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RESEARCH ARTICLE

Electrophysiological and molecular mechanisms of protection by iron sucrose against phosphine-induced cardiotoxicity: a time course study

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Abstract

The present study was designed for determining the exact mechanism of cytotoxic action of aluminum phosphide (AlP) in the presence of iron sucrose as the proposed antidote. Rats received AlP (12 mg/kg) and iron sucrose (5–30 mg/kg) in various sets and were connected to cardiovascular monitoring device. After identification of optimum doses of AlP and iron sucrose, rats taken in 18 groups received AlP (6 mg/kg) and iron sucrose (10 mg/kg), treated at six different time points, and then their hearts were surgically removed and used for evaluating a series of mitochondrial parameters, including cell lipid peroxidation, antioxidant power, mitochondrial complex activity, ADP/ATP ratio and process of apoptosis. ECG changes of AlP poisoning, including QRS, QT, P-R, ST, BP and HR were ameliorated by iron sucrose (10 mg/kg) treatment. AlP initiated its toxicity in the heart mitochondria through reducing mitochondrial complexes (II, IV and V), which was followed by increasing lipid peroxidation and the ADP/ATP ratio and declining mitochondrial membrane integrity that ultimately resulted in cell death. AlP in acute exposure (6 mg/kg) resulted in an increase in hydroxyl radicals and lipid peroxidation in a time-dependent fashion, suggesting an interaction of delivering electrons of phosphine with mitochondrial respiratory chain and oxidative stress. Iron sucrose, as an electron receiver, can compete with mitochondrial respiratory chain complexes and divert electrons to another pathway. The present findings supported the idea that iron sucrose could normalize the activity of mitochondrial electron transfer chain and cellular ATP level as vital factors for cell escaping from AlP poisoning.

Keywords

Complex IV, cytochrome C oxidase, iron sucrose, phosphine

History

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Introduction

Aluminum phosphide (AlP), so called rice tablet, is conventionally used as a fumigant or a rodenticide in rice storage places. Based on clinical trials, release of phosphine due to the contact of AlP with moisture (particularly gastric acid) causes severe effects on target organs, including the heart and lungs (Eddleston & Phillips, 2004). High mortality of AlP toxicity is mainly attributed to cardiovascular collapse within the first 12–24 h; but, no specific treatment is available to stop its toxicity and all of them are supportive (Anand et al., 2012; Singh et al., 2006; Soltaninejad et al., 2012; Tehrani et al., 2013). Based on previous documents, lipid peroxidation, disruption of membrane integrity, interference with the mitochondrial electron transport chain, decrease of ATP level and cytochrome C oxidase chain activity are the proposed mechanisms for phosphine toxicity in the literature (Dua et al., 2010; Yajima et al., 2009). Mitochondrion, the major source of energy production in cell, is the target organelle for the toxicity of many xenobiotics including AlP. Inhibition of the enzymes of mitochondrial respiration could lead to oxidative stress and activate apoptotic pathways, which ultimately leads to cell death. Besides, the redox energy gathered from mitochondrial metabolism is needed for establishing the proton gradient that is necessary for cell survival (Abdollahi et al., 2004; Dua & Gill, 2004; Mostafalou & Abdollahi, 2013).

As reported in previous studies, the highest reduction potential belongs to cytochrome C oxidase ($E0 = +0.29$ V). Thus, it is suggested as the primary target of phosphine in the electron transfer chain (complex IV). Phosphine ($E0 = -1.18$ V), as a known reducing agent, tends to lose electrons to reach the highest reduction potential according to electrochemical potential. Other studies have indicated that phosphine is bound to a metal ion (Fe) in the active site of

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cytochrome C oxidase (Nath et al., 2011; Solgi & Abdollahi, 2012).

Since mitochondria is the major source of energy production, inhibiting its activity in the cell play the key mediation of AlP toxic effects. It is well established that the heart is susceptible to free radical damage, due to its intrinsic elevated oxidative metabolic activity and its fragile antioxidant resistance, in comparison to other parts of the body (Zafiropoulos et al., 2014). This study attempted to investigate toxic mechanisms of AlP in the heart mitochondria by measuring different toxic parameters with a focus on inhibition of mitochondrial electron-transport chain. Regarding this perspective, iron sucrose was selected as an electron receiver in the exogenic form to be tested as a possible protector.

Materials

Aluminum phosphide (>95% purity) was purchased from Samiran Pesticide Formulating Co. (Tehran, Iran), iron sucrose (ferrovin®) from a local pharmacy (Tehran, Iran), mitochondria isolation kit from Bio-Chain Ins. (Newark, NJ), annexin V-FITC/PI from Beijing Biosea Biotechnology Co, Ltd (Beijing, China), adenosine diphosphate (ADP) sodium salt, adenosine triphosphate (ATP) disodium salt, tetrabutylammoniumhydroxide (TBAHS), methanol (HPLC grade), column (SUPELCOSIL™ LC-18-T) from Supelco (Antrim, UK), acetic acid, $FeCl_3$-$6H_2O$, sodium sulfate, trichloroacetic acid (TCA), potassium dihydrogen phosphate anhydrous (KH_2PO_4, analytical grade), 2,4,6-tripyridyl-s-triazine (TPTZ), 2-thiobarbituric acid (TBA), rotenone, 2,6-dichlorindophenol (DCIP), antimycin A and collagenase from Merck Chemical Co. (Darmstadt, Germany). All the chemicals were at the highest purity available.

Animals

Male Wistar rats were obtained from animal house of Faculty of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran). Animals were fed with standard chow diet and water *ad libitum*. The animal experimentation protocols were approved by the TUMS Ethics Committee on Medical Research with code number 91-03-33-19006.

Study design for determining ECG parameters

A pilot experiment was designed to find the optimum dose of iron sucrose to alleviate the toxicity of AlP on rat ECG parameters. For this purpose, oral LD_{50} of AlP was calculated as 12 mg/kg according to a Probit analysis and was used for inducing cardiac toxicity. The animals were randomly divided into seven groups ($n = 6$ in each group). AlP was weighted and dispersed in 2 mL of almond oil and then was administered by gavage. Iron sucrose was administered intra-peritoneally (IP) 30 min after the gavage in the groups receiving iron sucrose. The control group received only almond oil, AlP group received AlP (12 mg/kg), iron group received iron (10 mg/kg) + almond oil (2 mL), AlP + iron 5 group received AlP (12 mg/kg) + iron (5 mg/kg), AlP + iron 10 group received AlP (12 mg/kg) + iron (10 mg/kg), AlP + iron 20 group received AlP (12 mg/kg) + iron (20 mg/kg), and AlP + iron 30 group received AlP (12 mg/kg) + iron (30 mg/kg). After 10 min of gavage, the animals were anesthetized and rapidly connected to the PowerLab® device (AD Instruments Pty Ltd, Bella Vista, New South Wales, Australia) for complete monitoring of electrocardiogram (ECG) and blood pressure (BP). After 20 min of exposure, iron sucrose was administered intraperitoneally and then ECG and BP were recorded for 3 h. According to the data, iron sucrose (10 mg/kg) was selected as the most positive dose on cardiovascular symptoms.

Study design of mitochondrial activity

The rats were randomly divided into 18 groups ($n = 6$ in each group). The experiment was performed as a time course study (from 1 to 24 h). Therefore, the rats were grouped at six different time points (1, 2, 4, 8, 12 and 24 h). Each time point group included a control, an AlP and other treated groups. The control groups received only almond oil by gavage, AlP groups received only 0.5 LD_{50} AlP (6 mg/kg) that was dissolved in almond oil (via gavage). This was the dose of AlP that induced cardiotoxicity with least mortality, because we required the rats to be alive after 24 h to assay the protective effects of iron sucrose in their heart tissue, which is poisoned with AlP. Initially, the treated groups received AlP (6 mg/kg) by gavage, and after 30 min received one IP injection of iron sucrose (10 mg/kg). At the end of each time point, the rats were anesthetized and their hearts were surgically removed and washed in standard saline (4 °C). Fresh cardiac tissues (100–150 mg) were used according to the protocol of mitochondria isolation kit. Finally, the rest of the tissues and mitochondrial samples were immediately stored at −80 °C for further analysis. For biochemical assessment, 200 mg of cardiac tissues (stored at −80 °C) from each rat was homogenized (4 °C/3 min) in 0.5 mLof TCA (6%) and then centrifuged at 12 000*g* for 10 min at 4 °C. The supernatant was then removed and neutralized with potassium hydroxide (KOH, 4 M).

ECG and BP monitoring

ECG electrodes of PowerLab® device were placed on the right hand and both the legs of the anesthetized rat (Lead II) and continuous ECG data were recorded for 3 h. For each ECG tracing, QRS complexes and segments of QT, P-R and ST were measured. ECGs were analyzed by PowerLab® system software. The systolic BP was recorded for every 3 min using the tail cuff, which was connected to the tail of the anesthetized rats.

Determining LD_{50} of AlP

AlP (4–16 mg/kg) was orally administered by gavage to different groups (each with five rats). The mortality rate was recorded for 24 h, and LD_{50} was calculated by the Probit analysis (Baeeri et al., 2013).

Determining ferric reducing/antioxidant power

The ferric reducing/antioxidant power (FRAP) test was performed on the basis of the antioxidant power of plasma to reduce Fe^{3+} to Fe^{2+}. The reagents included 300 mM acetate buffer (pH 3.6) with 16 mL acetic acid per liter of buffer solution, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$.

Working FRAP reagent was prepared as required by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ solution. Ten microliters of H_2O diluted sample was then added to 300 mL fresh reagent warmed at 37 °C. The complex between TPTZ and Fe^{2+} had a blue color with absorbance at 593 nm (Baeeri et al., 2013).

Lipid peroxidation assay

One of the end products of the oxidation of polyunsaturated fatty acids is malondialdehyde (MDA) that reacts with thiobarbituric acid (TBA) to produce a complex called TBA reactive substance (TBARS), which is determined by a spectrophotometer (BioTek, Winooski, VT). Samples were diluted with buffered saline (1:5), aliquot (400 mL), and TCA (28% w/v, 800 mL) and centrifuged at 3000 *g* (30 min, 4 °C). Then, supernatant (600 mL) + TBA (1% w/v, 150 mL) were incubated for 15 min at 95 °C with *n*-butanol (4 mL). The solution was centrifuged and absorption of the supernatant was measured at 532 nm StatsDirect version 3.0.146 (Cheshire, UK). The method was calibrated by tetraethoxypropane standard solutions (Khoshakhlagh et al., 2007).

Activity assay of complex I (NADH–ubiquinone oxidoreductase)

Complex I activity assay was determined spectrophotometrically (340 nm) by monitoring oxidation sensitive to the rotenone of NADH to NAD^+ in the presence and absence of rotenone. Decrease in the absorbance of NADH at 340 nm was recorded as the total activity of complex I for 3 min by a spectrophotometer from BioTek® Instruments, Inc. (Winooski, VT). The enzyme activity was calculated at 340 nm and was reported as NADH μM/min/mg of mitochondrial protein (Sherwood & Hirst, 2006; William & Immo, 2009).

Activity assay of mitochondrial complex II

The 2,6-dichlorophenolindophenol (DCPIP) reduction showed complex II's (succinate–ubiquinone oxidoreductase) specific activity, which was determined by spectrophotometric analysis at 600 nm. The mitochondria were preincubated in potassium phosphate buffer, $MgCl_2$, and succinate and then, after adding antimycin A, rotenone, KCN and DCPIP, the baseline was recorded for 3 min. The reaction was started with ubiquinone and the enzyme-dependent reduction of DCPIP was measured for 3–5 min at 600 nm. Activity of complex II was calculated using a DCPIP standard curve and reported as DCIP μM/min/mg of mitochondrial protein (Pon & Schon, 2007).

Activity assay of complex IV (cytochrome C oxidase)

Firstly, cytochrome C was reduced by adding enough sodium hydrosulfite. The mitochondrial protein and Lubrol-PX in potassium phosphate buffer were then added to the reduced cytochrome C to start the reaction. According to the previously established spectrophotometric method, decrease in optical absorption at 550 nm was measured for 3–6 min. Data were presented as the natural logarithm of the absorbance divided by time and reported as the first-order rate constant [(*k*) min/mg] of mitochondrial protein (Cooperstein & Lazarow, 1951).

Activity assay of complex V (ATP synthesis)

The ATP-regenerating system indicates ATP synthesis activity. For this purpose, the buffer solution was prepared from tris-acetate, potassium acetate, sucrose, potassium chloride, $MgCl_2$, ATP, pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate and NADH. The reaction was initiated by adding mitochondrial protein and then read spectrophotometrically at 340 nm for 8 min by comparing the absorption change in the presence and absence of oligomycin (6 mg/mL). The results were reported as μM NADH/min/mg of mitochondrial protein (Karami-Mohajeri et al., 2014; Morava et al., 2006).

Determining the ADP/ATP ratio

In order to calculate the ratio of ADP/ATP, heart tissue of 300 mg of each rat was sonicated in 250 μL of TCA (6%) and then centrifuged at 12 000*g* for 10 min at 4 °C. The supernatant was removed and neutralized with potassium hydroxide (KOH; 4 M). The high-performance liquid chromatography (HPLC) was performed by a 510-pump and a solvent delivery system (Waters Chromatography Division, Milford, MA), column (SUPELCOSIL1 LC-18-T, Supelco, Inc., Bellefonte, PA) with a guard column, and 486 UV-Visible Detector (Waters Chromatography Division, Milford, MA).

Isocratic elution (flow: 1 mL min^{-1}; 254 nm) with tetrabutylammonium hydrogen sulfate (4 mM) in potassium phosphate buffer (0.1 M; pH = 5.5) and methanol (85:15 v/v) was used according to the protocol provided. Levels of ATP and ADP were determined by the standard curve and then the ratio was calculated (Hosseini et al., 2010).

Apoptosis and necrosis

The measurement of apoptosis and necrosis by flow cytometry needed single cells; thus, the rats were divided into 18 groups ($n = 3$ in each group) with six different time points (1, 2, 4, 8, 12 and 24 h) and received AlP and iron sucrose in the same manner as described before. The rats were anesthetized, then the left ventricles of the heart were rapidly removed from each rat, and they were digested using collagenase (Egorova et al., 2005; Pinto et al., 2013; Terzic et al., 1992). The cells were washed with phosphate-buffered saline (PBS) at room temperature and stained with annexin V-FITC and propidium iodide (PI) according to kit's instructions. The stained cells were incubated in binding buffer and analyzed for cell death by flow cytometry (Apogee Flow Systems, Hertfordshire, UK). Data were provided and analyzed using Apogee Histogram Software. Density plots of flow cytometry included percentage of live cells (lower left quadrant; annexin V−; PI−), percentage of cells in apoptosis (lower right quadrant; annexin V+; PI−), late apoptosis (upper right quadrant; annexin V+; PI+), and necrosis (upper left quadrant; annexin V−; PI+) (Krifka et al., 2012; Vermes et al., 1995).

Statistical analysis

Results were presented as mean ± SE. All the statistical analyses were performed using StatsDirect version 3.0.146

(Cheshire, UK). Assays were performed in triplicate and the mean was used for statistical analyses. Statistical significance was determined using a one-way ANOVA test, followed by the *post-hoc* Tukey test. $p < 0.05$ was considered statistically significant.

Results

Electrocardiogram parameters

As shown in Table 1, the P–R interval was significantly prolonged in segments 4, 5 and 6 (90–180 min) in the AlP group and in segments 5 and 6 (120–180 min) in the AlP+iron30 group compared to the control ($p < 0.05$); but, in other groups, no difference was detected. QRS interval in AlP + iron20 and AlP +iron30 groups was increased in almost all segments, whereas the increase was observed in segments 5 and 6 (120–150, 150–180 min) in the AlP group (Figure 1). In other groups, no difference was detected compared to the control ($p < 0.05$). All the treated groups, in all the segments (except 90–120 min), showed a significant difference from the control in QT values. ST height was unstable in all the treated groups during the test period, which indicated a significant difference from the controls in all segments (Table 1).

Heart rate

Heart rate was decreased in segments 5 and 6 (120–150, 150–180 min) in all groups that received AlP; whereas, it was significantly increased in AlP + iron5 and, to a more degree in AlP + iron10 groups compared with the AlP group ($p < 0.05$). Data are shown in Table 1.

Blood pressure

The blood pressure significantly dropped in all the groups after administration of AlP, except AlP + iron10 and AlP + iron20 groups, in which no decrease was detected in comparison with the AlP group (Table 1). Analysis of the results showed a maximum increase in heart rate and BP in AlP + iron10 group compared with the AlP group without any prolongation of the P–R interval ($p < 0.05$). Therefore, iron sucrose (10 mg/kg) can be considered the most effective dose.

Table 1. Electrocardiogram parameters in various groups.

		Group						
Time	Variable	Control	ALP	Iron	AlP + Iron 5	AlP + Iron 10	AlP + Iron 20	AlP + Iron 30
	P-R (ms)	46.6 ± 6.9	49.9 ± 5.2	44.3 ± 10.6	44.6 ± 10.7	48.2 ± 1.2	49.1 ± 1.9	48.6 ± 13
0–30 min	QRS (ms)	14.5 ± 9	14.6 ± 0.5	14.1 ± 0.6	14.3 ± 1	14.4 ± 0.8	16 ± 1.3[a]	15.8 ± 1.2[a]
	QTC (ms)	144 ± 8	117 ± 5[b]	122 ± 9[a]	121 ± 10[a]	138 ± 12	175 ± 21[c,f]	116 ± 11[b]
	ST (ms)	88 ± 7	117 ± 11[c]	115 ± 12[c]	115 ± 6[c]	150 ± 13[c,f]	93 ± 7[e]	86 ± 5[f]
	HR	335 ± 28	364 ± 30	342 ± 32	343 ± 28	341 ± 23	314 ± 22	384 ± 34
	BP	75.6 ± 9.7	72.1 ± 13.1	86.6 ± 14.3	89.5 ± 16.2	80.7 ± 12.9	90.7 ± 18.8	81 ± 13.6
30–60 min	P-R (ms)	47.8 ± 4.1	49.2 ± 5.7	48.4 ± 10.9	53 ± 17.3	44.1 ± 10.9	50.9 ± 5.8	55.4 ± 5.2
	ORS (ms)	14.1 ± 0.6	14.6 ± 1.1	14.3 ± 0.9	14.8 ± 1.2	14.9 ± 0.7	16.7 ± 1.4[a]	16.9 ± 1.3[a]
	QTC (ms)	136 ± 6	111 ± 12[b]	114 ± 9[b]	111 ± 8[b]	107 ± 13[c]	164 ± 10[f]	116 ± 11[a]
	ST (ms)	86 ± 9	127 ± 14[c]	104 ± 11[d]	101 ± 8[e]	136 ± 15[c]	104 ± 12[d]	64 ± 6[a,f]
	HR	318 ± 35	341 ± 28	321 ± 30	325 ± 33	333 ± 27	281 ± 21[d]	346 ± 30
	BP	96.8 ± 14.9	65.9 ± 9	94.2 ± 16.2	98.6 ± 136	86.3 ± 14.8	74.4 ± 15.7	102 ± 163[d]
60–90 min	P-R (ms)	47 ± 8.7	53.8 ± 18.7	54.3 ± 16.4	49.2 ± 18.7	49.7 ± 17.9	49.6 ± 14.1	52.4 ± 28.1
	QRS (ms)	14 ± 1.1	14.3 ± 0.9	14.2 ± 1.3	146 ± 0.7	14.6 ± 0.5	16.5 ± 31.2[b]	1.59 ± 1.5[a]
	QTC (ms)	152 ± 13	124 ± 12[b]	109 ± 8[c]	112 ± 11[c]	121 ± 14	180 ± 15[b,f]	114 ± 10[c]
	ST (ms)	92 ± 9	106 ± 8	103 ± 12	106 ± 11	149 ± 16[c,f]	101 ± 8	89 ± 9
	HR	343 ± 45	335 ± 77	3076 ± 68	303 ± 35	348 ± 57	270 ± 39[b,e]	308 ± 26
	BP	112.8 ± 24	58.5 ± 11.1[b]	104 ± 17.5[d]	101.49 ± 25.9[d]	99.6 ± 18.7	94.8 ± 22.2	108 ± 24.3[d]
90–120 min	P-R (ms)	48.4 ± 4.6	64.7 ± 6[b]	52.5 ± 9[d]	49.5 ± 4.1[e]	46.9 ± 8.1[f]	47.7 ± 3.3[f]	55.2 ± 7
	QRS (ms)	14.1 ± 2.3	14.6 ± 1.4	14.5 ± 2.5	14.8 ± 1.3	14.3 ± 1.7	16.4 ± 2.6[a]	17.1 ± 3.1[b,d]
	QTC (ms)	138 ± 9	121 ± 12	125 ± 11	112 ± 14[b]	150 ± 10[e]	166 ± 17[c,f]	11.1 ± 7[b]
	ST (ms)	89 ± 7	94.4 ± 8	92 ± 10	100 ± 12	168 ± 14[c,f]	102 ± 8	−10 ± 9[c,f]
	HR	304 ± 25	309 ± 18	305 ± 27	301 ± 33	306 ± 24	266 ± 16[b,e]	253 ± 22
	BP	109.1 ± 17.7	49 ± 9.1[c]	115 ± 28.4[f]	73.2 ± 17.9	112 ± 24.1[f]	86.5 ± 20.8[e]	74 ± 18.8
120–150 min	P-R (ms)	48.3 ± 8	62.9 ± 5.5[b]	47.3 ± 4.7[e]	49.3 ± 5.5[d]	47.9 ± 6[e]	52.1 ± 9.6[d]	60.3 ± 9.2[b]
	QRS (ms)	14 ± 0.7	16.5 ± 1.2[a]	14.3 ± 1.1	15.5 ± 14	15.2 ± 1.7	16.9 ± 1.1[b]	19.6 ± 1.6[c,e]
	QTC (ms)	148 ± 14	118 ± 13[c]	132 ± 11	124 ± 10[a]	127 ± 8[a]	163 ± 13[f]	101 ± 9[c]
	ST (ms)	88 ± 9	−47 ± 12[c]	136 ± 14[f]	106 ± 12[f]	148 ± 16[c,f]	106 ± 9[f]	11.3 ± 4.4[c]
	HR	325 ± 32	251 ± 19[c]	329 ± 31[f]	305 ± 25	335 ± 29[f]	[b]257 ± 33	234 ± 21[c]
	BP	121.5 = 25.5	39.3 ± 4.2[c]	1263 ± 19.9[f]	56.9 ± 17.7[c]	101.3 ± 29.7[f]	60.9 ± 7.6[c]	52.7 ± 7.8[c]
150–180 min	P-R (ms)	47 ± 5	66.1 ± 6.2[c]	45.74 ± 1.5[e]	51.2 ± 2.2[d]	41.6 ± 6.1[f]	56.3 ± 2.3[b,d]	58.9 ± 2.1[b]
	QRS (ms)	14.1 ± 0.9	16.2 ± 1.7[a]	14.4 ± 1.3	15.5 ± 1.4	14.6 ± 0.8	16.2 ± 1.8[a]	20.1 ± 1.1[c,f]
	QTC (ms)	156 ± 13	129 ± 14[b]	111 ± 11[c]	118 ± 10[c]	107 ± 8[c,e]	167 ± 12[f]	115 ± 10[c]
	ST (ms)	99 ± 18	39 ± 7[c]	139 ± 31[c,f]	109 ± 13[f]	144 ± 34[c,f]	103 ± 18[f]	22 ± 4[c,d]
	HR	354 ± 21	180 ± 12[c]	343 ± 31	304 ± 25[a,f]	358 ± 33	257 ± 30[c,f]	192 ± 16[c]
	BP	125.4 ± 28.6	31.3 ± 7.6[c]	118.9 ± 17.9[f]	39.8 ± 8.6[c]	98.5 ± 16.9[f]	60.1 ± 6.7[c,d]	48 ± 9.1[c]

Data are mean ± SE of six animals in each group. Significantly different from the control group at [a]$p < 0.05$, [b]$p < 0.01$ and [c]$p < 0.001$; Significantly different from the AlP group at [d]$p < 0.05$, [e]$p < 0.01$ and [f]$p < 0.001$. Control group receives only almond oil, AlP group receives aluminum phosphide (12 mg/kg), iron group receives iron (10 mg/kg)+ almond oil (2 mL), AlP + iron5 group receives AlP (12 mg/kg) + iron (5 mg/kg); AlP + iron10 group receives AlP (12 mg/kg) + iron (10 mg/kg), AlP + iron20 group receives AlP (12 mg/kg) + iron (20 mg/kg), and AlP + Iron30 group receives AlP (12 mg/kg) + iron (30 mg/kg).

Lipid peroxidation

A significant increase was found in lipid peroxidation after 2 h when TBARS values in AlP groups were compared with the same time point of control groups ($p < 0.05$). Meanwhile, TBARS was significantly decreased in the groups treated with iron sucrose (10 mg/kg) after 8 h compared with the same time point of AlP groups. These effects were time dependent (Figure 2).

Antioxidant powe

The FRAP levels in AlP and treated (iron sucrose; 10 mg/kg) groups after 4 h were decreased significantly compared with the same time point of control groups. A significant increase was found in the antioxidant power of iron sucrose (10 mg/kg) treated group after 8 h of treatment in comparison with the same time point of AlP groups (data were not shown).

AlP effects on mitochondrial respiratory chain

Mitochondrial activities of respiratory chain in each complex were separately evaluated. There were no significant changes ($p > 0.05$) in the activity of complex I in AlP groups at all-time points (data were not shown). Activity of complex II was significantly and time-dependently inhibited in AlP groups after 12 h compared with the same time of the control groups; whereas, it was significantly increased in the treated groups after 24 h (data were not shown). Activities of complexes IV and V and ATP/ADP ratio in all AlP groups were significantly inhibited in comparison with the same time of the control groups (Figures 3 and 4). Meanwhile, the increase in the activities of complexes IV and V and ATP/ADP ratio was shown after 2, 4 and 4 h, respectively, compared with the same time of AlP groups.

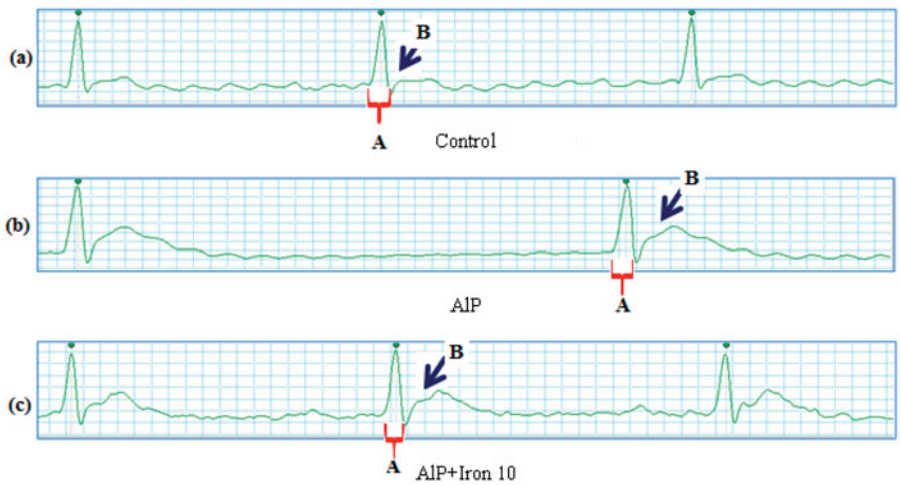

Figure 1. (a–c) Changes in ECG parameters (A) QRS interval and (B) ST height.

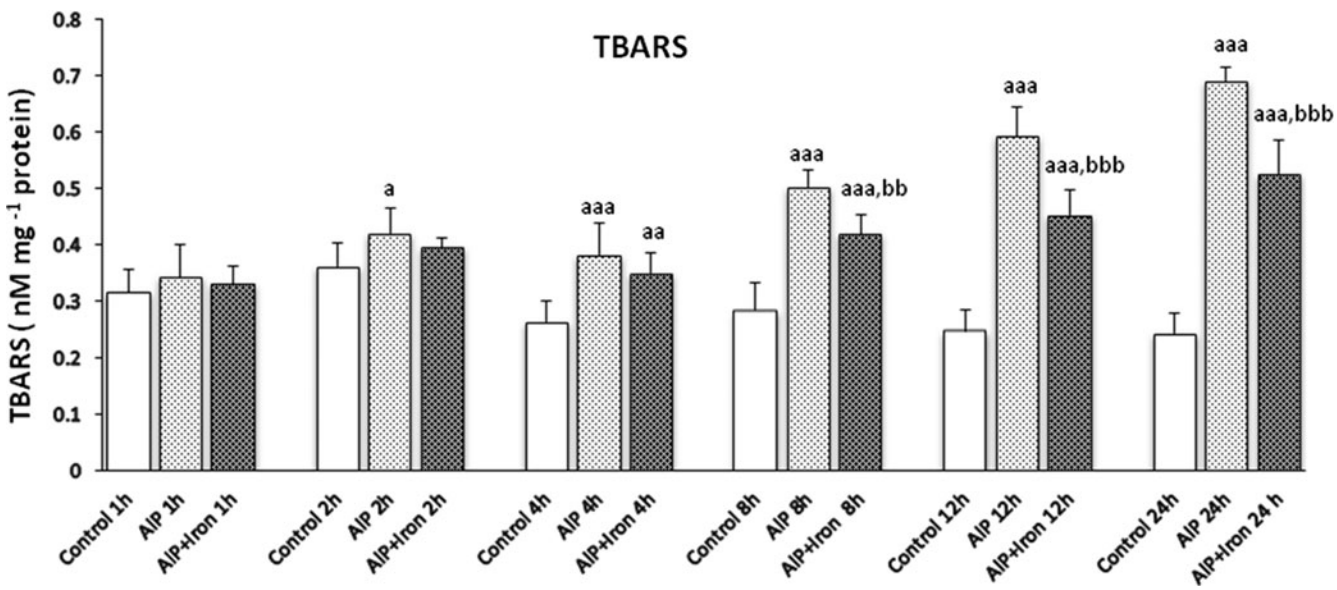

Figure 2. Changes in plasma lipid peroxidation as thiobarbituric acid reactive substance. Data are mean ± SE of six animals in each group. The AlP and AlP + iron groups are compared with the same control group at six times (1, 2, 4, 8, 12 and 24 h). Significantly different from the control group at $p < 0.001$ (aaa), $p < 0.01$ (aa), $p < 0.05$ (a); Significantly different from aluminum phosphide (AlP) group at $p < 0.001$ (bbb), $p < 0.01$ (bb), and $p < 0.05$.

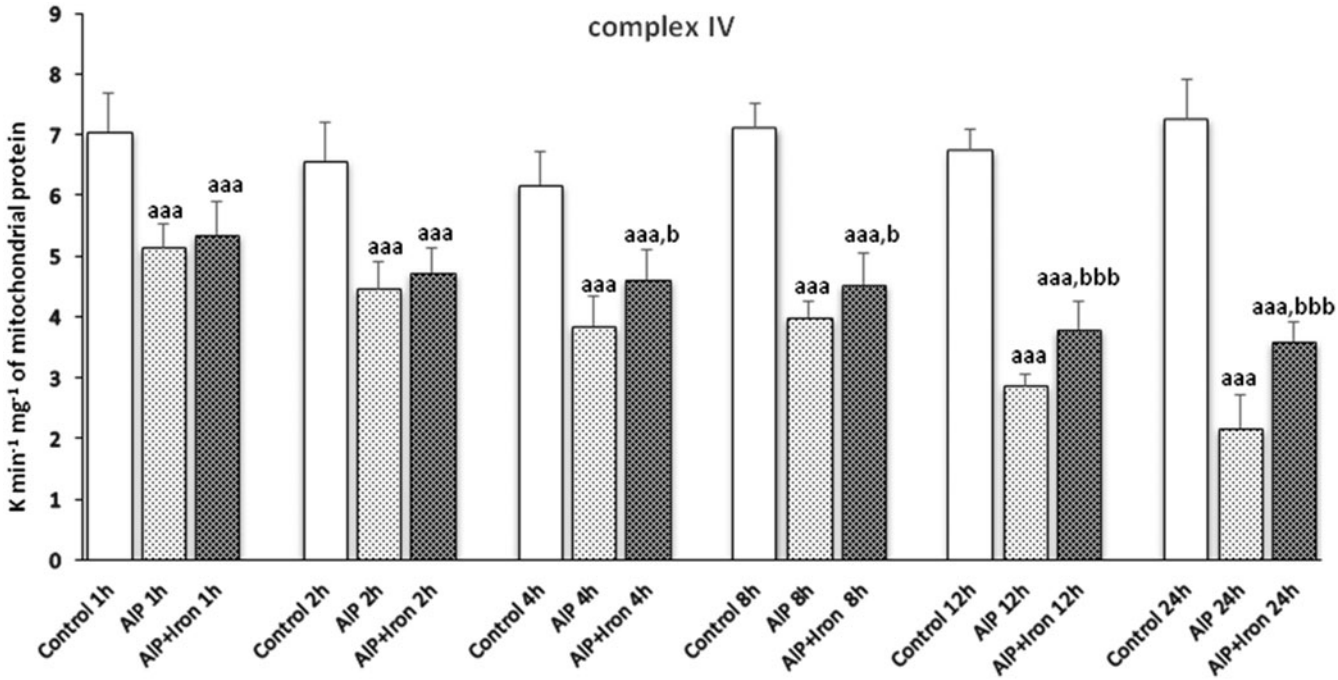

Figure 3. Changes in activity of cytochrome C oxidase in the cardiac muscle of rat in different groups. Data are mean ± SE of six animals in each group. The AlP and AlP + iron groups are compared with the same control group at six times (1, 2, 4, 8, 12 and 24 h). Significantly different from the control group at $p < 0.001$ (aaa); Significantly different from aluminum phosphide (AlP) group at $p < 0.001$ (bbb) and $p < 0.05$ (b).

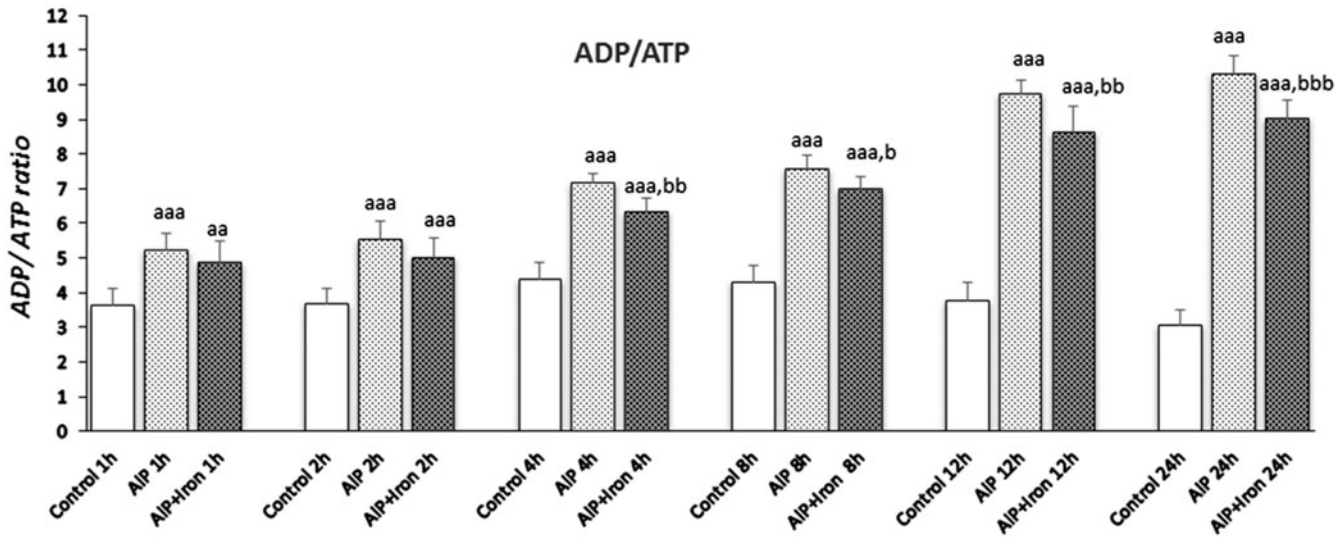

Figure 4. Changes in the rat cardiac muscle ADP/ATP ratio in different groups. Data are mean ± SE of six animals in each group. The AlP and AlP + iron groups are compared with the same control group at six times (1, 2, 4, 8, 12 and 24 h). Significantly different from the control group at $p < 0.001$ (aaa), $p < 0.01$ (aa), $p < 0.05$ (a); Significantly different from aluminum phosphide (AlP) group at $p < 0.001$ (bbb), $p < 0.01$ (bb), and $p < 0.05$ (b).

Apoptosis and necrosis by flow cytometry

Measurement of cell death was performed by flow cytometry using annexin-V-FITC and PI, staining phosphatidylserine and DNA residues, respectively. Flow cytometry results showed that the percentage of cell viability in AlP groups was time-dependently decreased in comparison with the same time point of control groups; whereas, it was shown in the treated groups after 2 h (Figure 5). The result also represented that the percent viability in AlP+Iron groups was significantly increased in comparison with the same time point of AlP groups after 12 h. Iron groups showed no significant difference compared with the controls [data were not shown (Figures 2–5)].

Discussion

The present results indicated that administration of AlP led to reducing in BP and heart rate, and subsequently prolonging in the QRS, P-R intervals and QT. ST depression associated with

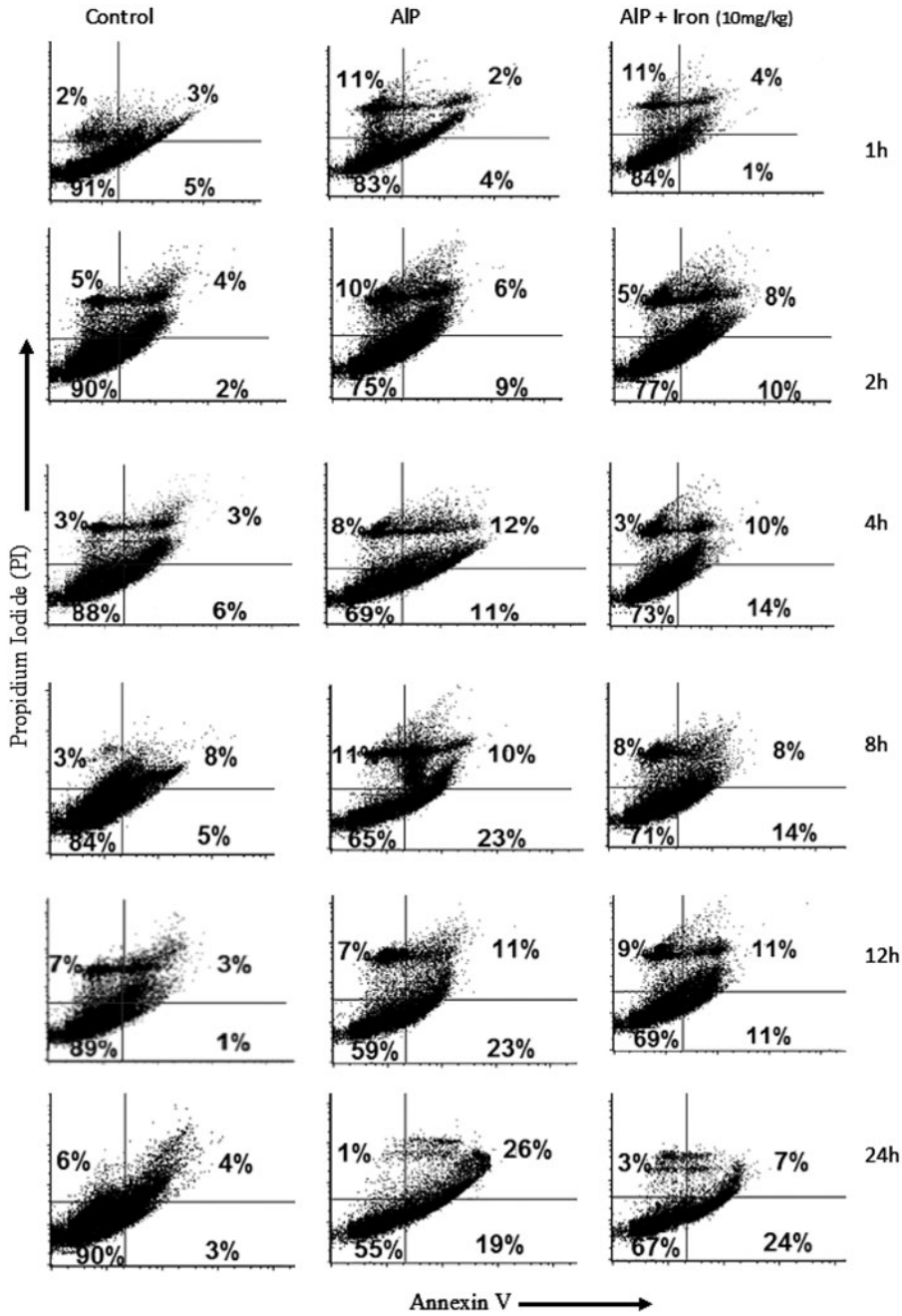

Figure 5. Flow cytometry of cardiac cells. The numbers at the bottom right quadrant of each dot plot represent the percentage of cells in earlyapoptosis (annexin V-positive, PI-negative). Numbers at the top right quadrant represent thepercentage of cells in late apoptosis and/or secondary necrosis (annexin V-positive, PI-positive). The data represent three separate experiments.

ischemia was also detected in AlP groups. Meanwhile, in the iron sucrose treated animals, BP, heart rate and P–R interval were similar to those in the control groups. Surprisingly, higher and lower doses of iron sucrose showed significant difference when compared with the control groups ($p < 0.05$) (Table 1). Due to the increment of BP and heart rate without any change in the P–R interval in iron sucrose (10 mg/kg) pilot study, a time course study was performed to identify the mechanism of action of phosphine and protective effects of iron sucrose.

According to the previous studies, it is expected to see the reduction of antioxidant power and increase of cellular lipid peroxidation by AlP. But, the present results confirmed that AlP-induced lipid peroxidation with no significant change at FRAP levels 2 h after exposure, even in the presence of antioxidants (Figure 2). This finding suggested the involvement of mitochondria as a vulnerable target of reactive lipid species and main source of ROS production (Karami-Mohajeri et al., 2013; Mehrpour et al., 2012).

To assess the involvement of mitochondria, mitochondrial electron transport chain function was evaluated after AlP exposure. The result showed that the activities of complexes II, IV and V significantly decreased in the heart mitochondria without change in the activity of mitochondrial complex I (NADH:ubiquinone oxidoreductase) following incubation of isolated heart mitochondria with AlP (Anderson et al., 2012; Bumbrah et al., 2012; Proudfoot, 2009; Woyda-Ploszczyca & Jarmuszkiewicz, 2012). It has been proven that complexes I, II and III are the main sites of the mitochondrial ROS generation involved in apoptosis (Grimm, 2013; Hosseini et al., 2014; Quinlan et al., 2012). The present results showed that AlP in the early hours of exposure (active complex)

decreased activities of mitochondrial complex II (SDH) and complex III (cytochrome bc1 complex). The most interesting finding was that complex IV (cytochrome C oxidase) was more vulnerable than other mitochondrial complexes to AlP toxicity, which was consistent with the findings of previous studies (Figure 3) (Luetjens et al., 2000; Singh et al., 2006). It is clear that interruption of electron transport in complex IV triggered inhibition of the chain activity and decreased electron transfer from complex IV.

Interruption of electron transfer in mitochondrial respiratory chain caused disruption of oxidative phosphorylation and then ameliorated ATP level and increased ADP/ATP ratio. Determining the ATP level and ADP/ATP ratio on isolated mitochondria is the only well-accepted biochemical approach that is able to distinguish between apoptotic and necrotic cell death. Thus, measurement of changes at ATP level and ADP/ATP ratio are important biochemical points for evaluating the interruption of mitochondrial respiratory chain (Hosseini et al., 2013). These results confirmed that impairment of ETC results in a reduced ability for ATP synthesis and increased mitochondrial ADP/ATP ratio (Figure 4). The present data might provide, an increase in ADP/ATP ratio in cells that could lead to ATP depletion and switching to necrosis, and would be expected to cause mitochondrial depolarization or MPT pores opening under critical conditions (e.g. brain injury, stroke and ROS) (Lemasters et al., 2009; Stavrovskaya & Kristal, 2005). These data suggested that exposure of the heart mitochondria to AlP interrupted electron transfer between mitochondrial complexes and protons in mitochondrial inter-membrane space, which led to the loss of proton gradient and finally, MPT pore opening and MMP decline. Therefore, catalytic activity of F1F0-ATP synthesis can reverse and be hydrolyzed to ADP and reduce the ATP level (Armstrong, 2006a,b; Hosseini et al., 2014). Under physiological conditions, the main source of ROS production is mitochondrial complexes I and III reinforced by impeding the flow of electrons (Jezek et al., 2010; Mráček et al., 2009). Then, electron transport into the final electron acceptor is inhibited by phosphine due to the high redox potential (Dua et al., 2010). Therefore, it seems that the primary target of phosphine is mitochondrial complexes IV and V and mitochondrial inter-membrane space due to the interruption of cell membrane potential, mitochondrial proton gradient, and ion cofactors (all of which are necessary for cell survival).

Mechanistically, it is proposed that phosphine toxicity is related to the redox potential in the mitochondrial electron transport chain by the interruption of membrane integrity with the release of cytochrome C and mitochondrial apoptosis factor to cytosolic space (Armstrong, 2006a,b; Dua & Gill, 2004). Considering the results, iron sucrose (as an electron receiver) can compete with mitochondrial respiratory chain complexes. Using an exogenic form of electron receiver (e.g. iron sucrose) diverts electrons from another pathway; therefore, Fe^{3+} was used in this experiment. Previous studies have indicated that iron sucrose in the selected doses has no role in inflammation, oxidative stress and lipid peroxidation (Vu'O'ng Le et al., 2011).

Strong relationship between ATP generation and cell viability has been reported in the literature. Therefore, ATP level is very important for cell survival and the present flow cytometry results demonstrated that an increase in the ADP/ATP ratio induced cell death. These results also demonstrated that phosphine could induce cardiac cell death in the first hours of exposure, which was probably due to a decrease in ATP content in cells in a time-dependent manner. Another important finding was an increase in the cell viability after 12 h of incubation with AlP (Figure 4), which was in agreement with previous studies, in which phosphine inhibits oxidative respiration by 70% and causes a severe drop in mitochondrial membrane potential (Proudfoot, 2009).

These findings supported that phosphine toxicity was related to alterations in metabolism, which plays a key role in cell death induction. Hence, inhibition of cell death by electron scavengers is possible. In the present study, iron sucrose was used as a ROS scavenger which did not lead to oxidative stress. Because of molecular weight and renal elimination, the iron sucrose complex did not get into Fenton reaction, which was the reason for its selection among iron complexes (Geisser, 1997; Legssyer et al., 2003).

It can be proposed that iron sucrose in a dose-dependent manner caused contradictory effects in the oxidative stress induction after exposure to phosphine. Further studies are required to clarify the role of iron sucrose in the increase of ATP content with more focus on electron receivers (e.g. Al^{+} and Cu^{+}).

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Declaration of interest

All authors declare no conflict of interest related to the present work.

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