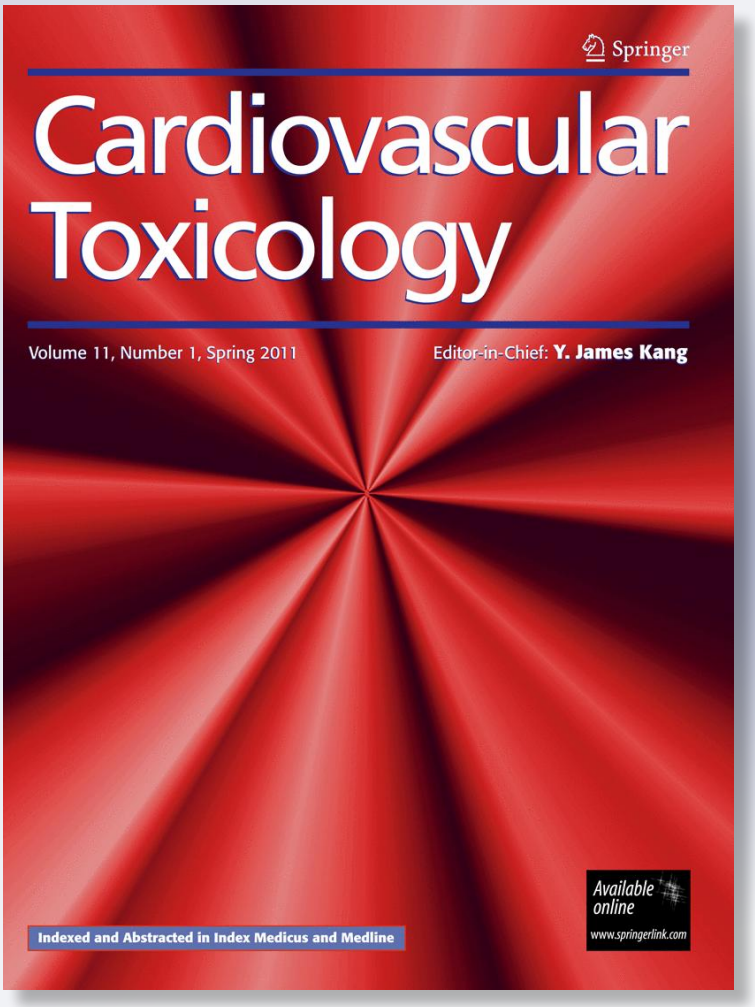
A Combination of Melatonin and Alpha Lipoic Acid has Greater Cardioprotective Effect than Either of them Singly Against Cadmium-Induced Oxidative Damage

Cardiovascular Toxicology

ISSN 1530-7905
Volume 11
Number 1

Cardiovasc Toxicol (2010)
11:78-88
DOI 10.1007/
s12012-010-9092-9



Springer

Cardiovasc Toxicol (2011) 11:78–88
DOI 10.1007/s12012-010-9092-9

A Combination of Melatonin and Alpha Lipoic Acid has Greater Cardioprotective Effect than Either of them Singly Against Cadmium-Induced Oxidative Damage

Raktim Mukherjee · Sudeep Banerjee · Niraj Joshi · Prem Kumar Singh · Darshee Baxi · A. V. Ramachandran

Published online: 3 November 2010

Abstract Present study evaluates cardioprotective role of melatonin (Mel), alpha lipoic acid (ALA), a combination of melatonin and alpha lipoic acid (Mel + ALA) against cadmium (Cd)-induced oxidative damage. Female albino rats were subjected to 15-day exposure to Cd (5.12 mg/kg bw) alone or treated with ML (10 mg/kg bw) + ALA (25 mg/kg bw) simultaneously. Plasma markers of cardiac damage, cardiac free radical generation, lipid peroxidation, endogenous antioxidant status, cadmium load, metallothionein induction, and histopathology were evaluated in various experimental groups. Combination of Mel + ALA significantly prevented leakage of marker enzymes of cardiac damage, changes in cardiac free radical generation, endogenous antioxidants, antioxidant status, structural alterations and augmented the degree of metallothionein (MT) induction. The results demonstrate that ML + ALA co-administration effectively protected against Cd-induced cardiac oxidative damage.

Keywords Cadmium · Metallothionein · Melatonin · Alpha lipoic acid · Cardiotoxicity

R. Mukherjee · S. Banerjee · N. Joshi · P. K. Singh · D. Baxi · A. V. Ramachandran (✉)
Division of Cardiotoxicity, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390 002, Gujarat, India
e-mail: avrcn2008@yahoo.co.in

Present Address:
R. Mukherjee
Shree PM Patel College of PG Studies and Research in Science, Affiliated to SP University, Anand, Gujarat, India

Introduction

Cardiovascular diseases are the most common cause of death in developed as well as developing countries [1]. Cadmium exposure has been considered to be the risk factor for various cardiovascular diseases [2] such as atherosclerosis [3], cardiomyopathy [4], and hypertension [5]. Cadmium-induced oxidative stress is the major factor responsible for cardiac tissue damage and related complications [6, 7]. According to Millis et al. [8], the consumption of vegetables constitutes the main source of Cd in humans. It has been demonstrated that populations who consume essentially locally grown vegetable produce from contaminated areas are at greater risk of dietary Cd exposure as Cd in their diet is not diluted by food from other non-contaminated areas [9]. This scenario is very common in the majority of developed countries, including India. Earlier work from our laboratory has demonstrated that cereals and vegetables grown on either side along the Baroda effluent channel contain cadmium seven times in excess of the World Health Organization (WHO)-recommended permissible limit [10].

In this context, it is worthwhile to focus on compounds having antioxidant properties to combat the cadmium-induced damage caused by the disruption in pro-oxidant to antioxidant balance in cells. Melatonin, an endogenous molecule from the pineal gland is a versatile antioxidant possessing metal-chelating properties and has been used in various forms of cadmium toxicity, ischemia reperfusion injury, and various other abnormalities in the cardiovascular system [11]. Further, melatonin has been shown to reduce oxidative stress-induced post-ischemic myocardial recovery, protect against ischemia reperfusion injury, reduce cardiac arrhythmia, and protect against various other forms of cardiovascular abnormalities [12–16]. In

addition to being an effective free radical scavenger, melatonin also enhances the activity of the membrane calcium pump [17] that regulates calmodulin [18] and decreases the intracellular calcium concentration [19]. Moreover, melatonin has been reported in several dietary plants and as a phytochemical in olive oil and, recently, it has been suggested that melatonin present in edible plants may improve human health by virtue of its biological activities and good bioavailability [20]. Yet another antioxidant, alpha lipoic acid (ALA) on the other hand is water- and fat-soluble sulfur-containing compound with already proven role in cardiotoxicity and as a metal chelator [21, 22]. ALA occurs naturally in the human diet and is found in abundance in animal tissues with high metabolic activity such as heart, liver and kidney, and to a lesser extent in fruits and vegetables [23]. Role of ALA has also been tried in various cardiovascular abnormalities [24].

Till date, there are no reports on the effect of a combination of melatonin + ALA on either models of cardiac oxidative stress or cadmium-induced cardiac damage. Hence, we were tempted to evaluate the beneficial effects of a Melatonin + ALA combination against Cd-induced cardiotoxicity in rats.

Materials and Methods

Chemicals

All chemicals used in the study were of highest purity and of analytical grade. The dosage of cadmium selected in the present study is an environmentally relevant realistic dose based on the actual concentration of cadmium found in the cereals and vegetables grown across the Baroda Effluent channel as reported in our earlier publication [10]. The actual cadmium content administered to animals was calculated on the basis of average feed consumption in rats empirically based on field values of routinely consumed cereals and vegetables grown along the Baroda effluent channel.

Experimental Animals

Healthy adult female albino rats of *Charles foster* strain weighing 100–150 gm (60 day old) were housed in polypropylene cages and maintained under conditions of controlled temperature (25 ± 2°C) with constant 12 h/12 h dark light cycle in the animal house of department of Zoology, The Maharaja Sayajirao University of Baroda. Animals were provided with standard rat pellet and water ad libitum. The metal content of feed and water was monitored on a regular basis. Animal experiments were conducted according to the guidelines of CPCSEA from the ministry of Social Justice and Empowerment, Government of India vide CPCSEA (827/ac/04/CPCSEA).

Cadmium-Induced Cardiotoxicity in Rats

A total of 48 rats were divided into 8 groups of 6 animals each as follows:

Group I (Control): rats treated with 0.9% sodium chloride.

Group II (Melatonin): rats treated with melatonin (10 mg/kg body weight, *p.o.*) daily at 19 h for 15 days.

Group III (ALA): rats treated with alpha lipoic acid (25 mg/body weight, *p.o.*) daily at 19 h for 15 days.

Group IV (Melatonin + ALA): rats treated with melatonin (10 mg/kg body weight, *p.o.*) and ALA (25 mg/kg body weight, *p.o.*) daily at 19 h for 15 days.

Group V (Cd): rats treated with cadmium chloride ($CdCl_2$; 5.2 mg/kg body weight, *p.o.*) daily at 19 h for 15 days.

Group VI (Cd + Mel): rats treated with cadmium chloride ($CdCl_2$; 5.2 mg/kg body weight, *p.o.*) and melatonin (10 mg/kg body weight, *p.o.*) daily at 19 h for 15 days.

Group VII (Cd + ALA): rats treated with cadmium chloride ($CdCl_2$; 5.2 mg/kg body weight, *p.o.*) and ALA (25 mg/kg body weight, *p.o.*) daily at 19 h for 15 days.

Group VIII (Cd + Mel + ALA): rats treated with cadmium chloride ($CdCl_2$; 5.2 mg/kg body weight, *p.o.*), melatonin (10 mg/kg body weight, *p.o.*), and ALA (25 mg/kg body weight, *p.o.*) daily at 19 h for 15 days.

At the end of experimental period, animals were fasted over night (12 h), and blood samples were collected from retro-orbital sinus under mild ether anesthesia. Animals were subjected to cervical dislocation under mild ether anesthesia (as per the CPCSEA guidelines), and hearts of control and experimental rats were excised, auricles and ventricles separated and immersed in ice-cold physiological saline. A 10% homogenate was prepared in ice-chilled phosphate buffer saline (pH 7.4). Aliquot of homogenate was used immediately for the estimation of thiobarbituric acid reactive substance (TBARS), hydroxyl radical, H_2O_2, and non-enzymatic antioxidants. The remaining homogenate was then centrifuged at 5,000 rpm for 20 min at 4°C, and the supernatant was used for the estimation of all enzymatic antioxidants and marker enzymes of cardiac damage.

Cardiac Lipid Peroxidation (LPO) and Reactive Oxygen Species (ROS)

Cardiac LPO was estimated according to the procedure of Beuge and Aust [25]. Malonaldehyde produced during peroxidation of lipids served as an index of LPO. Hydrogen

peroxide production was assessed by the spectrophotometry as per Holland and Storey [26] and expressed as mol/min/mg protein. Hydroxyl radical production was quantified by the method of Puntarulo and Cederbaum [27] and expressed as mol/min/mg protein.

Assay of Non-Enzymatic and Enzymatic Antioxidants

Cardiac total reduced glutathione (GSH) [28], vitamin C [29], vitamin E [30], superoxide dismutase (SOD) [31], catalase (CAT) [32], glutathione peroxidase (GPx) [33] and glutathione S-transferase (GST) [34] were estimated in tissue homogenate.

Enzymic Markers of Myocardial Damage

Serum and cardiac creatine kinase—MB isoform (CK-MB), creatine kinase (CK), and lactate dehydrogenase (LDH) were determined by diagnostic kits (Reckon Diagnostics Ltd., Baroda, India) as per manufacturer's instructions. Cardiac Troponin I (Tn-I) was assayed by ELISA, and absorbance was measured by a spectrophotometric method using microplate reader at 450 nm.

Cardiac Cadmium and Metallothionein Content

Samples of known weight (whole heart) were subjected to dry mineralization in an electric oven according to Zmudzki [35]. The ash was dissolved in a known volume of 1 N HNO_3. The concentration of cadmium (after appropriate dilution) was assessed by atomic absorption spectrophotometry (Thermo S series) with electrothermal atomization in a graphite cuvette. The cathode lamp was operated under standard conditions using appropriate resonance line (228.8 nm). The concentrations of Cd were expressed as μg/g of wet tissue.

Cardiac metallothionein (MT) was determined using Cd–heme method [36] as described by Chwelatiuk et al. [37].

Microscopic Evaluation of Cardiac Tissue

Myocardial tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (3 μm) were cut on glass slides and stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Estimation of Cardiac Protein Content

Protein content of homogenate was estimated by the method of Lowry et al. [38] using bovine serum albumin as a standard.

Statistical Analysis

One-way ANOVA with Bonferonni post-test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

The alterations in the level of lipid peroxidation and other free radicals are presented in Fig. 1. The administration of cadmium chloride caused significant increase in malondialdehyde ($P < 0.001$), hydroxyl radical ($P < 0.001$), and hydrogen peroxide ($P < 0.001$) compared to control group. The administration of melatonin and/or ALA together with Cd was able to reduce or prevent the increase of such radicals. The combination of Cd + Mel + ALA was more effective than either of these protectants with reference to hydroxyl radical.

The data on myocardial non-enzymatic antioxidants such as GSH, vitamin C, and vitamin E are presented in Fig. 2. The concentrations of GSH, vitamins C and E were significantly decreased ($P < 0.05$) in the heart of cadmium-exposed rats compared to controls. Simultaneous administration of cadmium along with Mel and/or ALA resulted in the restoration of GSH to control levels, whereas vitamin C and vitamin E appeared to increase as found compared to those found with treatment with cadmium alone. Thus, a combination of Cd + Mel + ALA was the most effective in offsetting the detrimental changes in glutathione content.

As shown in Fig. 3, there was a significant ($P < 0.001$) reduction in the activity levels of SOD (68%), CAT (43%), GPx (54%), and GST (34%) in the cadmium-treated group compared to control group. For all the antioxidant enzymes, Mel was able to restore the activity of GPx and CAT, whereas ALA significantly restored the level of antioxidant enzymes SOD and CAT, but the combination of Cd + Mel + ALA showed the greater degree of protection in restoring SOD and CAT to near control levels.

As shown in Fig. 4, cardiac cadmium load and metallothionein (MT) concentration were both significantly increased in Cd-treated group compared to control group. All protectant schedules recorded decrement in the cardiac cadmium load, and metallothionein induction was maximum in Cd + Mel + ALA group compared to control group.

A significant ($P < 0.001$) elevation in the serum levels of marker enzymes of cardiac damage (CPK, LDH, cTnI, and CK-MB) with corresponding decrease in tissue levels was the feature in cadmium-administered animals (Fig. 5). The combination of Cd + Mel + ALA maintained the

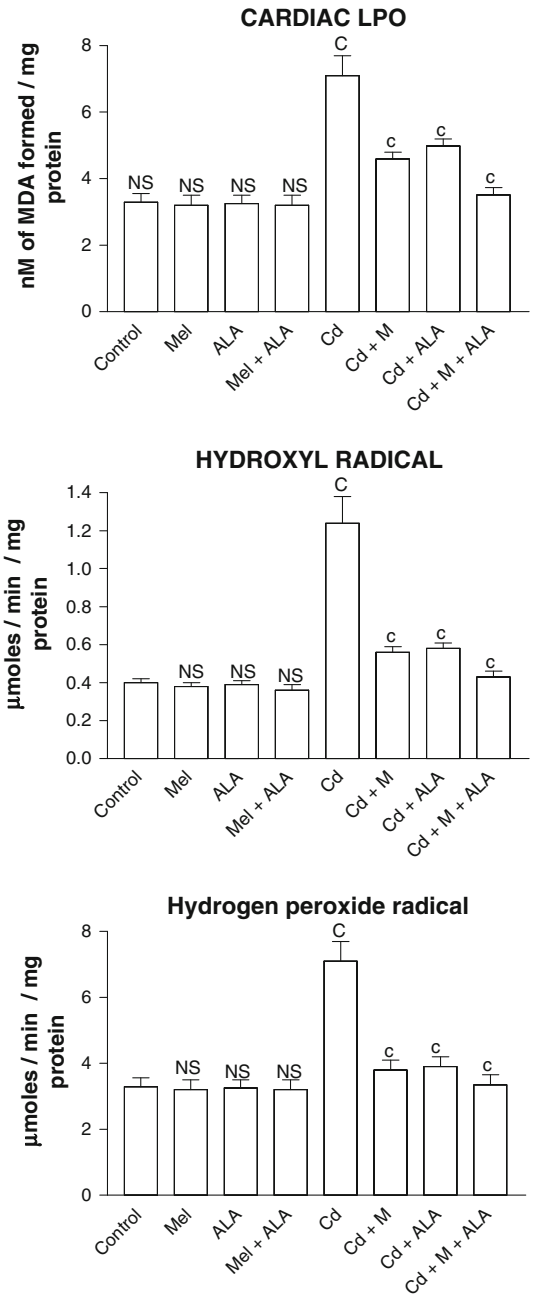


Fig. 1 Effect of cadmium exposure and simultaneous administration of melatonin + ALA treatment on cardiac lipid peroxidation, hydrogen peroxide, and hydroxyl radical. Values are expressed as mean ± SEM for $n = 6$, where, C ($P < 0.001$) and NS non-significant when control versus Mel, ALA, Mel + ALA, Cd and, c ($P < 0.001$) when Cd versus Cd + M, Cd + ALA, Cd + M + ALA

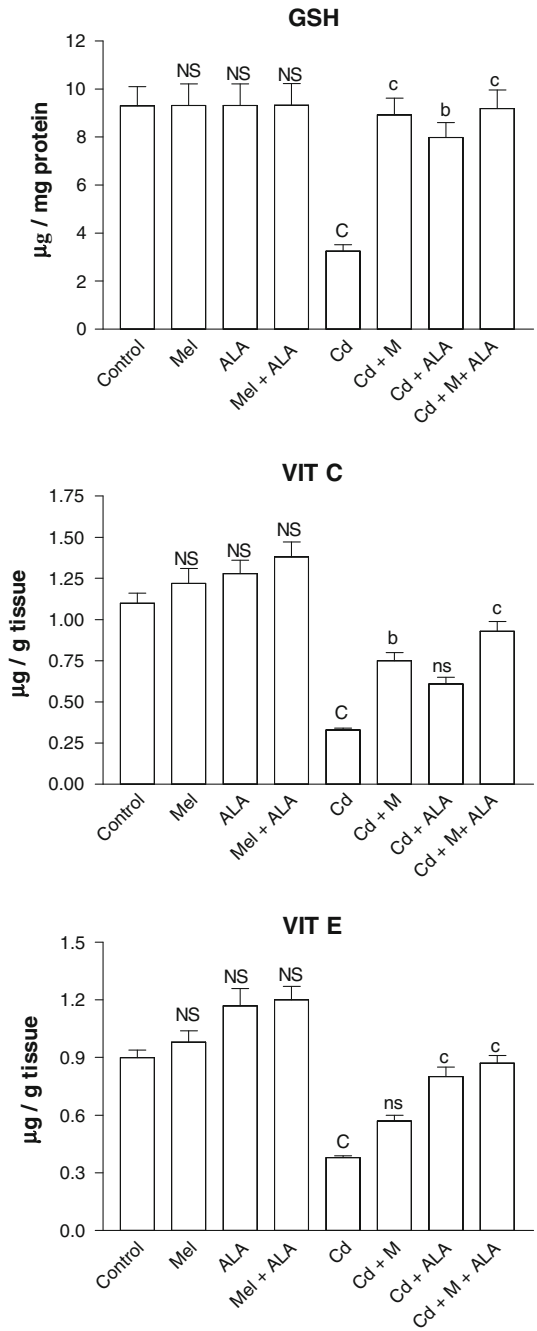


Fig. 2 Effect of cadmium and co-treatment with melatonin + ALA combination on cardiac non-enzymatic antioxidants of control and experimental groups of rat. Values are expressed as mean ± SEM for $n = 6$, where, C ($P < 0.001$) and NS non-significant when control versus Mel, ALA, Mel + ALA, Cd and, b ($P < 0.01$), c ($P < 0.001$) and *ns* non-significant when Cd versus Cd + M, Cd + ALA, Cd + M + ALA

levels of these marker enzymes close to normal levels in the cadmium-exposed group.

Figure 6 illustrates microscopic evaluation of different cardiac tissues of experimental animals. Cadmium intoxication resulted in severe damage and/or disruption of the cardiac muscle. Degree of necrotic damage is in the following order Cd > Cd + ALA > Cd + Mel > Cd + Mel + ALA. Reduction of cardiac damage in animals co-administered with either, melatonin, ALA, or both along with cadmium indicates their protective role against cadmium-induced cardiac damage.

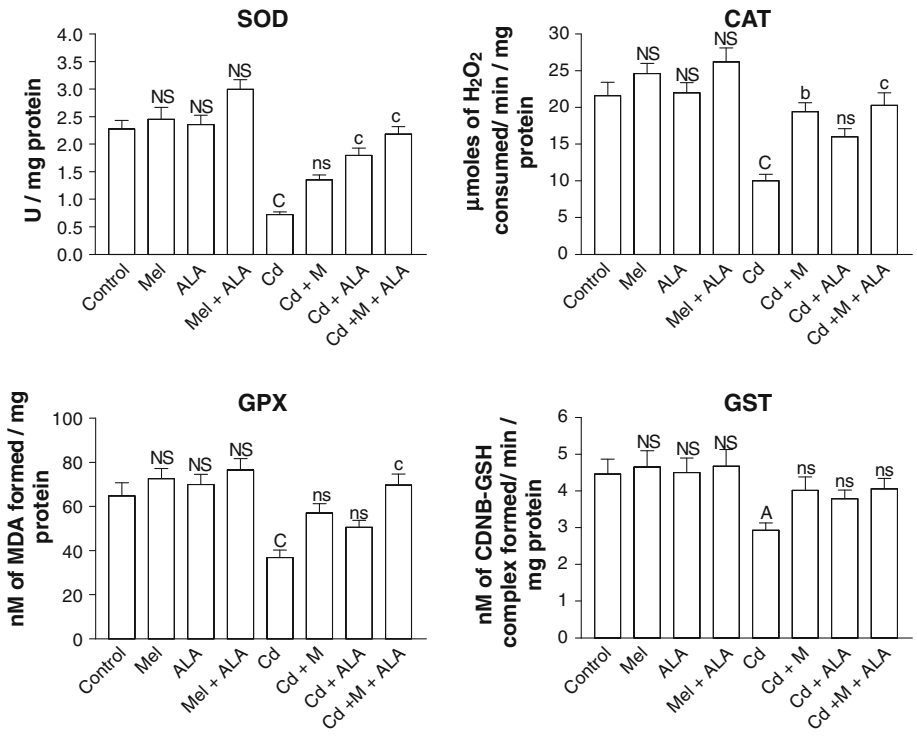


Fig. 3 Effect of simultaneous administration of melatonin + ALA combination on the status of enzymatic antioxidants in the heart of cadmium-treated animal. Values are expressed as mean ± SEM for $n = 6$, where, C ($P < 0.001$) and *NS* non-significant when control versus Mel, ALA, Mel + ALA, Cd and, a ($P < 0.05$), b ($P < 0.01$), C ($P < 0.001$) and *ns* non-significant when Cd versus Cd + M, Cd + ALA, Cd + M + ALA

Discussion

Administration of cadmium at realistic sublethal dosages corresponding to the actual concentration in the cereals and vegetables grown on either side of the Baroda effluent channel demonstrates compromised antioxidant defense, an increased accumulation of cardiac cadmium and cardiac damage manifested by increased levels of serum CK, LDH, and cardiac Troponin I. There is markedly enhanced protection by co-administration of melatonin and alpha lipoic acid (ALA) against these cadmium-induced alterations.

The results of our study indicate cardiac oxidative stress as evidenced by the increased levels of hydroxyl radicals and hydrogen peroxide. Similar rise in hydroxyl radical production has been reported in the testes of cadmium-treated mice [39] and in the liver of freshwater goldfish (*Carassius auratus*) when exposed to cadmium at a concentration of 5 mg/L [40]. Hydroxyl radicals are known to carry out oxidative modification of the mitochondrial and myofibrillar protein via oxidation of amino acid residues and have been implicated in the pathogenesis of myocardial injury [41]. This escalation of hydroxyl radical observed in the current study owes its origin to its precursor entity i.e. hydrogen peroxide (H_2O_2). It is said that hydrogen peroxide per se is not a free radical, but if allowed to remain in the cells for protracted period, it can lead to the formation of deadly hydroxyl radical. The data recorded in the present study adequately justify the contention of free radical-induced tissue oxidative damage as both hydroxyl radical and hydrogen peroxide are significantly elevated in the heart of cadmium-treated animals.

Level of MDA, a major oxidation product of peroxidized poly unsaturated fatty acids, has been considered to be an important indicator of lipid peroxidation [42]. In the current study, high levels of cardiac MDA in cadmium-exposed rats indicate heightened lipid peroxidation and prevalent oxidative stress as has been reported for cadmium and other heavy metal intoxication [43, 44].

Significant decline in non-enzymic antioxidants like glutathione, vitamin C, and vitamin E has been noted in the cadmium-exposed group. The sulphydryl reactive metals like cadmium have known to have high affinity for glutathione (GSH), which is the primary intracellular antioxidant and conjugating agent. A single atom of cadmium can bind to and cause the irreversible excretion of up to two GSH tripeptides [45]. Although this glutathione conjugation process helps in excretion of heavy metals, it nevertheless depletes the cell of its available pool of GSH. Cadmium-induced depletion in GSH recorded herein could be viewed in the context of free radical generation by cadmium through fenton reaction leading to peroxidative damage. This is apparently further aggravated due to the decrease in GSH content. GSH also functions in synchrony with other non-enzymatic antioxidants such as vitamins C and E. The sparing effect of GSH and vitamin C on each other is essentially related with the potential of regenerating each other [46]. The observed decrease in vitamin C can therefore be related with the depletion in GSH.

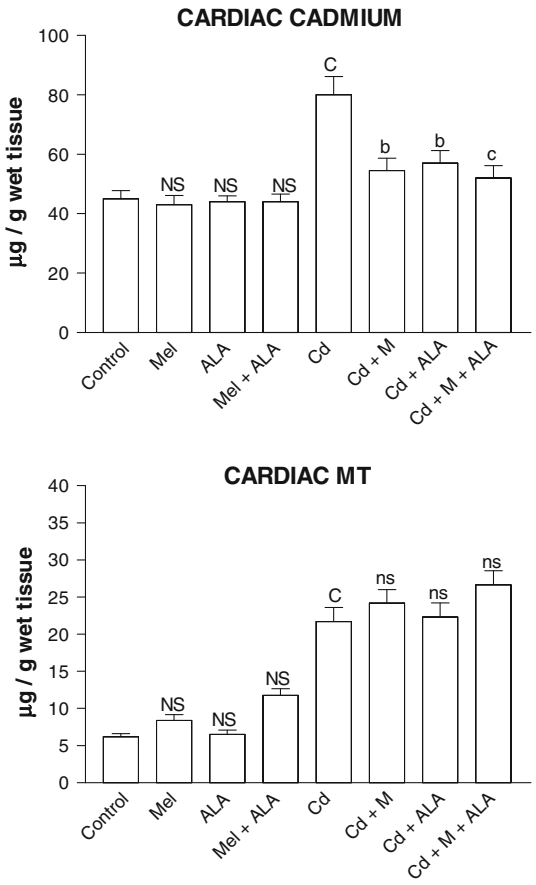


Fig. 4 Concentration of cadmium and induction of metallothionein in the heart of cadmium-treated animals. Values are expressed as mean ± SEM for $n = 6$, where, C ($P < 0.001$) and *NS* non-significant when control versus Mel, ALA, Mel + ALA, Cd and, b ($P < 0.01$), C ($P < 0.001$) and *ns* non-significant when Cd versus Cd + M, Cd + ALA, Cd + M + ALA

Vitamin C is a low molecular weight antioxidant that defends the cellular compartment against water-soluble oxygen and nitrogen radicals and hence serves as an effective antioxidant of the hydrophilic phase. Likewise, vitamin E is the major lipid-soluble antioxidant present in all cellular membranes affording protection against lipid peroxidation by reacting with lipid peroxyl radicals and conversion to a non-reactive tocophyryl radical [47, 48]. Our results point toward damage to both aqueous and lipid compartments of the myocardium as evidenced by the decline in the content of non-enzymatic antioxidants.

Cadmium-exposed rats show significant decrease in myocardial antioxidant enzymes as it has also been reported by Manna et al. [49]. Corroborating our results on the decrease in cardiac SOD and CAT are the reports on Cd-induced decrease in antioxidant enzyme in other organs [50, 51]. SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide, which is finally removed by CAT. Decrease in both SOD and CAT observed in our present study may therefore be related with their inactivation in their encounter with superoxide and hydrogen peroxide free radicals.

GPx is responsible for detoxification of increased lipid peroxides and hydrogen peroxides using GSH as reducing equivalent. Concomitant decrease in GPX and GSH in the context of increased hydroxyl radical and H_2O_2, is suggestive of this enzyme being consumed in detoxification of toxic hydroperoxides. GST, the enzyme responsible for the conjugation of GSH to foreign compounds, is also significantly reduced and can be accredited to the ability of cadmium to bind to the non-histone protein part of the enzyme [52].

The observed induction of metallothionein in parallel with cadmium accumulation seems to be an adaptive mechanism to sequester the metal in bound form. Tissue oxidative damage induced induction of metallothionein suggests non-MT-bound cadmium to be the damaging culprit [37].

Cadmium exposure is significantly known to increase the activities of serum CPK, LDH, and cardiac troponin I. Cardiac troponin T (cTnT) and troponin I (cTnI) have been shown to be highly sensitive and specific markers in the determination of myocardial cell injury [53, 54]. The high cTnT and cTnI values provide biochemical evidence for myocardial cell injury. O'Brien et al. [55] have shown cTnT is a powerful biomarker in laboratory animals for sensitive and specific detection of cardiac injury arising from various causes. Further, Vorderwinkler et al. [56] have found an increase in cTnI in parallel with cTnT in effluents from isolated perfused rat hearts after hypoxia-reoxygenation-induced myocardial infarction. Thus, the currently observed increase in the levels of serum marker enzymes of cardiac damage like CK and LDH indicates myocardial injury. These findings corroborate well with an earlier report on cadmium toxicity at sublethal dosage [57].

Simultaneous administration of a combination of Cd + melatonin + ALA offsets the cadmium-induced changes in antioxidant defense, and overall, the combination of protectants has better therapeutic efficacy in preventing Cd-induced compromised antioxidant status. A highlighting feature of our study is the ability of the combination to completely quench hydroxyl and superoxide radicals. A possible mechanism of action of this dual protectant regimen can be attributed to an additive action as both melatonin and ALA have been reported to scavenge hydroxyl radial [58]. With regard to the ability of this combination in scavenging superoxide free radicals, a reported complementary property provides adequate support. Melatonin is reported to be minimally reactive to superoxide anion [17], while ALA has much superior activity against this free radical. Thus, in combination, these protectants seem to complement each other leading to complete quenching of free radicals [59].

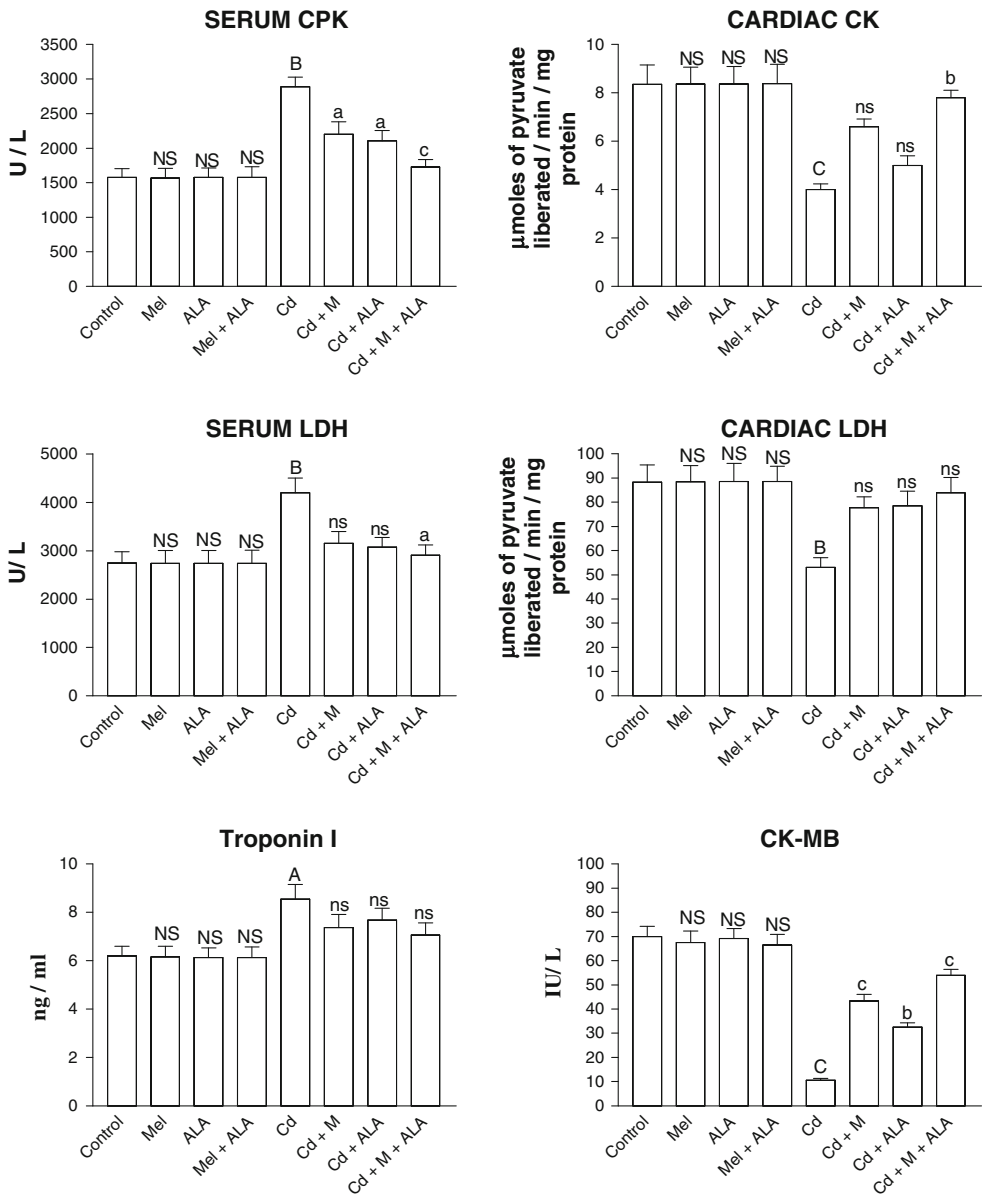


Fig. 5 Effect of cadmium treatment and simultaneous administration of melatonin + ALA on marker enzymes of cardiac damage in control and experimental animals. Values are expressed as mean ± SEM for $n = 6$, where, B ($P < 0.01$), C ($P < 0.001$) and *NS* non-significant when control versus Mel, ALA, Mel + ALA, Cd and, a ($P < 0.05$), b ($P < 0.01$), C ($P < 0.001$) and *ns* non-significant when Cd versus Cd + M, Cd + ALA, Cd + M + ALA

Apart from hydroxyl free radical and superoxide free radical, melatonin and ALA are also known to scavenge singlet oxygen, hydrogen peroxide, peroxyl radical, hypochlorous acid, peroxynitrite, and nitric oxide [60, 61].

The observed prevention of lipid peroxidation and corresponding conserved glutathione status under the influence of Mel + ALA combination seems more tilted in favor of melatonin. The decrease in lipid peroxidation is indicative of melatonin's role as a chain-breaking antioxidant. However, available reports suggest that this action is by inhibiting free radicals [62]. Hence, the possibility of a non-genomic mode of action of this combination cannot be ruled out. Our assumption stems from the fact that both melatonin and ALA have potential to get dissolved in both lipid and aqueous media and translocate to sites where –SH compounds are actually required [63, 64]. Additionally, lipoic acid has been reported to afford protection to cell membranes by possible interaction with the antioxidants glutathione and ascorbate via the vitamin E cycle [23]. Our biochemical data of marker enzymes of myocardial damage correlate well with the histological findings, and the combination of Mel + ALA seems potent to prevent the

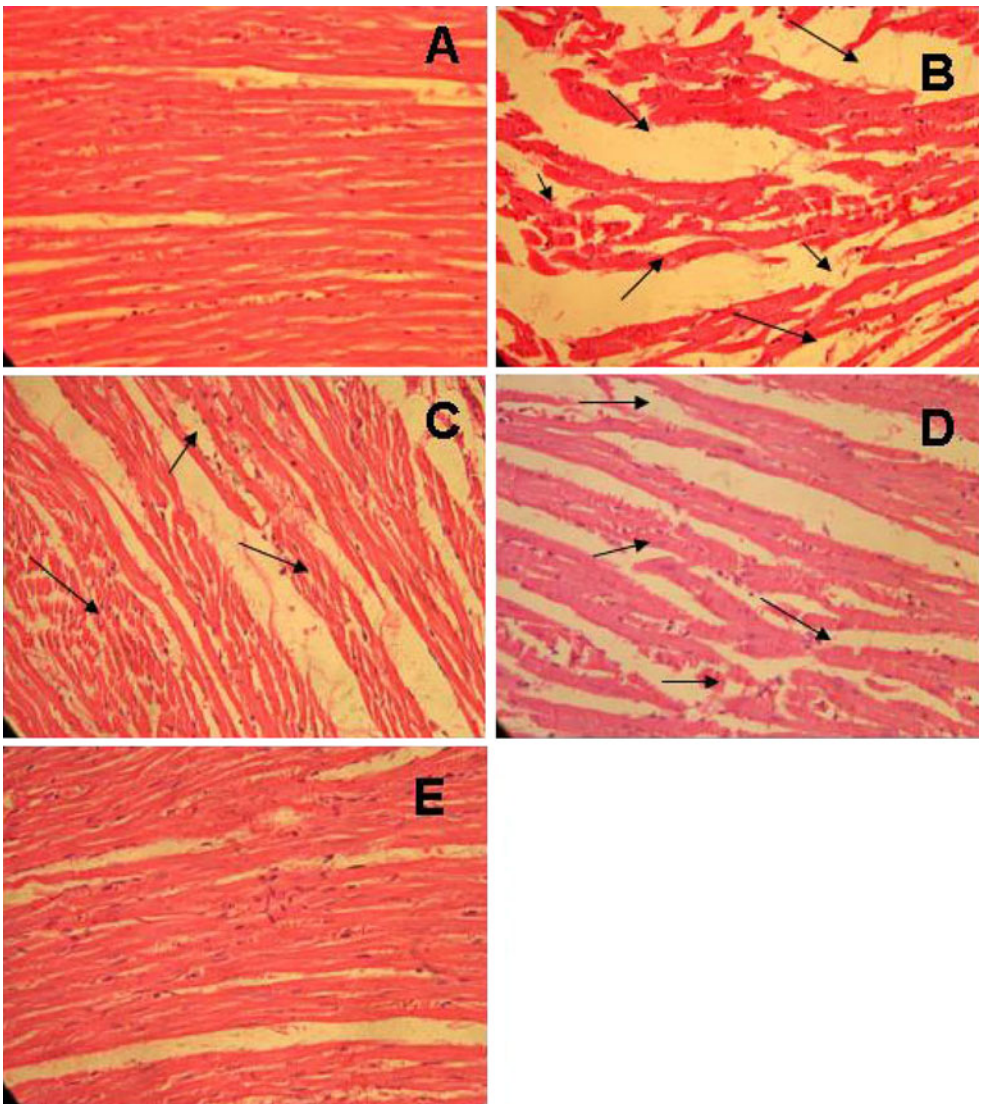


Fig. 6 Hematoxylin and eosin–stained cardiac sections of (**a**) control rat (100×) showing normal histoarchitecture of cardiac muscle, **b** cadmium-treated rat (100×) showing extensive degeneration in cardiac muscle (*marked with arrows*), **c** cardiac section simultaneously administered with Cd + melatonin (100×) showing occasional loss of muscle fiber, **d** cardiac section simultaneously administered with Cd + ALA (100×) showing occasional loss of muscle fiber > Cd + Mel group and **e** cardiac section simultaneously administered with Cd + ALA + melatonin (100×) showing normal structure of myocyte

otherwise observed cardiac myofibrillar damage due to necrosis.

Melatonin is shown to be more potent in preventing tissue accumulation of cadmium, and the combination of Mel + ALA maintains cadmium levels close to control values. The mechanistic basis behind this reduction in metal load can be co-related with the corresponding induction of MT. The dramatic induction of MT observed in our study can sequester Cd and render it toxicologically inert [65], but interaction of MT with Cd can also lead to generation of hydroxyl radicals [66]. Interestingly, administration of Mel + ALA combination further augmented the degree of metallothionein induction. Although this increment was non-significant, it is suggestive of metal sequestering property since 1 mol of MT (6,600) binds 7 mol of Cd [37]. In vivo studies on adult mice treated with melatonin 10 mg/kg body weight have shown similar increase in Cd-induced MT gene expression [67]. Melatonin besides being a Cd-binding protein is also a cardiac antioxidant [68, 69]. Hence, induction of metallothionein can essentially be seen as an adaptive mechanism to boost the antioxidant defense of the cardiac tissue. Additionally, lipoic acid and melatonin have been demonstrated to form stable complex with manganese, copper, and zinc besides chelating cadmium and iron [70]. Individually, the ability of both melatonin and ALA as potential as metal-chelating

agent against metal toxicity has been evaluated in experimental models [37]. Thus, the decrease in metal load can be possibly attributed to induction of metallothionein and the ability of this combination to form complexes.

The present formulation also has a significant sparing effect on non-enzymatic antioxidants, which suggests enhanced metal-chelating activity. Sulphydryl reactive metals are detoxified by glutathione transferase leading to depletion of glutathione, a major antioxidant in the cell. Both melatonin and ALA can maintain intracellular pool of glutathione necessary for maintaining balanced redox state of the cell as well as an additional reserve for carrying out detoxification reactions. The present results demonstrate for the first time the cardioprotective effect of Mel + ALA combination against cadmium toxicity in an additive manner and that the superior protection exerted by the combination is essentially by way of minimizing free radical formation and by increasing metal-chelating capacity. Apparently, the endogenous antioxidant defense of the body is insufficient to counteract the deleterious effects of metal-induced free radical damage, and hence exogenous antioxidant supplementation can afford therapeutic benefit against metal-induced tissue oxidative damage.

Conclusion

To the best of our knowledge, ours is the first in vivo report on a combination of melatonin and ALA in alleviating symptoms of cadmium-induced alterations in myocardial antioxidant system with little scope for comparison due to the lack of studies using such a combination. However, to have a greater insight on the mechanism of this protectant combination, it is worthwhile to discuss the role of a lone synthetic compound and a conjugate of melatonin and ALA. This compound named melatoninolipoamide was used to carry out pulse radiolysis study. The results indicate that melatonin moiety of the conjugate reacts preferably with oxidizing radicals, and the lipoic acid moiety exhibits preferential reaction with reducing radicals [71]. Thus, our rationale behind the choice of melatonin and ALA was essentially to quench all forms of free radicals leading to overall protection of myocardium. Our results suggest additive effect of melatonin with ALA as observed by the greater magnitude of protection provided by these in combination compared to either of them singly. Our results are in consonance with the reported synergism of melatonin with ALA in an in vitro study [72]. In view of these favorable results of this combination in reducing cadmium toxicity, it is worthwhile to consider melatonin + ALA as a over the counter antioxidant combination as a possible alternative therapeutant against cadmium-induced myocardial damage.

Acknowledgments Authors acknowledge Mr. Ravirajsinh N Jadeja, Department of Zoology, The M S University of Baroda, India for his help in preparing the manuscript.

References

1. Lee, J., Heng, D., Chia, K. S., Chew, S. K., Tan, B. Y., & Hughes, K. (2001). Risk factors and incident coronary heart disease in Chinese, Malay and Asian Indian males: The Singapore Cardiovascular Cohort Study. *International Journal of Epidemiolgy, 30*, 983–988.
2. Bhatnagar, A. (2006). Environmental cardiology: Studying mechanistic links between pollution and heart disease. *Circulation Research, 99*, 692–705.
3. Subramanyam, G., Bhaskar, M., & Govindappa, S. (1992). The role of cadmium in induction of atherosclerosis in rabbits. *Indian Heart Journal, 44*, 177–180.
4. Smetana, R., Glogar, D., Weidinger, F., & Meisinger, V. (1987). Heavy metal and trace element deviations. A comparison of idiopathic dilated cardiomyopathy and coronary heart diseases. *Wiener Medizinische Wochenschrift, 137*, 553–557.
5. Balaraman, R., Gulati, O. D., Bhatt, J. D., Bhatt, S. D., Rathod, S. P., & Hemavati, K. G. (1989). Cadmium induced hypertension in rats. *Pharmacology, 38*, 226–234.
6. Mollaoglu, H., Gokcimenb, A., Ozguner, F., Oktemd, F., Koyu, A., Kocak, A., et al. (2006). Caffeic acid phenethyl ester prevents cadmium-induced cardiac impairment in rat. *Toxicology, 227*, 15–20.
7. Manna, P., Sinha, M., & Sil, P. C. (2008). Amelioration of cadmium-induced cardiac impairment by taurine. *Chemico-Biological Interactions, 174*, 88–97.
8. Millis, P. R., Ramsey, M. H., & John, E. A. (2004). Heterogeneity of cadmium concentration in soil as a source of uncertainty in plant uptake and its implications for human health risk assessment. *Science of the Total Environment, 326*, 49–53.
9. Chary, N. S., Kamala, C. T., & Raj, D. S. (2008). Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. *Ecotoxicology and Environmental Safety, 69*, 513–524.
10. Ramachandran, A. V. (2003). *Aftermath of Baroda effluent channel: Impact assessment along the channel and the Mahi estuary with reference to heavy metals, environment global changes and challenges* (pp. 15–49). Jaipur: ABD Publishers.
11. Karbownik, M., & Reiter, R. J. (2000). Antioxidative effects of melatonin in protection against cellular damage caused by ionizing radiation. *Proceedings of the Society for Experimental Biology and Medicine, 225*, 9–22.
12. Lee, Y. M., Chen, H. R., Hsiao, G., Sheu, J. R., Wang, J. J., & Yen, M. H. (2002). Protective effects of melatonin on myocardial ischemia/reperfusion injury in vivo. *Journal of Pineal Research, 33*, 72–80.
13. Lochner, A., Genade, S., Davids, A., Ytrehus, K., & Moolman, J. A. (2006). Short- and long-term effects of melatonin on myocardial post-ischemic recovery. *Journal of Pineal Research, 40*, 56–63.
14. Reiter, R. J. (2000). Melatonin: Lowering the high price of free radicals. *News in Physiological Sciences, 15*, 246–250.
15. Sahna, E., Olmez, E., & Acet, A. (2002). Effects of physiological and pharmacological concentrations of melatonin on ischemia-reperfusion arrhythmias in rats: Can the incidence of sudden

cardiac death be reduced? *Journal of Pineal Research, 32*, 194–198.
16. Tengattini, S., Reiter, R. J., Tan, D. X., Terron, M. P., Rodella, L. F., & Rezzani, R. (2008). Cardiovascular diseases: Protective effects of melatonin. *Journal of Pineal Research, 44*, 16–25.
17. Chan, T. Y., & Tang, P. L. (1996). Characterization of the antioxidant effects of melatonin and related indoleamines in vitro. *Journal of Pineal Research, 20*, 187–191.
18. Anton-Tay, F., Martinez, I., Tovar, R., & Benitez-King, G. (1998). Modulation of the subcellular distribution of calmodulin by melatonin in MDCK cells. *Journal of Pineal Research, 24*, 35–42.
19. Vanecek, J. (1995). Melatonin inhibits increase of intracellular calcium and cyclic AMP in neonatal rat pituitary via independent pathways. *Molecular and Cellular Endocrinology, 107*, 149–153.
20. Iriti, M., Varoni, E. M., & Vitalini, S. (2010). Melatonin in traditional Mediterranean diets. *Journal of Pineal Research, 49*, 101–105.
21. Al-Majed, A. A., Gado, A. M., Al-Shabanah, O. A., & Mansour, M. A. (2002). Alpha-lipoic acid ameliorates myocardial toxicity induced by doxorubicin. *Pharmacological Research, 46*, 499–503.
22. Gurer, H., Ozgunes, H., Oztezcan, S., & Ercal, N. (1999). Antioxidant role of [alpha]-lipoic acid in lead toxicity. *Free Radical Biology and Medicine, 27*, 75–81.
23. Packer, L., Tritschler, H. J., & Wessel, K. (1997). Neuroprotection by the metabolic antioxidant alpha-lipoic acid. *Free Radical Biology and Medicine, 22*, 359–378.
24. Motawi, T. M. K., Sadik, N. A. H., & Refaat, A. (2010). Cytoprotective effects of DL-alpha-lipoic acid or squalene on cyclophosphamide-induced oxidative injury: An experimental study on rat myocardium, testicles and urinary bladder. *Food and Chemical Toxicology, 48*, 2326–2336.
25. Beuge, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*, 302–310.
26. Holland, M. K., & Storey, B. T. (1981). Oxygen metabolism of mammalian spermatozoa. Generation of hydrogen peroxide by rabbit epididymal spermatozoa. *Biochemical Journal, 198*, 273–280.
27. Puntarulo, S., & Cederbaum, A. I. (1988). Effect of oxygen concentration on microsomal oxidation of ethanol and generation of oxygen radicals. *Biochemical Journal, 251*, 787–794.
28. Beutler, E., Duron, O., & Kelly, B. M. (1969). Improved method for the reduced glutathione. *Journal of Laboratory and Clinical Medicine, 61*, 882–888.
29. Omaye, S., Turnbull, J. D., & Sauberlich, H. E. (1979). Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods in Enzymology, 62*, 3–11.
30. Desai, I. D. (1984). Vitamin E analysis methods for animal tissues. *Methods in Enzymology, 105*, 138–147.
31. Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry, 47*, 469–474.
32. Sinha, A. K. (1972). Calorimetric assay of catalase. *Analytical Biochemistry, 47*, 389–394.
33. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., & Hoekstra, W. G. (1973). Selenium biochemical role as a component of glutathione peroxidase. *Science, 179*, 588–590.
34. Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry, 249*, 7130–7139.
35. Zmudzki, J. (1977). Determination of lead in biological material by atomic absorption spectrophotometry (AAS). *Medycyna Weterynaryjna, 33*, 179–181.
36. Onosaka, S., & Cherian, M. G. (1982).The induced synthesis of metallothionein in various tissues of rats in response to metals. II. Influence of zinc status and specific effect on pancreatic metallothionein. *Toxicology, 23*, 11–20.
37. Chwelatiuk, E., Wlostowski, T., Krasowska, A., & Bonda, E. (2006). The effect of orally administered melatonin on tissue accumulation and toxicity of cadmium in mice. *Journal of Trace Element and Medical Biology, 19*, 259–265.
38. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.
39. Shen, Y., Sangiah, S., & Ye, M. Y. (1995). Determination of hydroxyl radical formation in the testes of cadmium-treated mice by high performance liquid chromatography. *Journal of Liquid Chromatography, 18*, 2217–2228.
40. Shi, H., Sui, Y., Wang, X., Luo, Y., & Ji, L. (2005). Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of *Carassius auratus*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 140*, 115–121.
41. Babusikova, E., Kaplan, P., Lehotsky, J., Jesenak, M., & Dobrota, D. (2004). Oxidative modification of rat cardiac mitochondrial membranes and myofibrils by hydroxyl radicals. *General Physiology and Biophysics, 23*, 327–335.
42. Jadeja, R. N., Thounaojam, M. C., Patel, D. K., Devkar, R. V., Ramachandran, A. V., et al. (2010). Pomegranate (*Punica granatum* L.) juice supplementation attenuates isoproterenol-induced cardiac necrosis in rats. *Cardiovascular Toxicology, 10*, 174–180.
43. Stohs, S. J., Bagchi, D., Hassoun, E., & Bagchi, M. (2000). Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology and Oncology, 19*, 201–213.
44. Stohs, S. J., Bagchi, D., Hassoun, E., & Bagchi, M. (2001). Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology and Oncology, 20*, 77–88.
45. Zalups, R. K., & Barfuss, D. W. (1996). Nephrotoxicity of inorganic mercury co-administrated with L-cysteine. *Toxicology, 109*, 15–29.
46. Martensson, J., & Meister, A. (1991). Glutathione deficiency decreases tissue ascorbate levels in newborn rats: Ascorbate spares glutathione and protects. *Proceedings of the National Academy of Sciences USA, 88*, 4656–4660.
47. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*, 44–84.
48. Clarke, M. W., Burnett, J. R., & Croft, K. D. (2008). Vitamin E in human health and disease. *Critical Reviews in Clinical Laboratory Sciences, 45*, 417–450.
49. Manna, P., Sinha, M., & Sil, P. C. (2009). Taurine plays a beneficial role against cadmium-induced oxidative renal dysfunction. *Amino Acids, 36*, 417–428.
50. Manca, D., Ricard, A. C., Trottier, B., & Chevalier, G. (1991). Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride. *Toxicology, 67*, 303–323.
51. Sarkar, S., Yadav, P., Trivedi, R., Bansal, A. K., & Bhatnagar, D. (1995). Cadmium-induced lipid peroxidation and the status of the antioxidant system in rat tissues. *Journal of Trace Elements and Medical Biology, 9*, 144–149.
52. Hidalgo, H. A., & Bryan, S. E. (1977). Cadmium-115 bound to nuclear and cytoplasmic proteins. *Toxicology and Applied Pharmacology, 42*, 319–327.
53. Adams, J. E., 3rd, Bodor, G. S., Davila-Roman, V. G., Delmez, J. A., Apple, F. S., Ladenson, J. H., et al. (1993). Cardiac troponin I.

A marker with high specificity for cardiac injury. *Circulation, 88*, 101–106.
54. Burlina, A., Zaninotto, M., Secchiero, S., Rubin, D., & Accorsi, F. (1994). Troponin T as a marker of ischemic myocardial injury. *Clinical Biochemistry, 27*, 113–121.
55. O'Brien, P. J., Dameron, G. W., Beck, M. L., Kang, Y. J., Erickson, B. K., Di Battista, T. H., et al. (1997). Cardiac troponin T is a sensitive, specific biomarker of cardiac injury in laboratory animals. *Laboratory Animal Science, 47*, 486–495.
56. Vorderwinkler, K. P., Mair, J., Puschendorf, B., Hempel, A., Schluter, K. D., & Piper, H. M. (1996). Cardiac troponin I increases in parallel to cardiac troponin T, creatine kinase and lactate dehydrogenase in effluents from isolated perfused rat hearts after hypoxia-reoxygenation-induced myocardial injury. *Clinica Chimica Acta, 251*, 113–117.
57. Kopp, S. J., Barany, M., Erlanger, M., Perry, E. F., & Perry, H. M., Jr. (1980). The influence of chronic low-level cadmium and/or lead feeding on myocardial contractility related to phosphorylation of cardiac myofibrillar proteins. *Toxicology and Applied Pharmacology, 54*, 48–56.
58. Haenen, G. R. M. M., Vermeulen, N. P. E., Timmerman, H., & Bast, A. (1989). Effect of thiols on lipid peroxidation in rat liver microsomes. *Chemico-Biological Interactions, 71*, 201–212.
59. Sumathi, R., Baskaran, G., & Varalakshmi, P. (1996). Effect of DL [alpha]-lipoic acid on tissue redox state in acute cadmium-challenged tissues. *The Journal of Nutritional. Biochemistry, 7*, 85–92.
60. Moini, H., Packer, L., & Saris, N.-E. L. (2002). Antioxidant and prooxidant activities of [alpha]-lipoic acid and dihydrolipoic acid. *Toxicology and Applied Pharmacology, 182*, 84–90.
61. Reiter, R. J., Tan, D. X., Manchester, L. C., Lopez-Burillo, S., Sainz, R. M., & Mayo, J. C. (2003). Melatonin: Detoxification of oxygen and nitrogen-based toxic reactants. *Advances in Experimental and Medical Biology, 527*, 539–548.
62. Reiter, R. J., Tan, D. X., Qi, W., Manchester, L. C., Karbownik, M., & Calvo, J. R. (2000). Pharmacology and physiology of melatonin in the reduction of oxidative stress in vivo. *Biological Signals and Receptors, 9*, 160–171.
63. Shida, C. S., Castrucci, A. M., & Lamy-Freund, M. T. (1994). High melatonin solubility in aqueous medium. *Journal of Pineal Research, 16*, 198–201.
64. Cao, Z., Tsang, M., Zhao, H., & Li, Y. (2003). Induction of endogenous antioxidants and phase 2 enzymes by [alpha]-lipoic acid in rat cardiac H9C2 cells: Protection against oxidative injury. *Biochemical Biophysical Research Communications, 310*, 979–985.
65. Klaassen, C. D., Liu, J., & Choudhuri, S. (1999). Metallothionein: An intracellular protein to protect against cadmium toxicity. *Annual Reviews in Pharmacology and Toxicology, 39*, 267–294.
66. O'Brien, P. J., Dameron, G. W., Beck, M. L., & Brandt, M. (1998). Differential reactivity of cardiac and skeletal muscle from various species in two generations of cardiac troponin-T immunoassays. *Research in Veterinary Science, 65*, 135–137.
67. Alonso-González, C., González, A., Mediavilla, D., Cos, S., Martínez-Campa, C., Sánchez-Barceló, E., et al. (2007). Melatonin prevents cadmium toxicity through activation of metallothionein I and II genes expression. *Toxicology Letters. Abstracts of the 44th Congress of the European Societies of Toxicology 172*, S205–S206.
68. Kang, Y. J. (1999). The antioxidant function of metallothionein in the heart. *Proceedings for Society of Experimental Biology and Medicine, 222*, 263–273.
69. Kang, Y. J. (2007). Antioxidant defense against anthracycline cardiotoxicity by metallothionein. *Cardiovascular Toxicology, 7*, 95–100.
70. Ou, P., Tritschler, H. J., & Wolff, S. P. (1995). Thioctic (lipoic) acid: A therapeutic metal-chelating antioxidant? *Biochemical Pharmacology, 50*, 123–126.
71. Venkatachalam, S. R., Salaskar, A., Chattopadhyay, A., Barik, A., Mishra, B., Gangabhagirathi, R., Priyadarsini, K. I., et al. (2006). Synthesis, pulse radiolysis, and in vitro radioprotection studies of melatoninolipoamide, a novel conjugate of melatonin and [alpha]-lipoic acid. *Bioorganic & Medicinal Chemistry. Tetrahedron Prize for Creativity in Organic Chemistry 14,* 6414–6419 (2005: B. Giese).
72. Lopez-Burillo, S., Tan, D. X., Mayo, J. C., Sainz, R. M., Manchester, L. C., & Reiter, R. J. (2003). Melatonin, xanthurenic acid, resveratrol, EGCG, vitamin C and alpha-lipoic acid differentially reduce oxidative DNA damage induced by Fenton reagents: A study of their individual and synergistic actions. *Journal of Pineal Research, 34*, 269–277.