young control (Bonferroni test).

$p < 0.001$ significantly different from aged control (Bonferroni test).

$p > 0.05$ non-significantly different from aged control (Bonferroni test).

$p < 0.001$ significantly different from POLY-MVA (0.05 ml/kg body weight).

*p 0.05 ml/kg body weight (which is equivalent to 0.38 mg complexed α-lipoic acid/kg).
mitochondrial inner membrane electron transport chain and cytosolic xanthine oxidase (Tsuttsui, 2001). Further, one electron reduction of oxygen producing ROS has been reported for at least nine mammalian mitochondrial enzymes. The locations as well as activities of these enzymes and/or complexes, mitochondrial cytochrome b5 reductase, monoamine oxidases, dihydroorotate dehydrogenase, mitochondrial dehydrogenase of α-glycerophosphate, succinate dehydrogenase complex, mitochondrial aconitase, α-ketoglutarate dehydrogenase complex, complex I and complex III are known (Starkov and Wallace, 2006). The excessive generation of free radicals led to peroxidative changes that ultimately result in enhanced lipid peroxidation (Rikans and Hornbrook, 1997). Significant decrease in the level of lipid peroxidation was observed in the POLY-MVA treated group. This could possibly explain the enhanced GSH level as well as GPx activity. Nevertheless, the significance of CAT in the myocardial mitochondrion, the elevated CAT activity resulting from the treatment of POLY-MVA can further explain the protection of lipid peroxidation. Since the palladium α-lipoic acid complex serves as a potent redox molecule, it may facilitate a chain breaking antioxidant effect on the lipid peroxidation process.

The heart is particularly vulnerable to damage induced by free radicals because protective enzymes such as Mn SOD and CAT are present at lower levels than other tissues of the body. Therefore, mitochondria of cardiac myocytes become less efficient with increasing age, leading to greater damage to DNA and proteins. Dietary supplementation of agents that protect the genomic and structural integrity of mitochondria against oxidative decline of energy may ultimately protect the body from age-associated heart diseases. Since palladium α-lipoic acid formulation is non-toxic to normal cells, the cells can actually benefit from the energy boost. The toxicological studies of POLY-MVA indicated that the LD50 of palladium α-lipoic acid formulation exceeded 5000 mg/kg. Unlike its relative platinum, no evidence of any mutagenic property was demonstrated for palladium. No mutagenic effect of the combination was observed in the Ames’ test (Bunger et al., 1996). The unique electronic and redox properties of palladium α-lipoic acid complex appear to be the key to its physiological effectiveness (Garnett, 1995, 1998; Krishnan and Garnett, 2006). Oxygen Radical Absorbance Capacity (ORAC) analysis of palladium α-lipoic acid formulation demonstrates that it is approximately five times more potent antioxidant than DL-α-lipoic acid (Antonawich and Valane, 2007). Results of the present study also demonstrate the better activity of palladium α-lipoic acid with respect to that of the DL-α-lipoic acid. Since the POLY-MVA contains minor constituents, absolutely reliable comparison with the positive control, DL-α-lipoic is not feasible. Results of this study reveal that palladium α-lipoic acid formulation is an effective agent to protect the age-linked decline of myocardial mitochondrial antioxidant status and thus is capable to enhance the energy production of normal cell mitochondria.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References


