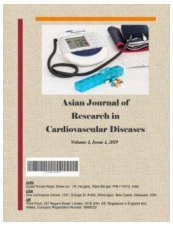

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A Combination of Melatonin and Alpha Lipoic Acid Abrogates Chromium-Induced Cardiac Oxidative Damage

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Authors' contributions

This work was carried out in collaboration between both authors. Author AV R designed the study and logically designed the experiments. Author RM executed all laboratory experiments, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

This study was designed to examine the cardioprotective effects of a combination of melatonin (10 mg / Kg BW) + alpha lipoic acid (25 mg / Kg BW) [M + A] against cardiac oxyradical alterations due to chromium toxicity in rats. Apart from monitoring electrocardiograph parameters, diagnostic cardiac marker enzymes, myocardial antioxidative parameters, trace element alterations and degree of metallothionein (MT) induction were also assessed. Administration of hexavalent chromium as Cr (VI) oxide (7.06 mg/kg for 15 days) to female *Wistar* rats induced marked alterations in ECG - patterns, significant increases in serum levels of creatine phosphokinase–MB (CPK-MB), lactate dehydrogenase (LDH) and cardiac troponin I (cTnI). Moreover, the level of myocardial thiobarbituric acid-reactive substances was significantly increased while, reduced glutathione content and activities of antioxidant enzymes [SOD, CAT, GPx, GR and GST] were significantly decreased. Administration of M + A significantly prevented leakage of marker enzymes of cardiac damage, changes in cardiac free radical generation, antioxidant status, histochemical changes and,

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augmented the degree of cysteine rich antioxidant and metal chelating protein, metallothionein (MT) induction,. These findings indicate that M + A exert an additive cardio protective effect on chromium – induced cardiac oxidative damage in rats.

Keywords: Cardiotoxicity; hexavalent chromium compounds; modulating antioxidants.

1. INTRODUCTION

The role of reactive oxygen species stands implicated in a vast majority of cardiovascular diseases [1]. Chromium, categorized as class C toxic metal is an essential in trace element. Class C elements are toxic at higher concentrations in keeping with the basic recognition of Paracelsus in the 16th century that "the right dose differentiates a poison from a remedy" [2]. Moreover, chromium reportedly generates free radicals leading to oxidative stress [3-4]. Oral intake through food and water apparently serves as the major mode of chromium exposure in humans. Cr (VI) entering via the oral route gets reduced to Cr (III) before entry into cells. Earlier work from our laboratory demonstrated that the content of Cr in cereals and vegetables grown on either side along the Baroda effluent channel to be seven times the World Health Organization (WHO)-recommended permissible limit [5]. Therefore, it becomes imperative to make a comprehensive assessment of oxidative changes induced by Cr (VI) via the oral route at a realistic dosage.

The reported oxidative stress as the possible mechanism of Cr toxicity necessitates the need to test the role of antioxidants as therapeutants. To this end, several antioxidants tested, such as vitamin E [6-8], garlic powder [9-10] selenium [11] aqueous extract of Phyllanthus amarus [12] and taurine [13], conferred protection against chromium mediated oxidative insult. In one of our earlier studies, combination of M+A demonstrated protective effect against cadmium-induced cardiac oxidative damage [14]. Both, hydrophilicity [15] and lipophilicity [16] of melatonin enables it to cross physiological barriers and permeate all subcellular compartments. This property confers an overt advantage to M over other antioxidants, as their solubility problems limit their partitioning between intra- and extra-cellular compartments [17]. Alpha lipoic acid, another antioxidant, is an integral ingredient of several multivitamin formulas, anti-aging supplements, and even pet food. It is a well-defined therapy for preventing diabetic polyneuropathies as it scavenges free radicals, chelates metals and, restores intracellular glutathione levels which otherwise declines with age [18]. Alpha lipoic acid quenches free radicals and is a well known cardioprotective agent [19]. In view of these aforementioned reports on the free radical generating ability of hexavalent chromium and the role of antioxidants in conferring adequate protection, we attempted the use of a combination of M + A with the following considerations.

1. The present inventory is the first report on M + A combination against chromium-induced cardiac oxidative damage. Since both melatonin and Alpha Lipoic acid are potent antioxidants, the combination could have strong potential to modulate antioxidant defense along with distribution of trace elements.
2. A vast majority of studies involving oral effects of hexavalent chromium toxicity involves potassium dichromate ($K_2Cr_2O_7$) as the test chemical while, there is no report of toxicity of chromium trioxide on cardiovascular system.
3. There are no available reports on alterations in antioxidant trace elements in cardiotoxicity associated with hexavalent chromium compounds.

2. MATERIALS AND METHODS

2.1 Chemicals

The dosage of chromium selected in the present study is an environmentally relevant estimated average dose based on the actual concentration of chromium found in the cereals and vegetables grown across the Baroda Effluent channel as reported in our earlier publication [5]. The concept of realistic dosage was proposed by Benoff et al. [20], and has formed the basis of our previous works on metal toxicity [14] [21]. Thus the actual chromium 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 as was done for cadmium [14].

2.2 Experimental Animals

Healthy adult female albino rats of *Wistar* strain weighing 100 – 150 gm (60 day old) were housed in polypropylene cages and maintained under conditions of controlled temperature (25 ± 2 ºC) and photoperiodicity (LD 12:12) in the animal house of the 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).

2.3 Experimental Design

A total of 24 rats were divided into 4 groups of 6 animals each as follows:

Control: rats administered with 0.9% sodium chloride.

Melatonin + Alpha Lipoic Acid (M + A): rats administered 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.

Chromium (Cr): rats administered with hexavalent chromium trioxide, (CrO_3) 7.06 mg / Kg body weight, *p.o.*) daily at 19 hr for 15 days.

Cr + Melatonin + Alpha lipoic acid (Cr + M + A): Rats treated with hexavalent chromium trioxide (CrO3 (VI)); 7.06 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 overnight (12 h) and blood samples collected from retro-orbital sinus under mild ether anaesthesia. Auricles and ventricles, separated from hearts excised from animals subjected to cervical dislocation under mild ether anaesthesia (as per the CPCSEA guidelines), were immersed in ice cold physiological saline. A 10 % homogenate of ventricles was prepared in chilled phosphate buffer saline (pH 7.4). Required 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 5000 rpm for 20 min at 4ºC and the supernatant was used for the estimation of all enzymatic antioxidants and marker enzymes of cardiac damage.

2.4 Rat ECG

ECG electrodes (SS2L, Biopack systems) were placed in lead II position. The ECG data was acquired using BIOPAC MP 30 data acquisition system (Biopac systems, Santa Barbara, CA).

The ECG parameters were analyzed using BIOPAC student Lab software (version 3.6.7). The changes in these parameters at the intervals were studied.

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 a microplate reader at 450 nm.

Cardiac Lipid peroxidation (LPO) and reactive oxygen species (ROS): Cadiac LPO was estimated according to the procedure of Beuge and Aust [22] and malonaldehyde produced during peroxidation of lipids served as an index of LPO. Hydrogen peroxide production was assessed by the spectrophotometric method of Holland and Storey [23] and expressed as µmol/min/mg protein. Hydroxyl radical production was quantified by the method of Puntarulo and Cederbaum [24] and expressed as µmol/min/mg protein.

Assay of non-enzymatic and enzymatic antioxidants: Cardiac total reduced glutathione (GSH), [25] Vitamin C [26], Vitamin E [27] and Vitamin A [28] were quantified by the respective calorimetric methods. Superoxide dismutase (SOD), [29]; catalase (CAT), [30] glutathione peroxidase (GPx), [31] glutathione reductase (GR), [32] and glutathione-S-transferase (GST), [33] were estimated in tissue homogenates. Mitochondrial SOD (MnSOD) activity was distinguished from total SOD activity by the addition of potassium cyanide (1 mM) to the assay buffer. Cytosolic SOD (CuZnSOD) activity was calculated by subtracting MnSOD activity from total SOD activity. One unit of SOD activity

is defined as the amount of sample needed for 50% inhibition of pyrogallol oxidation.

Cardiac chromium, trace elements and metallothionein content: Samples of whole heart of known weight were subjected to dry mineralization in an electric oven as per Zmudzki [34]. The ash was dissolved in a known volume of 1 N HNO_3. Concentrations of chromium and the levels of trace elements (after appropriate dilution) were assessed by atomic absorption spectrometry (Thermo S series) with electrothermal atomization in a graphite cuvette (Cr) or flame atomization in an air–acetylene burner (Fe, Cu, Zn, Mn). Selenium (Se) concentration was determined using the hydride generation atomic absorption spectrometry (Atomic Absorption Spectrophotometer, Shimadzu AA 6701 HVG-1; Hydride Vapor Generator). Sodium borohydride solution (3 g $NaBH_4$, 1 g NaOH in 100 ml of Milli-Q water) was used as a reducing agent. All samples and standards were analyzed in duplicate. The cathode lamps of respective elements were operated under standard conditions using their respective resonance lines: Cr, 357.9 nm; Zn, 213.9 nm; Cu, 324.75 nm; Fe, 248.3 nm; Mn, 279.5 nm; Se, 196.0 nm. The concentrations of metals were expressed as µg/g wet tissue.

Cardiac metallothionein (MT) was determined according to the silver saturation method of Scheuhammer and Cherian [35] as described by Heredia et al. [36]. Silver was measured in an atomic absorption spectrophotometer (AAS) equipped with a graphite furnace (Thermo S Series). For control MT analysis, an aliquot of pooled heart was analyzed in each run and less than 10% of variation coefficient was obtained.

Cardiac collagen content: The non-infarcted cardiac tissue was analyzed for micrograms of collagen content per milligram of non-collagenous protein according to the method of Lopez-de Leon and Rojkind [37]. In brief, tissue sections were deparaffinized and stained for 30 minutes with a mixture of Sirius red F3BA and fast green FCF in saturated picric acid. After numerous washings in water, the stain was eluted from the tissue with 2 ml of 0.1N NaOH in absolute methanol (1: 1 v/v), and the optical density of the eluted color was read immediately in a spectrophotometer at 605 and 540 nm, which are the respective maximal absorbency wavelengths of fast green FCF and Sirius red F3BA. The collagen content was expressed as micrograms of collagen per milligram protein.

TTC staining and determination of infarct volume: The heart was excised and ice cold 0.9 % saline was poured over it. Tissue was blotted free of blood and stored at -20 ^{o}C preventing freeze drying. 2 mm thick sections were cut using a sterile blade and these slices were incubated in TTC (0.5 mg / mL for 30 min at 37 $^{\circ}$C). Tissue slices were scanned and the infract area were determined by computer based Image J software 1.30 V (rsb.info.nih/ij.).

Estimation of cardiac protein content: Protein content of homogenates was estimated by the method of Lowry et al. [38] using bovine serum albumin as the standard.

2.5 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. A Bonferroni test is a series of t-tests performed on each pair of groups and is a very useful statistical tool in comparing results from multiple groups.

3. RESULTS

3.1 Electrocardiographic Studies

ECG tracing showed normal cardiac activity in all rats in the control and M + A groups with a mean heart rate of 332 ± 10 and 320 ± 14 beat/min, respectively. Chromium exposed rats showed several ECG changes including bradycardia, ST segment elevation and prolongation QT intervals. Such ECG abnormalities were obviously improved in the M + A group as evidenced by normalization of heart rate, ST segment and both R-amplitude and QT intervals (Table 1; Fig. 1).

3.2 Enzymic Indices of Cardiac Damage

Administration of chromium trioxide induced severe biochemical changes as well as oxidative damage in cardiac tissue. Table 2 shows the abnormally elevated activities of enzymic indices of cardiac damage in the form of significant ($P< 0.001$) elevation in the activities of serum CPK (97 %), LDH (56%), CK-MB (86%) and cTnI (50%). Simultaneously, there was significant decrease in the activities of cardiac CK (56%) and LDH (47%). Activities of these marker enzymes were restored to near normalcy after M + A administration.

Table 1. The effect of melatonin + alpha lipoic acid (M + A) combination on chromium (Cr)-induced alterations in ST segment, R-amplitude, QT interval and heart rate

Parameters	Experimental groups			
	Control	M + A	Cr	Cr + M + A
ST-segment	0.2543 ± 0.004	0.2512 ± 0.006^{NS}	0.2957 ± 0.004 ^{C}	0.2760 ± 0.004^{a}
R- amplitude	0.9634 ± 0.007	0.9635 ± 0.009^{NS}	0.7881 ± 0.005^{A}	0.9002 ±0.007^{a}
QT interval	0.0650 ± 0.001	0.0680 ± 0.002^{NS}	0.0790 ± 0.003^{B}	0.0680 ± 0.002^{a}
Heart Rate	332 ± 10	330 ± 15 ^{NS}	252 ± 12^{B}	320 ± 14^{b}

Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and ^{NS} nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ^{ns} nonsignificant
Units: ST-segment (mV), R-Amplitude (mV), QT-interval (ms) and heart rate (beats / min)

Table 2. Effect of melatonin + alpha lipoic acid (M + A) combination on chromium (Cr)-induced changes in the level cardiotoxicity enzymatic indices in both serum and tissue

Parameters	Experimental groups			
	Control	M + A	Cr	Cr + M + A
Serum / plasma				
CPK	1575 ± 130	1570 ± 140^{NS}	3100 ± 160^{C}	1850 ± 170^{c}
LDH	2743 ± 235	2740 ± 260^{NS}	4300 ± 300^{C}	2950 ± 240^{a}
CK-MB	70.00 ± 4.20	67.50 ± 4.80^{NS}	130.50 ± 9.00^{C}	57.00 ± 3.10^{c}
cTnI	6.20 ± 0.40	6.15 ± 0.45^{NS}	9.30 ± 0.40^{C}	7.37 ± 0.55^{c}
Heart (Ventricles)				
CK	8.35 ± 0.80	8.36 ± 0.70^{NS}	3.70 ± 0.40^{B}	7.30 ± 0.32^{b}
LDH	88.40 ± 7.00	88.42 ± 6.80^{NS}	47.00 ± 0.40^{B}	81.00 ± 4.5^{b}

Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and NS nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ns nonsignificant
Units: Serum CPK (U / L), Serum LDH (U/L), Serum CK-MB (IU / L), cardiac troponin I (ng / ml), Cardiac CK (µM of phosphorous liberated / mg protein/ min), Cardiac LDH (µM of pyruvate liberated / mg protein/ min)

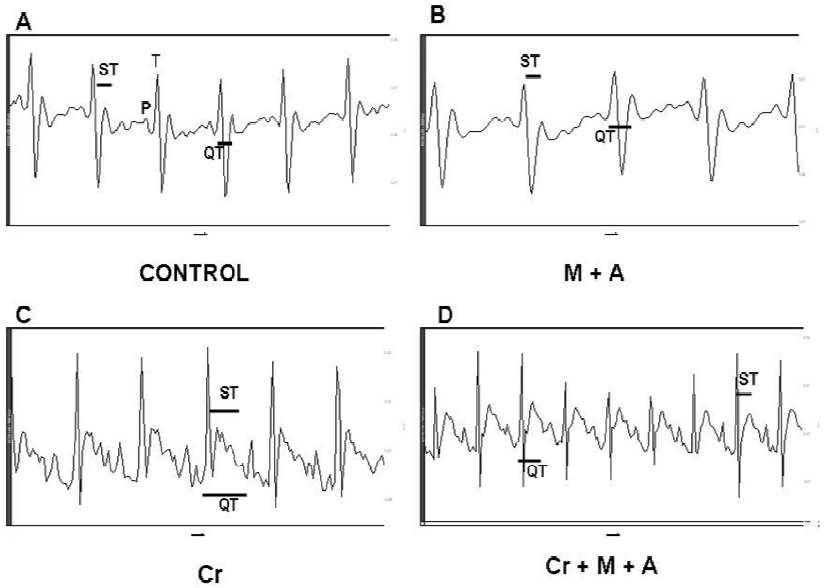


Fig. 1. Electrocardiogaphic Patterns of control (A), M + A (B), Cr- treated (C) and rats simultaneously administered with Cr + M + A (D)

3.3 Degree of Lipid Peroxidation and Levels Free Radicals

The alterations in the level of lipid peroxidation and other free radicals are presented in Table 3. The administration of hexavalent chromium caused significant increase in malondialdehyde ($P < 0.001$), hydroxyl radical ($P < 0.001$), and hydrogen peroxide ($P<0.001$) generation compared to control group. The administration of M + A together was able to reduce or prevent the increase of such radicals.

3.4 Cardiac Non-enzymic Antioxidants

The data on myocardial non-enzymatic antioxidants (GSH, vitamin A, and vitamin C and vitamin E) are presented in Table 4. The concentrations of GSH and vitamins A, C and E were significantly decreased ($P < 0.05$) in the heart of chromium-exposed rats compared to controls. Simultaneous M + A administration resulted in restoration of these non-enzymatic antioxidants to their control levels.

3.5 Cardiac Enzymic Antioxidants

The activities of enzymic antioxidants in the heart of normal and chromium exposed rats are shown in Table 5. Chromium exposed rats exhibited significant ($P < 0.001$) decrease in the activities of cardiac Cu-ZnSOD (40%), MnSOD (67.5%), CAT (44%), GPx (39%), GR (35%) and GST (49%) compared to the control rats. The decline in mitochondrial SOD was 27.5% more than its cytosolic form. Administration of M + A significantly prevented the Cr induced decline in the activities of these enzymes.

3.6 Cardiac Contents of Chromium and Trace Elements and, Degree of Metallothionein Induction

Table 6 presents the data on the status of chromium accumulation, degree of metallothionein induction and alterations in trace elements in the heart of control and experimental animals. The administration of Cr resulted in increase in its concentration in the heart. The cardiac Cr concentration in rats not exposed to Cr was negligible while it was 16.40 µg/gm wet tissue in rats exposed to Cr. Treatment with M + A prevented the accumulation of cardiac chromium to a significant extent. Cardiac levels of metallothionein were higher in rats receiving Cr (4 fold induction) than in Control rats while Cr+M+A rats depicted significantly lesser induction. However, control rats receiving M + A showed no change in metallothionein levels. With respect to bioelements, Cr exposed rats showed significantly increased cardiac iron (62%) and decreased Cu (58%), Zn (55%), Mn (67%) and Se (60%) contents. Simultaneous administration of Cr exposed animals with M + A offset the chromium induced alterations in trace element load.

3.7 Cardiac Fibrosis

Fig 2 depicts the degree of fibrosis in ventricular portion of rat heart. The degree of fibrosis was significantly increased ($P < 0.001$) in chromium administered rats compared to normal rats. Co-administration of M+A combination to rats treated with hexavalent chromium was associated with a reduction in ventricular fibrosis ($P < 0.001$ vs. Cr).

3.8 TTC Staining

The histochemical detection of myocardial damages in control and experimental rats through macroscopic enzyme mapping assay (TTC test) as depicted in Fig. 3A showed higher intensity of staining. Fig. 3B shows the corresponding infarct volume in control and experimental rats. Chromium exposure showed higher percentage of mean infarct size and simultaneous administration of M+A reduced the infarct volume significantly.

4. DISCUSSION

The study clearly shows chromium induced cardiotoxicity to be mediated through oxidative stress as marked by increased generation of hydrogen peroxide and hydroxyl radicals leading to ECG alterations, decline in the activity of the antioxidant enzymes (SOD, CAT, GPx, GR and GST), depletion of GSH and antioxidant vitamins (Vitamin C, E and A), induction of metallothionein and enhancement of lipid peroxidation. Further, co-administration of M+A effectively prevents the Cr induced alterations.

ECG is a quick and painless procedure based on changes in electrical charge in skin cells when the heart muscles contract. In routine clinical practice, ECG is considered as the initial test for diagnosis of cardiovascular abnormalities due to its noninvasive nature. Rats in the chromium group showed decreased heart rate, ST segment

elevation, decreased R-amplitude and prolongation of QT intervals. These changes reflect arrhythmia, conduction abnormalities and attenuation of left ventricular function. During the ST segment, the atrial cells are relaxed and the ventricles are contracted and so electrical activity is not visible. The ST segment is normally isoelectric and ST segment elevation occurs with recent cardiac injury, ventricular aneurysms, Prinzmetals angina, or pericarditis. The QT interval on the other hand represents the time duration from depolarisation to repolarisation of the ventricles. It begins at the onset of the QRS complex and ends at the endpoint of the T wave. Cai et al. [39] has demonstrated the utility of QT interval as a tool to evaluate the cardiotoxic activity of drugs. Moreover, the autonomic tone has its signature on the QT interval, which is primarily determined by the parasympathetic branch, since the cholinergic blockade was impressively related to QT prolongation [40]. In coronary disease, when the vagal activity is reduced, the QT interval prolongation is a predictor of arrhythmias [41-42] and sudden death [43-44]. In this context, M + A combination presents potential cardioprotective effect by its ability to prevent increase in the QT interval characteristic of Cr toxicity. Patel et al. [45] have incidentally shown favorable influence of melatonin in offsetting the abnormalities in ECG tracing associated with isoproterenol induced myocardial necrosis.

The aforementioned ECG abnormalities stand further strengthened by the biochemical data showing significant increase in the serum levels of enzymatic indices of cardiotoxicity (CK, LDH, CK-MB and cTnI). All these enzymes are specific for myocardial damage [46] with cTnI in particular, the structural protein of fetal and adult skeletal muscle, is a highly sensitive marker of cardiac damage as the absence of this structural protein confirms its cardiac specificity [47]. Thus an increase in CK and LDH in serum and a corresponding decrease in heart suggest the leaching of these enzymes from the cardiac myocytes into the blood stream [48]. Alternatively, it is suggestive of secondary events following chromium induced lipid peroxidation of cardiac membranes. Such alterations in enzymatic indices of cardiac damage have been reported in heavy metal induced cardiotoxicity and other experimental models of cardiac damage [49] [50] [14].

Table 3. The effect of melatonin + alpha lipoic acid (M + A) combination on chromium (Cr)-induced alterations in the level of lipid peroxidation and free radicals

Parameters	Experimental groups			
	Control	M + A	Cr	Cr + M + A
LPO	3.29 ± 0.27	3.20 ± 0.30^{NS}	7.30 ± 0.60^{C}	3.70 ± 0.25^{c}
H_2O_2	3.15 ± 0.24	3.00 ± 0.30^{NS}	6.60 ± 0.50^{C}	3.60 ± 0.30^{c}
Hydroxyl radical	0.40 ± 0.02	0.38 ± 0.02^{NS}	1.35 ± 0.14^{C}	0.50 ± 0.04^{c}

Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and NS nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ns nonsignificant
***Units**: LPO (nM of MDA formed per mg tissue), H_2O_2 (µmoles / min / mg protein), Hydroxyl radical (µmoles / min / mg protein).*

Table 4. Effect of chromium (Cr) and melatonin + alpha lipoic acid (M + A) combination on the levels of non-enzymic antioxidants of rat heart

Parameters	Experimental groups			
	Control	M + A	Cr	Cr + M + A
GSH (µg / g tissue)	9.30 ± 0.80	8.70 ± 0.30^{NS}	2.40 ± 0.30^{C}	7.00 ± 0.80^{c}
Vitamin A (µg / g tissue)	1.30 ± 0.10	1.34 ± 0.09^{NS}	0.60 ± 0.05^{C}	1.18 ± 0.10^{c}
Vitamin C (µg / g tissue)	1.30 ± 0.07	1.38 ± 0.09^{NS}	0.28 ± 0.01^{C}	1.15 ± 0.05^{c}
Vitamin E (µg / g tissue)	0.70 ± 0.05	0.88 ± 0.06^{NS}	0.28 ± 0.01^{C}	0.62 ± 0.04^{c}

Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and NS nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ns nonsignificant
Units: GSH, Vit A, Vit C and Vit E – µg per mg tissue.

Table 5. Effect of chromium and melatonin + alpha lipoic acid (M + A) combination on the activities of the antioxidant enzymes in the rat cardiac tissues

Parameters	Experimental groups			
	Control	M + A	Cr	Cr + M + A
CuZn SOD	1.36 ± 0.09	1.58 ± 0.12^{NS}	0.81 ± 0.06^{B}	1.20 ± 0.10^{b}
Mn SOD	0.90 ± 0.08	1.00 ± 0.09^{NS}	0.13 ± 0.01^{C}	0.75 ± 0.05^{c}
CATALASE	64.87 ± 5.80	72.62 ± 4.70^{NS}	36.40 ± 3.50^{B}	57.08 ± 4.10^{c}
GPx	21.61 ± 1.80	25.20 ± 1.40^{NS}	13.10 ± 0.85^{B}	20.00 ± 1.40^{a}
GR	31.63 ± 2.80	34.20 ± 3.10^{NS}	20.49 ± 1.70^{A}	30.50 ± 2.10^{ns}
GST	4.47 ± 0.40	6.10 ± 0.40^{A}	2.30 ± 0.20^{B}	4.20 ± 0.30^{b}

Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and NS nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ns nonsignificant
Units: SOD (units per mg protein where one unit is equal to the amount of enzyme required to inhibit auto oxidation of pyrogallol by 50%), CAT (µmoles of H_2O_2 consumed per min per mg protein), GPx (µg of reduced glutathione utilized /min/ mg protein), GR (nmoles of NADPH oxidized/min/mg protein), GST (nmoles of CDNB-GSH conjugate formed / min/mg protein)

Table 6. Heart tissue concentrations (µg/g tissue) of chromium, trace and major elements and degree of metallothionein induction

Parameters	Experimental groups			
	Control	M + A	Cr	Cr + M + A
Cr	1.80 ± 0.24	1.75 ± 0.32^{NS}	16.40 ± 2.04^{C}	10.80 ± 0.90^{a}
Metallothionein	3.00 ± 0.20	2.28 ± 0.30^{NS}	12.80 ± 0.30^{C}	8.20 ± 0.65^{c}
Cu	3.80 ± 0.30	3.90 ± 0.35^{NS}	1.60 ± 0.15^{C}	3.00 ± 0.25^{a}
Zn	12.00 ± 0.90	15.20 ± 1.10^{NS}	5.40 ± 0.40^{C}	10.80 ± 0.80^{b}
Mn	0.30 ± 0.02	0.35 ± 0.02^{NS}	0.10 ± 0.01^{C}	0.25 ± 0.01^{c}
Fe	8.40 ± 0.70	7.20 ± 0.06^{NS}	13.60 ± 1.10^{B}	9.20 ± 0.85^{b}
Se	2.05 ± 0.30	2.10 ± 0.28^{NS}	0.80 ± 0.25^{A}	1.90 ± 0.20^{a}

Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and NS nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ns nonsignificant
Units: For all parameters (µg / gm wet tissue)

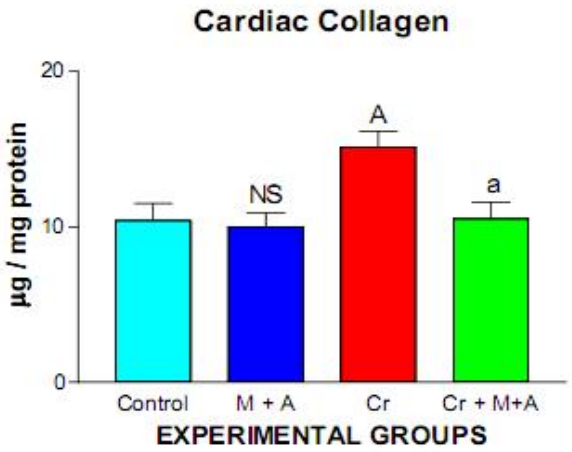
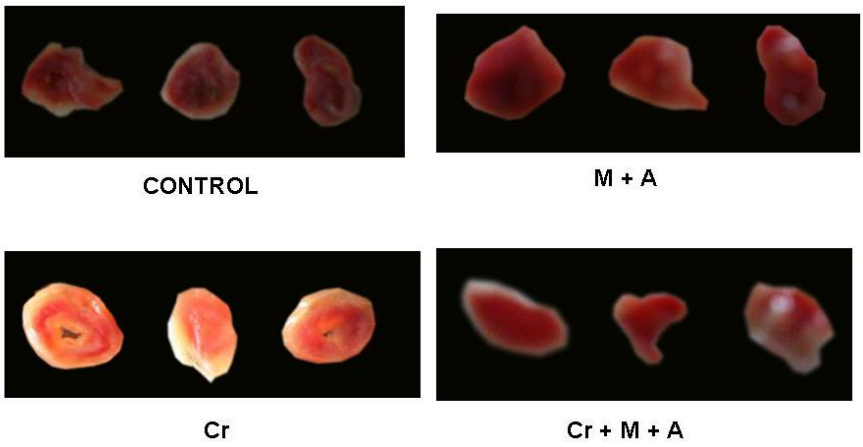


Fig. 2. Collagen concentration in control, M + A, Cr and Cr + M + A groups
Data are expressed as mean ± SEM. n = 6 for each groups
Significant different from controls: ^{A} P <0.05, ^{B}P < 0.01, ^{C} P<0.001 and NS nonsignificant
Significant different from chromium: ^{a} P <0.05, ^{b} P < 0.01, ^{c} P<0.001 and ns nonsignificant

A



B

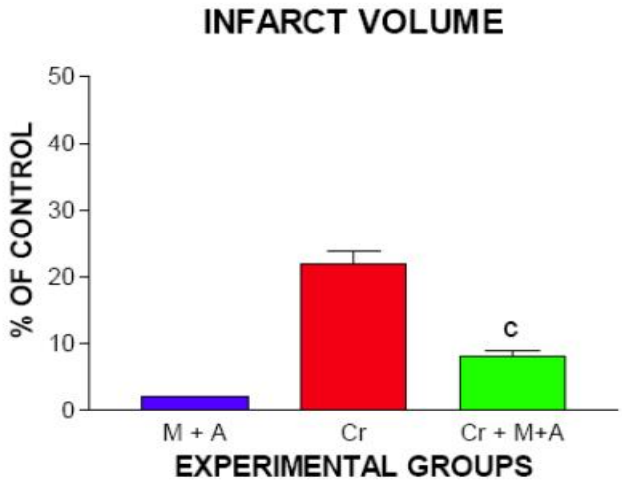


Fig. 3. Macroscopic enzyme mapping assay (TTC test) of heart tissue (ventricular sections) in control, melatonin + alpha lipoic acid, chromium and chromium + melatonin + alpha lipoic acid (Cr + M + ALA) and experimental rats (A). The corresponding degree of infarct volume relative shows significant increase in chromium-exposed rat. Simultaneous administration of M + A and chromium prevents the increase in infarct volume (B)

.

Our results also demonstrate an increase in myocardial TBARS levels in the chromium exposed rats, indicating oxidative stress as the possible mechanism underlying the central effects of cardiotoxicity associated with hexavalent chromium compounds [11] giving rise to alterations in ECG as observed herein. Incidentally, Cr toxicity induced oxidative stress has also been demonstrated in epididymis [51] ovary [6-7] and kidney [9]. Oxidative damage primarily occurs through production of reactive oxygen species, including hydroxyl radicals and hydrogen peroxide that are generated during the interaction between metals and biomolecules, eventually damaging membranes and other tissues [52]. The data on free radicals in the present study supports this contention marked by the increased hydrogen peroxide and hydroxyl radicals. This is in keeping with the observations of Aruldhas et al. [53] in the testis of Cr VI toxicated monkeys.

Additionally, all the cardiac non-enzymatic antioxidants studied also show significant decrement in Cr exposed animals. GSH is a major non-enzymatic antioxidant in the heart and

its decline can be catastrophic to the cardiomyocytes. Glutathione scavenges ROS directly or in a reaction catalyzed by glutathione peroxidase through the oxidation of 2 molecules of glutathione to a molecule of glutathione disulphide (GSSG) [52]. Thus the decrease in GSH level leads to a net suppression in the total antioxidant capacity since it plays a key role as a substrate for the enzyme glutathione S-transferase (GST) and as a cofactor for a variety of enzymes including glutathione peroxidase (GPx). Moreover, GSH depletion reportedly intensifies lipid peroxidation and predisposes cells to further oxidative damage [54]. Amongst the antioxidant vitamins, vitamin E is the most effective chain-breaking lipid soluble antioxidant that inhibits lipid peroxidation induced by ROS [55]. Vitamin C (ascorbic acid) is a well-established water- soluble antioxidant which scavenges free radicals and protects against oxidative damage [56]. The antioxidant activity of vitamin A and carotenoids is accredited to the hydrophobic chain of polyene units that can quench singlet oxygen, neutralize thiyl radicals and combine with and stabilize peroxyl radicals [57]. In light of the known specific antioxidant roles of these vitamin moieties, their decrease in the heart of chromium treated rats can result in escalated levels of free radicals and lipid peroxidation. Our contention stands validated by the reported competence of these vitamins to offset the detrimental effects of free radicals under various conditions of chromium toxicity [3] [6-8].

Along with the non-enzymatic antioxidants, enzymatic antioxidants too depict significant depletion due to Cr exposure. This needs to be discussed in the backdrop of the status of cardiac antioxidant metals that serve as essential cofactors of these enzymes. Many trace elements exert protection against free radicals and zinc and copper in particular are co-factors associated with the apoenzyme superoxide dismutase [58]. Decreased cardiac SOD activity in Cr(VI) treated rats suggests increased generation of superoxide radical. Alternatively, decreased SOD is also attributable to a decrease in Cu and Zn as depleted status of these trace elements can effectively compromise the activity of enzymatic antioxidants and render the tissues susceptible to oxidative stress [59]. Catalase is responsible for detoxification of H_2O_2 and hence decreased catalase activity reflects ineffective scavenging of H_2O_2 that can lead to increased lipid peroxidation through the production of hydroxyl radicals. Although Fe is a co-factor for catalase, the increased Fe content in the ventricles is possibly not associated with the alterations in catalase activity. It is highly probable that the increased pool of iron reflects decreased tissue ferritin due to a surge in superoxide free radicals. The basis behind this assumption stems from an available report that demonstrates down-regulation of ferritin heavy chain (FHC), the active center of ferritin, in rat models of heart failure [60]. In view of the popular concept of 'iron-heart disease' hypothesis, the increased load of cardiac iron assumes added significance. The iron–heart disease hypothesis presumes a defect in the iron control system and consequent iron overload and increased susceptibility to pathological progression of coronary heart disease [61]. Free iron in its reduced (ferrous) state is the more damaging culprit that exacerbates oxidative damage and compromises cellular integrity [62]. One limitation in the methodology adapted in the present study is that AAS measures the total cellular iron which is a summation of both ferrous and ferric forms. However, based on our results on other alternative parameters of oxidative stress, it can be presumed that the majority of iron is possibly in its ferrous state. In this context, measuring the exact level of ferrous and ferric ions in conditions of chromium toxicity can shed additional information on the mechanism of chromium-induced cardiac damage. The herein observed cardiac Se depletion appears well corroborated by the concurrent decrease in GPx activity as Se is its active co-factor [11]. Considering that glutathione S-transferases are detoxifying enzymes that catalyze the conjugation of a variety of electrophilic substrates to the thiol group of GSH producing less toxic forms [63], the significant decrease in cardiac GST activity on chromium exposure indicates insufficient detoxification of chromium. MnSOD, present in the inner membrane of mitochondria dismutates the superoxide anion radical to hydrogen peroxide. An important function of Mn is to act as co-factor for SOD activity and, reportedly, Mn deficiency stimulates lipid peroxidation [64]. The present study demonstrates relatively greater decline in MnSOD than in Cu-Zn isoform and, as MnSOD provides primary defense against mitochondria derived reactive oxygen species, its decline in the chromium exposed cardiac tissue portends mitochondrial dysfunction. Further, the observed decrease in the activity of GR corroborates the report of Aruldhas et al. [53]. Soudani et al. [11] in this context have recorded increased oxidative stress with increase in LPO and decrease in non-

enzymatic antioxidants in the cardiac tissue of Cr exposed female Wistar rats. While the above workers demonstrated an increase in enzymatic antioxidants (GPx, SOD and CAT), our study features a significant decline of both enzymatic and non-enzymatic antioxidants. The observed discrepancy in the result of antioxidant enzymes can be attributed to factors like, form of hexavalent chromium ($K_2Cr_2O_7$ v/s CrO_3), dosage, duration of treatment and route of administration. Bagchi et al. [4] have demonstrated enhanced formation of ROS, including superoxide anion, hydroxyl radicals and nitric oxide, enhanced intracellular oxidized state and membrane damage with leakage of LDH in hexavalent Cr induced oxidative stress. Thus the decreased levels of antioxidant enzymes recorded in the present study is attributable to its inactivation due to quenching generated free radicals and also from the displacement of trace element apoproteins.

Chromium induced induction of cardiac-specific metallothionein (MT) is perhaps the most important finding of our study. Earlier, Solis-Heredia et al. [36] had reported the ability of Cr (VI) salts in inducing pancreatic MT in rats. To the best of our knowledge, the present study is the first report on chromium induced cardiac MT expression. Our result assumes greater significance considering the fact that MT besides being a metal binding protein, is also a proven cardiac antioxidant [65-66] with important roles in detoxifying harmful heavy metals, scavenging oxygen free radicals and stabilizing biomembranes [67]. Interestingly, co-administration of Cr and M+A significantly minimizes the induction of MT. The impact of Cr+M+A to minimize the increase in metallothionein induced by Cr is contrary to our observation of no effect of M+A on Cd induced metallothionein induction [14]. In the context of oxidative stress, maintenance of a critical balance between pro-oxidants and antioxidants is a physiological feature of cells to sustain survival [68-69]. The observed increase in MnSOD together with decrease in metallothieonein in Cr exposed animals given M+A perhaps suggests an adaptive tilt by the myocardium towards more favorable oxidative environment possibly to counteract the deleterious effect of free radicals. An alternative explanation in this regard could be the active involvement of metallothionein in quenching the superoxide free radicals to the total exclusion of SOD as also indicated by Tamai et al. [70].

The observed increase in collagen content in the Cr-treated animals is a reflection of oxidant induced myocardial fibrosis. Administration of M+A together with Cr prevents this Cr induced fibrosis. Thus the decreased fibrosis in the experimental animals treated with M+A can be ascribed to the antioxidant function of this combination. More direct evidence for the role of ROS comes from experimental studies in which antioxidants attenuate cardiac remodeling. Treatment with ROS scavenger such as dimethyl thiourea (DMTU) or antioxidant probucol was found to increase left ventricular (LV) function, prevent LV dilation, wall thinning, and reduce cardiac fibrosis after myocardial infarction in rats [71-72]. Pinealectomized rats show characteristic myocardial fibrosis suggesting the potent role of melatonin in curbing oxidant induced cardiac fibrosis [73]. Further, it is important to note that myocardial fibrosis contributes to ventricular stiffening and progressive heart failure [74]. In this context, the ability of M+A to prevent fibrosis can be seen in the light of its beneficial ability to prevent cardiovascular dysfunction.

In our earlier study, we demonstrated for the first time the superior cardioprotective effect of M+A combination in an additive manner [14]. It is worthwhile to discuss some of the antioxidant properties of these individual antioxidants to get an insight on the mechanism of their additive action. Melatonin *per se* does not react directly with the superoxide anion radical, although the indoyl cation radical, which is generated from melatonin when it donates an electron, may do so [75]. On the other hand, ALA has been reported to prevent an increase in heart mitochondrial superoxide anion production [76]. In the present study, rats simultaneously administered with Cr(VI) and M+A combination show near control level of CuZn-SOD and increased MnSOD. This strengthens the contention that the present antioxidant formulation exerts superior protection against chromium induced mitochondrial dysfunction. The oxidized and reduced forms of ALA bind a number of metal ions, but with different properties depending on the metal chelated. *In vitro* studies show the preferential binding of LA to $Cu^{2+,}$ Zn^{2+} and Pb^{2+}, but not Fe^{3+} while, DHLA forms complexes with Cu^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+} and Fe^{3+} [77]. Melatonin on the other hand has potent metal chelating activity and can chelate iron as well and prevent downregulation of tissue ferritin level [78]. Taken together, the results of the present study and available literature clearly

suggest the mutually complementary actions of M and A.

Fu et al. [79] reported increased synthesis endogenous melatonin and alterations in the expression of MT_1 and MT_2 receptors suggestive of the involvement of melatonin as a natural cardioprotectant along with melatonin receptors against myocardial infarction. Results of TTC staining in the present study confirm myocardial necrosis leading to possible rise in endogenous melatonin due to Cr administration. Interestingly, despite this speculated increase in endogenous melatonin, excess generation of free radicals overwhelms the natural protection rendered by this pineal indolamine. Our results on the ability of M+A to prevent depletion of antioxidant enzymes is attributable to the genomic mode of action of melatonin as, melatonin reportedly stimulate the expression of antioxidant genes [80-83]. Likewise, alpha lipoic acid has also been reported to ameliorate oxidative stress and enhance gene expression of antioxidant enzymes [84]. Hence, the exogenous administration of M+A abrogates this elevated oxidative stress and prevents the down regulation of antioxidant defense essentially by virtue of their additive effect.

In summary, the present study demonstrates chromium induced multiple cardiac structural and functional alterations that are effectively prevented by co-treatment with M+A.

5. CONCLUSION

To our knowledge, this is the first study that demonstrates unequivocally the cardioprotective effect of M+A against Cr stimulated oxidative stress and associated cardiac dysfunction. The study also projects a special relevance in the context of ongoing effort to integrate complementary and alternative medications into the practice of conventional medicine for the treatment of serious cardiovascular disorders and for all diseases that involve free radicals as causative factors. While several thousands of antioxidants find mention in peer reviewed journals, very few of these published products find recognition as over the counter pharmaceutical products. Greater efforts need to be made by concerned agencies to carry forward the findings to the level of clinical trials on antioxidant combinations such as M+A in the prevention/amelioration of metal and other oxidant induced oxyradical overload and cardiac disorders.

DISCLAIMER

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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