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MOLECULAR TOXICOLOGY

On the mechanisms of melatonin in protection of aluminum phosphide cardiotoxicity

Mohammad Hossein Asghari[1,2] · Milad Moloudizargari[3] · Maryam Baeeri[4] · Amir Baghaei[5] · Mahban Rahimifard[4] · Reza Solgi[6] · Abbas Jafari[7] · Hamed Haghi Aminjan[1] · Shokoufeh Hassani[4] · Ali Akbar Moghadamnia[2] · Seyed Nasser Ostad[1] · Mohammad Abdollahi[1,4]

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Abstract Aluminum phosphide (AlP), one of the most commonly used pesticides worldwide, has been the leading cause of self-poisoning mortalities among many Asian countries. The heart is the main organ affected in AlP poisoning. Melatonin has been previously shown to be beneficial in reversing toxic changes in the heart. The present study reveals evidence on the probable protective effects of melatonin on AlP-induced cardiotoxicity in rats. The study groups included a control (almond oil only), ethanol 5% (solvent), sole melatonin (50 mg/kg), AlP (16.7 mg/kg), and 4 AlP + melatonin groups which received 20, 30, 40 and 50 mg/kg of melatonin by intraperitoneal injections following AlP treatment. An electronic cardiovascular monitoring device was used to record the electrocardiographic (ECG) parameters. Heart tissues were studied in terms of oxidative stress biomarkers, mitochondrial complexes activities, ADP/ATP ratio and apoptosis. Abnormal ECG records as well as declined heart rate and blood pressure were found to be related to AlP administration. Based on the results, melatonin was highly effective in controlling AlP-induced changes in the study groups. Significant improvements were observed in the activities of mitochondrial complexes, oxidative stress biomarkers, the activities of caspases 3 and 9, and ADP/ATP ratio following treatment with melatonin at doses of 40 and 50 mg/kg. Our results indicate that melatonin can counteract the AlP-induced oxidative damage in the heart. This is mainly done by maintaining the normal balance of intracellular ATP as well as the prevention of oxidative damage. Further research is warranted to evaluate the possibility of using melatonin as an antidote in AlP poisoning.

Keywords Aluminum phosphide · Melatonin · Apoptosis · Oxidative stress · Mitochondrial dysfunction

✉ Mohammad Abdollahi
Mohammad@TUMS.Ac.Ir;
Mohammad.Abdollahi@UToronto.Ca

[1] Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Pharmacology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran

[3] Student Research Committee, Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[4] Toxicology and Diseases Group, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran

[5] Department of Toxicology and Pharmacology, Faculty of Pharmacy, Alborz University of Medical Sciences, Karaj, Iran

[6] Legal Medicine Research Center, Legal Medicine Organization of Iran, Hamedan, Iran

[7] Department of Occupational Health, School of Public Health, Urmia University of Medical Sciences, Urmia, Iran

Introduction

Aluminum phosphide (AlP), a lethal solid pesticide, is frequently used to protect stored food products and during food transformation processes (Bumbrah et al. 2012). This pesticide has no effects on seed viability and leaves minimal residues on food products. Moreover, its usage is cost-effective and has shown high efficacy against all life stages of insects (Anand et al. 2011; Bumbrah et al. 2012). Although this pesticide is a high-risk agent for non-target species including humans, the advantages outweigh the risks of usage and due to the aforementioned

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advantages, it is still widely used by farmers (Mostafalou et al. 2013). The availability of this chemical in Asian pesticide markets has made it a favorable agent for those who intend to commit suicide. It is the most commonly used suicidal poison in India. An increasing number of self-poisonings with AlP are being reported among Iranians (Mehrpour et al. 2012; Moghadamnia 2012). More than 70% of individuals exposed to AlP die from its toxic effects in different body organs. Cardiotoxicity is the primary cause of death in AlP-poisoned cases. Dysrhythmias, congestive heart failure (CHF) and refractory hypotension have been shown to be the most important cardiovascular disturbances induced by AlP (Bogle et al. 2006).

AlP reacts to the presence of hydrochloric acid or water in the stomach by releasing the fatal phosphine gas. The precise mechanisms of AlP toxicity are not yet known; however, studies on animals show that it possibly works via inhibiting cytochrome oxidase in the mitochondria (Anand et al. 2011; Moghadamnia 2012).

Unfortunately, there is no specific antidote or effective drug to manage the cardiotoxic effects of AlP. The protective effects of some drugs such as triiodothyronine (Abdolghaffari et al. 2015), vasopressin (Jafari et al. 2015), iron sucrose (Solgi et al. 2015), Mg nanoparticle (Baeeri et al. 2013) and acetyl-L-carnitine (Baghaei et al. 2016) have been previously investigated; however, the results were not promising enough due to various reasons such as low safety profiles. Melatonin, as an amphiphilic molecule, crosses all morphophysiological barriers and can be particularly found in mitochondria, potentially protecting it against oxidative stress (Govender et al. 2014; Yang et al. 2014). This indoleamine is a free radical scavenger with good solubility in both aqueous and organic phases which maintaining a high capacity to modulate homeostasis mitochondrial (Paradies et al. 2015). Additionally, melatonin is shown to be advantageous to classic antioxidants, such as vitamin E, and vitamin C (Korkmaz et al. 2009). A huge body of evidence has been studied on the toxicity of pesticides and the correlation of exposure to these compounds with many disorders (Mostafalou and Abdollahi 2017). The protective actions of melatonin have been previously reported against various pesticide and metal toxicities (Asghari et al. 2017b; Romero et al. 2014). The results of studies on the antioxidant, anti-apoptotic and antiarrhythmic properties of melatonin give rise to the opinion that it can be highly potential in counteracting the underlying mechanisms of AlP-induced cardiotoxicity. Also, it may probably improve the clinical manifestations following AlP exposure, due to its pain relieving and anxiolytic properties (Asghari et al. 2017a). On such a basis, we decided to examine the possible cardioprotective effects of melatonin and the underling mechanisms in AlP-poisoned rats.

Materials and methods

Chemicals

ELISA kits for the evaluation of oxidative stress biomarkers and mitochondria isolation kit were obtained from Cayman Chemical Co. (USA) and BioChain Inc. (USA), respectively. AlP was obtained from Samiran Pesticide Formulating Co. (Iran). All the other chemicals were obtained from Sigma-Aldrich (GmbH, Munich, Germany).

Animals

Guidelines for the ethical use of animals were followed and a certification code was deposited by the institute committee of ethics (IR.TUMS.REC.1394.1432), approving all the animal experiments conducted in this study. For the animal studies, 200–250 g male Wistar rats were acclimatized in a room with 50–55% humidity, temperature of 20–25 °C and the light/dark cycle of 12 h, during which standard rat diet was available ad libitum.

Protocols

Determination of AlP LD100

In the previous studies, the LD50 and LD100 of AlP were reported to be 12.5 (Jafari et al. 2015) and 20 mg/kg (Anand et al. 2012) of rat body weight, respectively. We also tried to determine the LD100 of AlP for each experiment of our study. To do this, different doses of AlP between 12 and 20 mg/kg were selected. Almond oil was used as the solvent and the selected doses were orally given to the animals. The control group was solely administered with the same amount of almond oil used as the solvent. In each group, four rats were placed. The mortality was recorded 24 h post-treatment. Ultimately, the LD100 of AlP was calculated as 16.7 mg/kg, using the probit test. Since most doses of AlP that are deliberately ingested by individuals for self-harm intentions are supra-lethal, a single LD100 was considered. The present study was conducted to obtain data on the biochemical changes at the time of death following AlP poisoning. This could thus mimic a real case of human AlP poisoning.

Experimental design

Considering the hemodynamic parameters, four different doses of melatonin (20, 30, 40, 50 mg/kg) were chosen. After the determinations of LD100 of AlP (16.7 mg/kg) and doses of melatonin (MLT), the rats were randomly

allotted into eight groups, including Control, Ethanol 5%, AlP, MLT 50, AlP + MLT 20, AlP + MLT 30, AlP + MLT 40, and AlP + MLT 50, each in which six rats were placed. Almond oil was used as the solvent for AlP which was given orally (through gavage). Melatonin was dissolved in ethanol 5% and administered intraperitoneally. The animals in the groups 1 and 2 were only treated with appropriate volumes of almond oil and 5% ethanol, respectively. To measure the hemodynamic parameters, general anesthesia was induced and maintained by injecting 60.6 mg/kg of ketamine/xylazin and three repetitive injections (30.3 mg/kg), 45, 90 and 150 min after AlP treatment (LD100 dose). After anesthesia was induced, a PowerLab system (PowerLab 4/35 Data Acquisition Systems, AD Instruments, Australia) was used to noninvasively record the parameters including heart rate (HR), electrocardiogram (ECG) and blood pressure (BP). The animals received intraperitoneal injections of melatonin 30 min after treatment with AlP. In order for the biochemical studies, the heart was surgically isolated and the blood was washed out by rinsing in ice-cold saline and was then instantly freezed at −80 °C for biochemical evaluation. The flowchart of the experimental study design is shown in Fig. 1.

Determination of electrocardiogram (ECG) parameters

The anesthetized rats were immobilized and the ECG was monitored after attaching the electrodes to both hands and paws of the animals. PowerLab system software was used for data analysis and QRS complexes as well as the segments of QTc, and ST were measured. The tail cuff of PowerLab was also placed on the external part of the tail, where the pulse can be detected, to record the systolic BP and HR.

Tissue sampling and mitochondrial isolation

The AlP and the other treatment groups were observed till death, following the administrations. The animals were killed, the heart tissues were isolated, washed with ice-cold saline and were then cut into several sections. All the sections were stored at −80 °C various biochemical studies except for one small section (100 mg) which was used for mitochondrial complex assays. The mitochondria isolation kit protocol was then followed.

Activity assessment of complex I

NADH consumption is the principal of this assay, which is characterized by the translocation of electrons initially too complex I and then to an electron acceptor, i.e., synthetic ubiquinone. The method was followed to measure the activity of complex I in the heart homogenate. Briefly, an approximate amount of total mitochondrial protein (100–200 µg) was mixed with the reaction media and the changes in the absorbance of NADH were determined during 3 min at 340 nm. Rotenone was then added to measure the rotenone-unaffected activity of NADH–cytochrome *b* oxidase. The overall net activity was finally determined

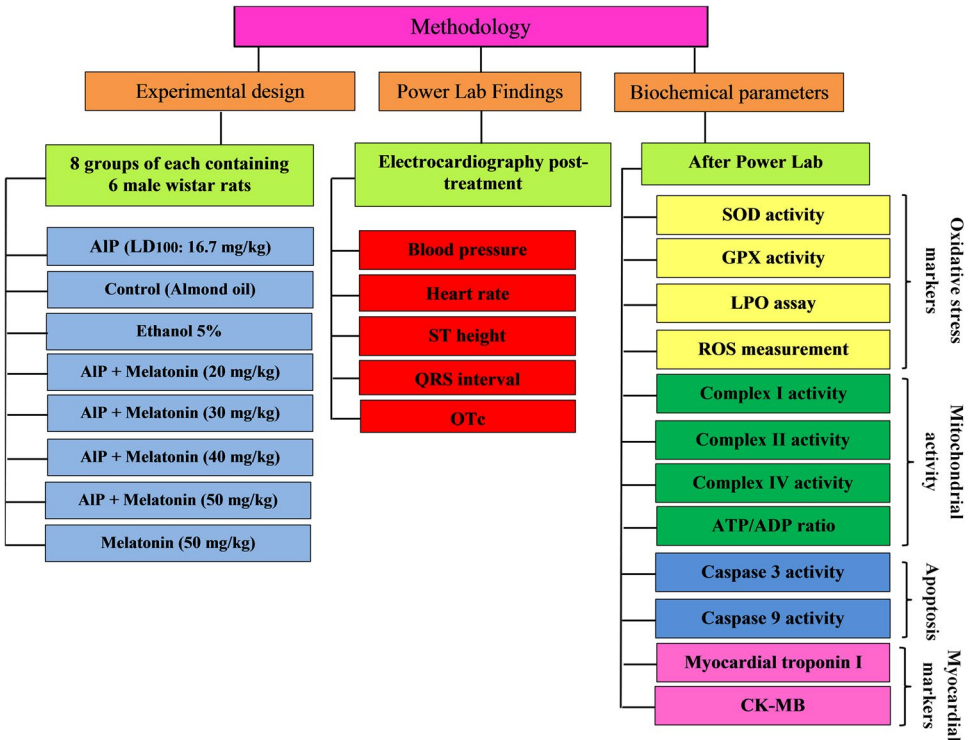

Fig. 1 The methodology of the experimental study is illustrated

by subtracting the rotenone-unaffected activity from the entire activity. The unit, nmol/min/mg protein, was used to express the results (Sherwood and Hirst 2006).

Activity assessment of complex II

The oxidation of 2,6-dichlorophenolindophenol (DCPIP) by complex II (succinate dehydrogenase) decreases the absorbance at 600 nm which can be used as an indicator of its activity. In brief, after the addition of a specific amount of total mitochondrial protein (10–50 µg), the baseline and the ubiquinone-initiated activity of the complex were both determined for 3 and 3–5 min, respectively. A standard curve of DCPIP was then used to calculate the final net activity of complex II which was then expressed as nmol DCIP/min/mg of mitochondrial protein (Karami-Mohajeri et al. 2014).

Activity assessment of complex IV

Shortly, after the addition of mitochondrial protein the reaction media, the decline in the absorbance at 540 nm was measured during 3–6 min as the reduced form of cytochrome c was being converted to the oxidized form by cytochrome-*c* oxidase. The unit, K/min/mg mitochondrial protein was used to express the results (Cooperstein and Lazarow 1951).

Assessment of ADP/ATP ratio in the heart

The ratio was determined based on a study by Hosseini et al. (Hosseini et al. 2010). The frozen samples were thawed and immediately homogenized (4 °C). The homogenized sample was centrifuged at 2000*g* for 10 min, the supernatant was gathered and was then applied to an HPLC system (Waters Chromatography Division, Milford, MA, USA) at a flow rate of 1 ml/min to do the detection. After the quantification of ATP and ADP levels, the ADP/ATP ratio was calculated using a standard curve.

Activity assessment of glutathione peroxidase (GPX)

After homogenizing the heart tissue based on the kit protocol, GPX activity was measured in the heart tissue and the serum samples. A Cayman's kit (Cayman Chemical Co., Ann Arbor, MI, USA) was used to assess the activity of GPX, based on a glutathione reductase-coupled procedure. The oxidized form of glutathione is formed when the hydroperoxide molecule is reduced by GPx.

Activity assessment of superoxide dismutase (SOD)

To assess the activity of SOD, the method of a study by Pourkhalili et al. in which the production of a red formazon dye acts as an indicator of the activity of this enzyme, was followed (Pourkhalili et al. 2011).

Lipid peroxidation (LPO) assessment

The principle for the assessment of LPO is based on a method in which the amount of produced malondialdehyde (MDA) as an end product of LPO corresponds to the extent of peroxidation. This is determined using a spectrophotometer. To do this, the method of Ranjbar et al. was followed (Ranjbar et al. 2010).

Reactive oxygen species (ROS) assessment

DCF-DA was used to measure the amount of peroxides. Five micrometres of DCF-DA was added to the supernatant and incubated at 37 °C for 30 min. The amount of DCF, the fluorescent end product, was then determined at excitation and emission wavelengths of 488 and 525 nm, respectively (Momtaz et al. 2010).

Activity assessment of caspases 3 and 9

The method of Hosseini et al. was followed to assess the activities of caspases 3 and 9. Shortly, the heart samples were rinsed with ice-cold saline after weighing. Homogenates of the tissues were prepared using lysis buffer containing $MgCl_2$ (2 mM), KCl (50 mM), EDTA (2 mM), Triton X100 (%1), HEPES (50 mM; pH 7.4) and were centrifuged at 12,000*g* for 20 min. The substrates of caspases 3 and 9 were added to specific amounts of the supernatant and were incubated at 37 °C for 4 h. Absorbance was determined at a wavelength of 405 nm and the results were shown as nanomoles per hour milligrams of protein (Hosseini et al. 2013).

Determination of CK-MB activity and troponin I level in heart tissue

The activity of creatinine kinase-MB (CK-MB) and troponin-I levels, as cardiac markers, was determined in frozen homogenates of the heart tissue using commercial kits from ZellBio GmbH, Germany. The absorbance of samples was checked by an enzyme linked immunosorbent assay (ELISA) reader.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). One-way ANOVA tests were used to perform the statistical analysis. Tukey was used as the post hoc test. p values less than 0.05 were considered significant.

Results

No significant differences were observed between the ethanol 5% and the control group (results not shown).

Survival time

We started to monitor the ECG abnormalities, 15 min after AlP treatment. Death was the final outcome in all the animals in the AlP and the treatment groups. The median survival time of the AlP group was 55 min [95% confidence interval (CI) 36.99–73]. In the groups receiving melatonin following AlP administration, the median survival times were 90 min (95% CI 69.59–110.4), 85 min (95% CI 66.99–103), 180 min (95% CI 122.38–237.61) and 320 min (95% CI 266.1–373.9) at doses of 20, 30, 40 and 50 mg/kg, respectively.

ECG, HR and BP

AlP-poisoned rats treated with melatonin showed improved hemodynamic parameters. The BP and HR started to decrease significantly 30 min after AlP treatment (Tables 1, 2). Interestingly, melatonin mitigated the progressive decrease of BP in the AlP group; however, BP did not change significantly in the sole melatonin group. ECG abnormalities including widened QRS, elevated ST and prolonged QTc were also observed in the AlP group (Table 3). Melatonin administration vanished the aforementioned ECG abnormalities.

Activity of mitochondrial respiratory complexes

The activities of mitochondrial complexes were separately evaluated to analyze the cardiac mitochondrial function. None of the treatment groups showed significant changes in the activity of complex II. However, the activities of complexes I and IV decreased significantly in the AlP group compared to the control group ($p < 0.05$). Melatonin at doses of 40 and 50 mg/kg was able to drastically increase the activities of these two complexes in comparison to the AlP group (Table 4).

ADP/ATP ratio assessment as an indicator of cardiac energy

A significant increase in the ADP/ATP ratio was observed following AlP treatment ($p < 0.05$). The administration of 50 mg/kg melatonin could decrease this ratio considerably (Table 4).

The activities of GPX and SOD

Cardiac SOD and GPX activities in the AlP group showed a significant decrease compared to the control

Table 1 Changes in blood pressure in various groups

	Time (min)					
	0–30	30–60	60–90	90–120	120–150	150–180
Control	99 ± 4	97 ± 5	97 ± 4	97 ± 6	96 ± 5	96 ± 4
MLT	100 ± 7	99 ± 6	97 ± 8	97 ± 6	100 ± 7	100 ± 6
AlP	99 ± 8	70 ± 8[a]	48 ± 4[a]	D	D	D
AlP +MLT 20	104 ± 7	95 ± 6	83 ± 6	74 ± 7[a]	67 ± 4[a]	D
AlP + MLT 30	102 ± 7	91 ± 5	80 ± 3[a]	71 ± 2[a]	67 ± 4[a]	D
AlP + MLT 40	100 ± 7	93 ± 7	87 ± 6	79 ± 3	73 ± 3	74 ± 4
AlP + MLT 50	98 ± 5	92 ± 6[b]	83 ± 5[b]	77 ± 4	73 ± 2	71 ± 1[a]

Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg)

D died

[a] Significantly different from control groups at $p < 0.05$

[b] Significantly different from AlP group at $p < 0.05$

Table 2 Changes in heart rate in various groups

	Time (min)					
	0–30	30–60	60–90	90–120	120–150	150–180
Control	279 ± 25	276 ± 21	272 ± 23	272 ± 34	270 ± 36	257 ± 30
MLT	278 ± 19	291 ± 34	289 ± 26	296 ± 16	280 ± 31	279 ± 16
AlP	253 ± 12	139 ± 36[a]	46 ± 11[a]	D	D	D
AlP + MLT 20	254 ± 11	194 ± 15	134 ± 13[a]	80 ± 9[a]	47 ± 12[a]	D
AlP + MLT 30	268 ± 27	197 ± 93	166 ± 22[ab]	93 ± 21[a]	49 ± 15[a]	D
AlP + MLT 40	258 ± 10	202 ± 7	155 ± 14[a]	110 ± 15	73 ± 6	72 ± 6[a]
AlP + MLT 50	292 ± 28	225 ± 25[b]	173 ± 20[ab]	111 ± 19	65 ± 7	75 ± 8[a]

Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg)

D died

[a] Significantly different from control groups at $p < 0.05$

[b] Significantly different from AlP group at $p < 0.05$

Table 3 Changes in ECG parameters of various groups

Time (min)	Variable	Control	MLT	AlP	AlP + MLT 20	AlP + MLT 30	AlP + MLT 40	AlP + MLT 50
0–30	QRS (ms)	11.64 ± 0.32	12.67 ± 0.23	23.88 ± 0.73[a]	21.41 ± 0.90[a]	21.12 ± 0.55[a,b]	17.43 ± 0.48[a,b]	13.17 ± 0.41[b]
	QTc (ms)	96.73 ± 2.49	89.70 ± 1.97	155.44 ± 7.02[a]	103.50 ± 1.93	99.51 ± 2.37[b]	114.70 ± 3.27[a,b]	101.56 ± 2.53[b]
	ST (μv)	35.89 ± 2.04	39.92 ± 0.77	80.59 ± 2.05[a]	69.25 ± 1.26[a,b]	67.57 ± 1.04[a]	73.80 ± 2.63[a,b]	43.58 ± 2.22[a,b]
30–60	QRS (ms)	14.47 ± 1.08	14.44 ± 0.35	26.96 ± 1.47[a]	23.12 ± 1.83[a]	24.08 ± 1.75[a]	20.97 ± 1.47	15.54 ± 0.49[b]
	QTc (ms)	81.84 ± 1.30	80.18 ± 1.70	203.82 ± 4.33[a]	189.88 ± 1.78[a]	148.09 ± 1.73[a,b]	142.79 ± 2.00[a,b]	88.77 ± 2.81[a,b]
	ST (μv)	31.25 ± 0.91	30.22 ± 0.99	138.42 ± 2.42[a]	121.48 ± 2.01[a]	138.57 ± 3.51[a]	92.36 ± 2.75[a,b]	38.64 ± 2.32[b]
60–90	QRS (ms)	14.53 ± 0.41	16.56 ± 0.45	59.90 ± 1.11[a]	45.93 ± 2.70[a,b]	38.79 ± 0.69[a,b]	21.54 ± 1.77[b]	15.03 ± 0.65[b]
	QTc (ms)	95.17 ± 2.72	103.91 ± 4.17	239.27 ± 2.53[a]	183.86 ± 3.01[a,b]	166.53 ± 2.38[a,b]	146.65 ± 2.77[a,b]	110.85 ± 6.28[b]
	ST (μv)	39.44 ± 1.32	39.50 ± 0.78	171.20 ± 1.33[a]	137.60 ± 2.04[a,b]	122.13 ± 0.80[a,b]	70.74 ± 2.91[a,b]	43.21 ± 1.17[b]
90–120	QRS (ms)	12.37 ± 0.84	12.49 ± 0.97	D	48.27 ± 3.16[a]	30.00 ± 0.79[a]	19.18 ± 1.14	15.25 ± 0.58
	QTc (ms)	103.01 ± 3.59	101.86 ± 1.75	D	192.88 ± 3.34[a]	174.00 ± 2.17[a]	138.55 ± 4.47[a]	113.77 ± 4.11
	ST (μv)	34.79 ± 1.26	31.07 ± 0.72	D	83.24 ± 2.28[a]	85.15 ± 1.45[a]	50.11 ± 2.06[a]	35.77 ± 1.47
120–150	QRS (ms)	15.77 ± 0.77	15.41 ± 0.65	D	33.60 ± 1.97[a]	31.02 ± 1.27[a]	17.81 ± 0.65	15.89 ± 0.60
	QTc (ms)	98.02 ± 2.07	97.90 ± 1.15	D	208.70 ± 1.48[a]	216.10 ± 3.16[a]	142.19 ± 7.30[a]	109.19 ± 8.27
	ST (μv)	20.99 ± 0.89	23.54 ± 0.92	D	72.92 ± 2.26[a]	74.05 ± 1.67[a]	48.18 ± 3.57[a]	26.56 ± 3.87
150–180	QRS (ms)	16.54 ± 0.58	16.32 ± 0.38	D	D	D	20.07 ± 1.12	20.54 ± 1.86
	QTc (ms)	88.92 ± 0.81	93.73 ± 3.11	D	D	D	133.66 ± 4.52[a]	96.68 ± 2.98
	ST (μv)	29.34 ± 1.09	27.56 ± 1.35	D	D	D	43.54 ± 2.38[a]	33.21 ± 2.62

Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg)

D died

[a] Significantly different from control groups at $p < 0.05$

[b] Significantly different from AlP group at $p < 0.05$

group ($p < 0.05$). Nevertheless, melatonin administration at doses of 40 and 50 mg/kg enhanced the activity of SOD. It also increased the activity of GPX at a dose of 50 mg/kg (Fig. 2).

Results of oxidative stress assessment

LPO levels in the AlP group were higher than those of the control group ($p < 0.05$). However, rats treated with 40 and 50 mg/kg melatonin showed lower MDA levels. ROS

Table 4 Effects of various treatments on the activity of mitochondrial complexes and ADP/ATP ratio in heart tissue

	Control	MLT	AlP	AlP + MLT20	AlP + MLT30	AlP + MLT40	AlP + MLT50
Complex I (nmol/min/mg)	230 ± 2.83	227 ± 5.32	148 ± 17.52[a]	155 ± 8.43[a]	168 ± 7.43[a]	218 ± 9.38[b]	223 ± 9.27[b]
Complex II (nmol/min/mg)	85.21 ± 2.14	82.35 ± 2.48	83.74 ± 5.29	78.98 ± 2.37	79.36 ± 3.71	79.63 ± 3.91	81.93 ± 6.37
Complex IV (K/min/mg)	515.1 ± 17.97	516.7 ± 18.02	335.9 ± 35.87[a]	375.7 ± 11.55[a]	385.8 ± 10.94[a]	424.9 ± 13.20[a, b]	493.9 ± 7.07[b]
ADP/ATP ratio	1.25	1.54	2.79[a]	2.57[a]	2.93[a]	2.25[a]	1.71[b]

Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg)

[a] Significantly different from control groups at $p < 0.05$

[b] Significantly different from AlP group at $p < 0.05$

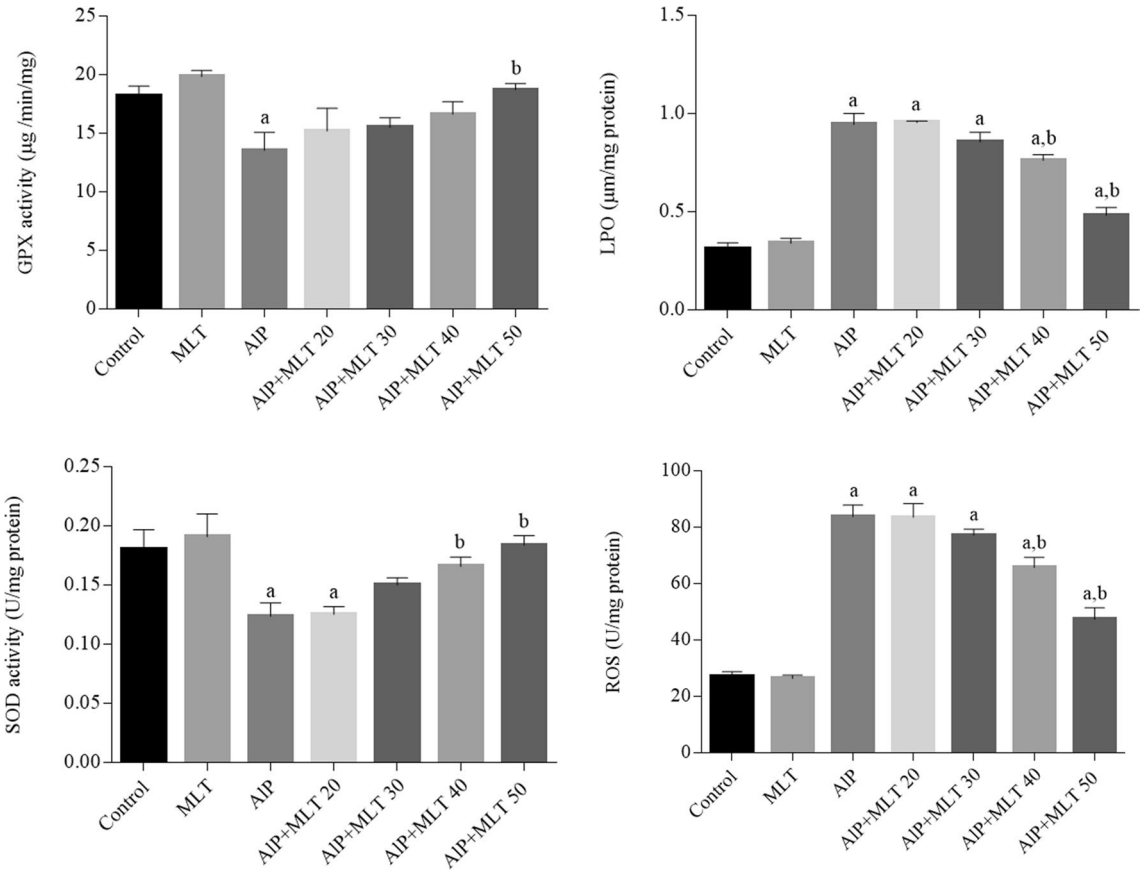

Fig. 2 Effects of treatments on oxidative stress parameters in rat heart tissue. Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg). *a* Significantly different from control groups at $p < 0.05$. *b* Significantly different from AlP group at $p < 0.05$

production in the AlP group was significantly higher than its production in the control group ($p < 0.05$). Melatonin administration at doses of 40 and 50 mg/kg decreased the amount of ROS significantly (Fig. 2).

Caspase-3 and 9 activities

Exposure to AlP increased the activities of caspases 3 and 9 in heart tissue compared to control group ($p < 0.05$).

Administration of melatonin at doses of 30 and 40 mg/kg significantly reduced the activity of caspase 3, while caspase 9 was inhibited at the doses of 40 and 50 mg/kg (Fig. 3).

Cardiac biomarkers (cTnI and CK-MB)

The animals in the AlP group showed an increase in CK-MB activity, compared to control group ($p < 0.05$). Melatonin treatment at doses of 30, 40 and 50 mg/kg diminished the increased activity of this enzyme. Moreover, the levels of troponin I increased in the AlP group and were then declined following the administration of melatonin at doses of 30, 40 and 50 mg/kg (Fig. 4).

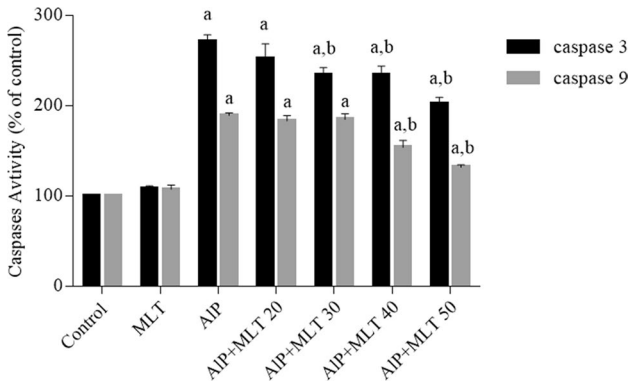

Fig. 3 Effects of treatments on caspase-3 and -9 activities in rat heart tissue. Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg). *a* Significantly different from control groups at $p < 0.05$. *b* Significantly different from AlP group at $p < 0.05$

Discussion

The present study was carried out to examine the cardioprotective effects of melatonin in acute AlP poisoning and the underlying mechanisms. The potential of melatonin prevents oxidative damage and apoptosis and to restore the activities of mitochondrial enzymes and cellular ATP storage makes it a potential candidate to reverse the toxic effects induced by AlP in the heart. Research has shown that AlP-induced alterations in the functions of cardiovascular system such as severe hypotension and decreased HR account for the high rates of mortality in AlP-poisoned patients (Moghadamnia 2012). A key finding of the present study was that melatonin can halt the progressive drop in the BP of AlP-poisoned rats without exerting any significant changes in the BP of healthy controls.

In a previous study on the effects of melatonin on nocturnal BP, the administration of melatonin versus placebo did not induce any significant changes (Laudon and Zisapel 2011). It has been shown that the decreased BP as a result of melatonin administration is associated with a vasodilatation induced by melatonin in a receptor-independent manner. Controversial results have been reported in many of the studies conducted so far indicating that the activation of melatonin receptors causes reduced cAMP levels and induces phosphatidylinositol-4,5-bisphosphate hydrolysis which consequently leads to vasoconstriction (Paulis and Simko 2007). However, it is also shown that the activation of endothelial MT_2 receptors increases intracellular Ca^{2+} in these cells (Pogan et al. 2002).

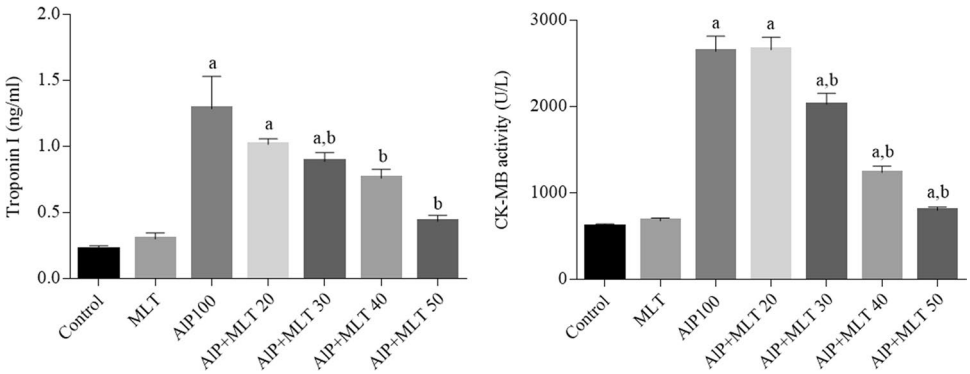

Fig. 4 Effects of treatments on cardiac biomarkers. Data are mean + SD of six animals in each group. The control group received almond oil alone; AlP group received only aluminum phosphide (LD100); MLT 50 group received only melatonin (50 mg/kg); AlP + MLT 20 group received AlP + melatonin (20 mg/kg); AlP + MLT 30 group received AlP + melatonin (30 mg/kg); AlP + MLT 40 group received AlP + melatonin (40 mg/kg); AlP + MLT 50 received AlP + melatonin (50 mg/kg). *a* Significantly different from control groups at $p < 0.05$. *b* Significantly different from AlP group at $p < 0.05$

Treatment of the rats with 50 mg/kg of melatonin induced no significant changes in their BP. These results indicate that melatonin has no effect on BP at its basal levels during healthy state. This is while previous studies have shown that melatonin decreases BP of humans and animal models at hypertensive state (Simko and Paulis 2007). It can be inferred that this substance regulates BP via a modulatory mechanism rather than producing a constant hypotensive effect. Our findings confirm the modulatory role of melatonin in BP regulation since melatonin administration to the AlP-treated rats prevented the severe drop in their BP. Moreover, melatonin prevented AlP-induced bradycardia and improved the function of cardiomyocytes through its antioxidant and antiarrhythmic actions.

As mentioned earlier, AlP induces several ECG abnormalities such as prolongation of QTc, ST alterations, and QRS widening (Baghaei et al. 2014). ST-segment represents the end of ventricular depolarization and the initiation of repolarization and is shown to be either elevated or depressed by AlP administration, signifying myocardial and pericardial damage (Shah et al. 2009; Soltaninejad et al. 2012). ST elevation is correlated closely with mortality rate of acute AlP poisoning and its improvement can be considered as an indicator of therapeutic success in ameliorating AlP poisoning (Karami-Mohajeri et al. 2013). In the present study, fAlP poisoning was followed by ST elevation which showed a significant reduction in response to melatonin treatment. Melatonin also alleviated the QRS widening observed as a result of AlP toxicity. Prolonged QTc was another noticeable change in the ECG of AlP-treated rats which was mitigated by melatonin administration as well. Melatonin might have exerted its protective roles at least partially via the modulation of exaggerated production of oxidative and nitrosative stress markers and alleviating the electrophysiologic dysfunction in the heart (Bertuglia and Reiter 2007; Sahna et al. 2002). Melatonin has been shown to decrease vasoconstriction and the permeability of vessels and leukocyte adhesion. This is while it also has the ability to enhance capillary perfusion in ischemia/reperfusion models. Reducing the incidence of ventricular tachycardia as well as complete removal of ventricular fibrillation is among other effects of melatonin in the heart (Bertuglia and Reiter 2007).

It is shown that AlP can cause considerable alterations in oxidative stress biomarkers. An assessment of MDA content in different human and animal samples showed that AlP significantly increases levels of LPO. Moreover, AlP is shown to increase ROS levels via disruption of the electron transfer chain (ETC) which leads to the overproduction of free radicals along with some alterations in the antioxidant mechanisms (Anand et al. 2013; Kariman et al. 2012; Tehrani et al. 2013). One study reported the efficacy of melatonin in protecting against LPO in many animal tissues studied under numerous oxidizing conditions; however, its exact mechanism is not yet clear (García et al. 2014). The results of studies on phospholipid-containing vesicles demonstrate that melatonin is found near the polar heads of these molecules in membrane lipid layers (Reiter et al. 2014). Such specific positioning of melatonin gives this molecule a higher chance of protecting phospholipids against free radicals. Melatonin can easily penetrate into subcellular compartments because of its small size and amphiphilic properties. Among subcellular organelles, endogenous levels of melatonin are higher in membranes and the mitochondria even at low blood levels of this indoleamine (Venegas et al. 2012). It is also found that melatonin is a potent lipid peroxyl radical (LOO·) scavenger. In the recent years, the melatonin metabolites, c3OHM and AMK, have been introduced to be highly effective LOO· scavengers (Marchetti et al. 2011; Mekhloufi et al. 2007). Our results showed that melatonin treatment (40 and 50 mg/kg) can decrease LPO and ROS levels in AlP-poisoned rats.

Although there is no general agreement on the relationship between AlP and SOD activity, there are controversial reports indicating that AlP affects SOD activity, an effect which is responsible for the dismutation of superoxide radicals into H_2O_2. Some studies have reported that phosphine induces cellular toxicity through the inhibition of SOD activity and via influencing cellular antioxidant defense (Ayobola 2012; Mehrpour et al. 2012). However, others claim that phosphine increases H_2O_2 levels through enhancing SOD activity (Anand et al. 2011; Gurjar et al. 2011). Thus, H_2O_2 overload leads to protein denaturation, lipid peroxidation in cell membranes and increased MDA levels (Anand et al. 2011). It has been also shown that melatonin contributes to Glutathione (GSH) recycling and maintaining high GSH/GSSG ratio through facilitating gene expression of GPX, SOD and catalase, and promoting de novo synthesis of GSH through stimulating the activity of γ-glutamyl-cysteine synthase (Escames et al. 2010). Our findings showed that melatonin administration at doses of 40 and 50 mg/kg increases the activities of GPX and SOD.

Studies show that complex IV (cytochrome *c* oxidase) is the primary site where phosphine interferes with ETC and dysregulates the ATP levels and energy requirements of the cell (Dua et al. 2010; Nath et al. 2011; Singh et al. 2006). Phosphine seems to interact with any enzyme and macromolecule containing heme groups and is a nonspecific cytochrome inhibitor. Through decreasing heme structure in hemoglobin, it induces methemoglobinemia (Anand et al. 2012; Lall et al. 2000; Shadnia et al. 2011). Despite the results of some previous studies, our finding did not show any significant reduction in the activity of complex II. However, complex I and IV activities were significantly reduced in heart tissue of AlP-poisoned rats. Interestingly,

such a decrease in the activities of complexes I and IV was absent in the rats treated with melatonin.

Thanks to its specific molecular characteristics, melatonin is a highly lipophilic molecule and easily crosses cell membranes to accumulate in subcellular compartments, especially in the mitochondria. It affects mitochondrial homeostasis through several mechanisms, finally leading to improved ETC activity (Leon et al. 2005). In one study, the protective role of melatonin on mitochondrial and oxidative damage induced by ruthenium red was evaluated. Melatonin could increase the activities of complexes I and IV, whereas the activities of complexes II and III remained unaffected (Martin et al. 2000). Reports have shown that the antioxidant action of melatonin is not the only responsible mechanism through which melatonin regulates the activities of the complexes I and IV. A unique feature of melatonin is that it can enhance electron flow in the ETC complexes via functioning as an electron donor as well as an electron acceptor due to its high redox potential (Paradies et al. 2015).

The apoptotic and necrotic effects of phosphine have been evaluated in several studies (Anand et al. 2012; Shah et al. 2009). Electron microscopy of some tissues showed that AlP exposure induces mitochondrial swelling due to the release of pro-apoptotic factors such as cytochrome c from the intermembraneous space which gives rise to the activation of caspase-3 and 9 (Heusch et al. 2010). Activity assessment of caspases 3 and 9 in the present study showed an increase in the activities of these two enzymes, which was more pronounced in case of caspase 3. This implies that mechanisms other than mitochondrial involvement are engaged in AlP toxicity. Our result also showed that the activity of caspase-9 at doses of 40 and 50 mg/kg and that of caspase-3 at doses of 30, 40 and 50 mg/kg was reduced by melatonin. Melatonin also acts as an anti-apoptotic agent via inhibiting the dimerization/activation of Bax, a pro-apoptotic protein. It also antagonizes the effect of Bax at the mitochondrial level via inducing significant re-localization of Bcl-2 (Radogna et al. 2008, 2015). Melatonin, due to its structural features, inhibits mitochondrial permeability transition pores and is accumulated in the mitochondria (Andrabi et al. 2004).

There are a number of diagnostic markers which are released into the blood stream following myocardial infarction (Patel et al. 2010). Enzymes are among the best of these markers which are desirable indicators of heart tissue damage mainly due to their hight tissue specificity. CK-MB is a specific enzyme abundantly found in the myocardium and is widely used as a diagnostic marker. Its usage as an early indicator of myocardial damage has made this enzyme an important diagnostic marker (Farvin et al. 2004). CK-MB levels were dramatically increased in the AlP-treated rats, while melatonin administration significantly decreased the activity of this enzyme. It was concluded that melatonin might have inhibited the leakage of CK-MB via maintaining the integrity of cellular plasma membranes.

Although it is claimed that troponin I (cTnI) is highly sensitive and a specific marker in myocardial cell injury detection (Acikel et al. 2003), there are scant data confirming the relationship between the observed increases in cTnI and histological findings in myocardial tissue following AlP-induced cardiotoxicity in rats. According to the findings of the current study, it was revealed that AlP exposure increased troponin I levels in the heart tissue. Also, cardiac troponin I levels significantly decreased as a result of melatonin administration.

Based on the valuable properties of melatonin, it can be potentially used for the management of AlP-induced clinical manifestations via reversing the molecular mechanisms of its toxicity. Commonly, AlP-poisoned patients are presented with anxiety and agitation. AlP intake by humans can cause epigastric pain (Moghadamnia 2012). It has been revealed that melatonin can play an antinociceptive role in a variety of experimental animal models and in humans; therefore, it is concluded that melatonin and its analogs may be also applied in pain management. Despite the lack of evidence on the effectiveness of melatonin as a premedication, it has been used as an alternative to midazolam in adults and children (Marseglia et al. 2015). According to the available data, there is a possibility of using melatonin and its analogs as anesthetic agents or as adjuvants to common anaesthetics. It is worth mentioning that these properties of melatonin have been exploited in diagnostic situations requiring sedation. In another study, using melatonin as a premedication prevented postoperative early agitation in children after sevoflurane anesthesia (Özcengiz et al. 2011).

Although melatonin administration resulted in prolonged survival of the treated animals in comparison to the untreated controls, death was the ultimate outcome in all groups at various time points following melatonin administration. One reason that might justify this is the single administration of melatonin which might interfere with the acute cardiotoxicity of AlP but not with its more long-lasting effects due to its limited half-life. It is also possible that the toxicity of AlP in other organs such as the kidney and liver is the cause of death, since the acute toxicity of AlP in the heart had been already blocked by melatonin administration. Further research is warranted in which multiple doses of melatonin, based on its half-life, may at least partially clarify the ambiguities herein. The administration of melatonin via systemic infusion may also aid in sustaining a constant baseline concentration of this substance, possibly resulting in longer survival times. Moreover, the use of other protective agents

which have the potential to counteract the AlP-induced toxicity in other organs, in combination with melatonin, may clarify the hypothesis that toxicity in other organs might have been the cause of eventual death in the animals.

Conclusion

The protective role of melatonin in various ailments has been previously shown. The present study was carried out to evaluate its probable protective effects against aluminum phosphide-induced cardiotoxicity and the underlying mechanisms. Based on the results, the average survival time increased following melatonin administration in AlP-poisoned rats which might be attributable to the protective effects of melatonin against various mechanisms underlying cardiotoxicity of this agent. Electrocardiographic abnormalities and mitochondrial dysfunction including oxidative stress, ATP depletion and apoptosis were observed following AlP poisoning. Melatonin proved helpful in ameliorating these changes and showed therapeutic effects by inhibiting mainstream pathways of oxidative stress and cell injury. Our results, along with the safe history of melatonin administration, invite for further assessment of its therapeutic effects in clinical settings of AlP poisoning.

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Author contributions MA gave the idea, AAM, and SNO were advisors; MHA did the study and participated in the literature search and drafted the article; MM participated in drafting and editing the article. MB, AJ, MR, HH, SH AB, and RS helped in performing the experimental part of the study. All authors were involved in data analysis and interpretation. MA supervised whole study. All authors read and approved the final version.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest

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