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An electrocardiographic, molecular and biochemical approach to explore the cardioprotective effect of vasopressin and milrinone against phosphide toxicity in rats

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ABSTRACT

The present study was conducted to identify the protective effect of vasopressin (AVP) and milrinone on cardiovascular function, mitochondrial complex activities, cellular ATP reserve, oxidative stress, and apoptosis in rats poisoned by aluminum phosphide (AlP). Rats were divided into five groups (n = 12) including control, AlP (12.5 mg/kg), AlP + AVP (2.0 Units/kg), AlP + milrinone (0.25 mg/kg) and AlP + AVP + milrinone. After treatment, the animals were connected to an electronic cardiovascular monitoring device to monitor electrocardiographic (ECG) parameter. Finally, oxidative stress biomarkers, mitochondrial complex activities, ADP/ATP ratio and apoptosis were evaluated on the heart tissues. Results indicated that AlP administration induced ECG abnormalities along with a decline in blood pressure and heart rate. AVP and milrinone significantly ameliorated these changes in all treated groups. Considerable protective effects on oxidative stress biomarkers, complex IV activity, ADP/ATP ratio and caspase-3 and -9 activities in treated groups were also found. These findings were supported by flow cytometry assay of cardiomyocytes. In conclusion, administration of AVP and milrinone, not only improve cardiovascular functions in AlP poisoned rats in the short time, but after a long time can also restore mitochondrial function and ATP level and reduce the oxidative damage, which prevent cardiomyocytes from entering the apoptotic phase.

1. Introduction

Aluminum phosphide (AlP), a solid fumigant insecticide and rodenticide, is usually used to protect food products from pest during the storage and transportation processes (Bumbrah et al., 2012; Mostafazadeh, 2012; Proudfoot, 2009). This agent is known as rice tablet in Iran and extensively used by farmers despite restricted sale (Mehrpour et al., 2012; Moghhadamnia, 2012; Mostafalou et al., 2013). This may be due to its special properties such as being highly potent against all stages of insects and cost beneficial, having no effect on seed viability, and leaving little residue on food products (Anand et al., 2011; Bumbrah et al., 2012; Moghhadamnia, 2012). Despite all good properties, it is very dangerous for non-targeted species such as humans so that more than 70% of the acute intoxicated patients die following AlP ingestions (Anand et al., 2011; Mostafalou et al., 2013; Singh et al., 1989). Poisonous effects of AlP tablet are due to fatal phosphine gas released when it comes into contact with water or hydrochloric acid in the stomach (Gurjar et al., 2011; Moghhadamnia, 2012). The exact mechanism of phosphine is still unknown; however, the results of some animal studies showed that oxidative stress, inhibition of cytochrome oxidase and cellular oxygen utilization in mitochondria, denaturation of oxyhemoglobin molecule and interfering with several enzymes or ion channels are plausible mechanisms of AlP toxicity (Anand et al., 2011; Mehrpour et al., 2012; Moghhadamnia, 2012; Mostafazadeh, 2012; Nath et al., 2011). The signs and symptoms of phosphine toxicity are nonspecific and most organs are affected and usually result to multi-organ failure (Bumbrah et al., 2012; Gurjar et al.,

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2011). However, the heart is the predominantly affected organ and most of intoxicated patients die of cardiovascular complications and intractable hypotension (Bogle et al., 2006; Chugh et al., 1991; Moghhadamnia, 2012; Mostafazadeh, 2012). Severe and refractory hypotension usually occurs following many poisonings and overdoses.

Conventional vasopressors, especially those having direct effects on alpha receptors, may not regularly improve hypotension in the setting of acute poisonings. There are several reports indicating that vasopressin (AVP) has had beneficial effects on severe hypotension and shock. Barry et al. have reported about the successful use of intravenous AVP in a case of a patient who had ingested a bottle of amitriptyline and showed hypotension unresponsive to conventional vasopressors and pH manipulation (Barry et al., 2006). In patients with massive calcium channel blockers and caffeine overdoses, AVP was successfully used to treat refractory hypotension which was unresponsive to calcium, glucagon, insulin, and conventional vasopressor therapies (Holstege et al., 2003; Kanagarajan et al., 2007). Also, low-dose AVP leads to significant increases in vascular tone in septic shock and in late vasodilated hemorrhagic shock and improves response to infused catecholamines (such as norepinephrine) (Russell, 2007). This hormone probably causes vasoconstriction and thereby increases systemic vascular resistance and blood pressure by the two main mechanisms. First, activation of V_1 receptors in vascular smooth muscles by AVP increases cytoplasmic Ca^{2+} through the phosphotidyl-inositol-biphosphate (PIP2) cascade. Second, blockage of K_{ATP} channels within the smooth muscle cell membrane by AVP facilitates myocyte depolarization and thus vasoconstriction (Dünser et al., 2001; Russell, 2007).

Another complication of AlP intoxication is cardiac dysfunctions such as lethal heart failure. Phosphodiesterase III inhibitors, especially milrinone, can potentially improve the hemodynamic status of acute heart failure in the setting of acute poisoning because these agents have potent inotropic effects via the increase of intracellular cAMP levels in myocytes (Feneck, 2007; Lescan et al., 2013; Rahimi et al., 2010). These inotropic compounds increase myocardial contractile force with less cardiac oxygen demand than catecholamines (Monrad et al., 1986; Satoh and Endoh, 1990; Tosaka et al., 2007). Unlike catecholamines, phosphodiesterase III inhibitors increase the intracellular cAMP levels by non-adrenergic pathways (Lescan et al., 2013; Monrad et al., 1986; Yano et al., 2000).

It was hypothesized that milrinone, a phosphodiesterase III inhibitor, might improve myocardial contractility accompanied by AVP induced vasoconstriction and correction of hypotension in acute AlP poisonings. The present study, therefore, aimed to investigate the protective effect of AVP and milrinone alone and in combination on hemodynamic (BP, HR, ECG), molecular and biochemical properties (oxidative stress biomarkers, mitochondrial complex activities, ADP/ATP ratio and apoptosis) in a rat model of AlP poisoning.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (GmbH, Munich, Germany) unless otherwise mentioned. AlP from Samiran Pesticide Formulating Co. (Tehran, Iran), Primacor® (Milrinone) from Sepaco Darou Pharmaceutical Center Ssk (Tehran, Iran), and Hypress® (Arginine Vasopressin) from Exir Pharmaceutical Co. (Tehran, Iran) were used in this study. The mitochondria isolation kit was purchased from BioChain Inc. (Newark, New Jersey, USA). Annexin V-FITC/PI was obtained from Beijing Biosea Biotechnology Co, Ltd (Beijing, China). ELISA kits for oxidative stress biomarkers from Cayman Chemical Co. (Michigan, USA) were used in this study.

2.2. Study design and methods

2.2.1. Animals

All experiments were done on animals, according to the ethical guidelines on the use of animals and were approved by the Ethics Committee of Tehran University of Medical Sciences with code number 92-01-33-21796. All male Wistar rats weighing 200–250 g were obtained from animal house of Faculty of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran) and housed in controlled environmental conditions of 20 to 25 °C temperature, relative humidity (50–55%), and 12-h light/dark cycle with free access to stock laboratory diet and water.

2.2.2. Determination of AlP LD50

Based on previous studies, AlP LD50 includes a wide range (8.7–12 mg/kg). This may be due to the continuous decomposition of AlP during shelf life. Thus, it was necessary to determine the LD50 of AlP for each experiment. The doses more than 15 mg/kg of AlP usually results in 100% mortality and no mortality is observed in doses less than 8 mg/kg; hence doses ranging between 8 and 15 mg/kg were used and dissolved in almond oil and administered to rats by gavage. Control group received only equivalent amount almond oil. Four rats per group were used at each dose level. Twenty four hours after treatment, mortality was recorded. Finally, AlP LD50 was determined at 12.51 mg/kg by using probit (Baeeri et al., 2013).

2.2.3. Study design

A pilot experiment was designed to determine the optimum dose of AVP and milrinone in rats poisoned with an LD50 dose of AlP. Five doses of AVP (1.0, 2.0, 3.0, 4.0, 8.0 U/kg) and milrinone (0.125, 0.25, 0.5, 0.75, 1.0 mg/kg) were chosen and based on hemodynamic parameters (electrocardiogram [ECG], Blood pressure [BP], heart rate [HR]); the optimum doses were determined. These doses, which decrease cardiovascular complications of AlP with less adverse effects, were used in the next step of the study. After determining LD50 of AlP (12.5 mg/kg) and optimum doses of AVP (2.0 U/kg) and milrinone (0.25 mg/kg), the animals were randomly categorized into five groups of twelve rats each, including group 1 (control), group 2 (AlP), group 3 (AlP + AVP), group 4 (AlP + milrinone), group 5 (AlP + AVP + milrinone). AlP was dissolved in almond oil and administered by gavage. AVP and milrinone were dissolved in saline and administered intraperitoneally. Control animals received only almond oil in appropriate volume. Each group was further divided into two subgroups of six rats each. In one group hemodynamic parameters were recorded while another group was sacrificed at 24 hours after treatment for biochemical studies (oxidative stress analyses, mitochondrial complex activity analyses, ADP/ATP ratio, caspase-3,-9 activity assays and flow cytometry assays). To measure hemodynamic parameters, AlP (LD50 dose) was administered intra-gastrically to all animals except the control. After 30 min, the animals were anesthetized by intraperitoneal injection of ketamine/xylazin (60/6 mg/kg) which was repeated at 30/3 mg/kg post 45 min, 1.5 h and 2.5 h, respectively, to maintain full general anesthesia until the completion of the experiment (3.0 h). After induction of anesthesia, the animal was quickly connected to a PowerLab system (PowerLab 4/35 Data Acquisition Systems, AD Instruments, Australia) to monitor electrocardiogram (ECG), blood pressure (BP), and heart rate (HR), noninvasively. AVP and milrinone were administered by intraperitoneal injection 60 min after AlP administration. But for biochemical studies, 24 hours after treatments, animals were sacrificed and the heart was dissected out and rinsed in ice-cold saline to remove the blood and immediately frozen and stored at −80 °C for various biochemical assays. It should be noted that for biochemical assays, 0.25 median lethal dose [LD50] of AlP was administered by gavage to all animals except the control group, and treatment groups received AVP and milrinone after 60 min. Based on our previous studies, this dose of AlP can induce cardiotoxicity without mortality (Baeeri et al., 2013) and we needed all animals alive after 24 hours to assay the cardioprotective effects of AVP and milrinone in rats poisoned with AlP.

2.2.4. ECG, BP, and HR

After induction of anesthesia, the electrodes were subcutaneously connected to the right hand and both right and left paws of the immobilized rat, the ECG data were obtained for 3 hours. The obtained data were analyzed by PowerLab system software and QRS complexes and the segments of QTc, PR, and ST were measured. In addition to the ECG, the systolic BP and HR was recorded by the tail cuff of PowerLab which was connected to the rat's external tail where the pulse was detected.

2.2.5. Tissue sampling and mitochondrial isolation

As mentioned above, twenty four hours after treatments, the animals were sacrificed and the heart was removed and rinsed in ice-cold saline to remove the blood. The heart tissue was divided into several sections. A small section of tissues (100 mg) was taken for mitochondrial complex assays and the rest of the tissues were stored in −80 °C for other various biochemical studies. For preparation of heart mitochondria, 100 mg of heart tissues was processed according to mitochondria isolation kit protocol.

2.2.6. Determination of NADH dehydrogenase activity

The principle of this assay is based on the consumption of NADH, which passes electrons to complex I, which are then passed to synthetic ubiquinone, as the electron acceptor. NADH dehydrogenase (complex I) activity was assayed in heart homogenate according to the method of Sherwood and Hirst (2006). The mitochondria (100–200 μg of total mitochondrial protein) were added to the reaction mixture containing potassium phosphate buffer (25 mM; pH = 7.4), 25% bovine serum albumin, magnesium chloride (MgCl2; 5 mM), decylubiquinone (2.8 mM), NADH (5.7 mM), antimycin A (3.7 mM), and potassium cyanide (KCN; 2 mM) to start the reaction and the alteration in NADH absorbance was measured at 340 nm for 3 min before the addition of rotenone. After adding rotenone (0.36 mM) to the reaction mixture, the

rotenone-insensitive activity of NADH-cytochrome b oxidoreductase was determined. Finally, the rotenone-insensitive activity was subtracted from total activity to determine the overall net rate. The results were expressed as nanomoles of NADH per minute per milligram of mitochondrial protein (nmol/min/mg protein).

2.2.7. Determination of succinate dehydrogenase activity

Succinate dehydrogenase (Complex II) activity was measured by following the decrease in absorbance due to the oxidation of 2, 6-dichlorophenolindophenol (DCPIP) at 600 nm. Briefly, the mitochondria (10–50 μg of total mitochondrial protein) were added in potassium phosphate buffer (25 mM; pH 7.2) containing MgCl2 (5 mM), and Succinate (20 mM) and incubated for 10 min at 30 °C. Then antimycin A, rotenone, KCN, and DCPIP were added and the baseline was noted for 3 min. The reaction was initiated by adding ubiquinone (65 mM), and the enzyme-catalyzed reduction of DCPIP is measured for 3–5 min (Karami-Mohajeri et al., 2013a). The succinate dehydrogenase activity was calculated using a DCPIP standard curve and was expressed as nmol DCIP/min/mg of mitochondrial protein.

2.2.8. Determination of cytochrome-c oxidase activity

Cytochrome-c oxidase (Complex IV) activity was measured in heart homogenate as per the method of Cooperstein and Lazarow (1951). Briefly, the reaction was started by the addition of 5–15 μg of mitochondrial protein to a solution containing reduced cytochrome c and lubrol PX (0.45 mM) in potassium phosphate buffer (25 mM), pH = 7.4 and then the decrease in absorbance was recorded at 550 nm for 3–6 min. The results were expressed as the natural logarithm of the absorbance divided by time and reported as the first-order rate constant (k) per minute per milligram of mitochondrial protein (K/min/mg mitochondrial protein).

2.2.9. Measurement of cardiac ADP/ATP ratio

ADP/ATP ratio was evaluated in heart tissue according to the method of Hosseini et al. (2010). The heart tissue (100 mg) was homogenized in 1 ml of an ice-cold 6% trichloroacetic acid (TCA) and then the homogenate was centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was neutralized to a pH 6.5 with potassium hydroxide (4 M). After that it was filtered through a Millipore filter (pore size 0.45 mm), and the neutralized extract was used to determine the concentrations of ATP and ADP (μg/ml per mg of tissue) using ion pair-high performance liquid chromatography (IP-HPLC).

2.2.10. Determination of catalase (CAT) activity

CAT activity was determined in supernatants of heart homogenates by measuring the absorbance decrease at 240 nm in a reaction medium containing 10 mM H_2O_2, and 50 mM sodium phosphate buffer (pH 7.0). One unit of the enzyme is defined as 1 mol H_2O_2 consumed/min, and the specific activity is reported as units/mg protein (Ranjbar et al., 2010).

2.2.11. Determination of superoxide dismutase (SOD) activity

Level of SOD activity was determined according to the kit protocol. Xanthine and xanthine oxidase generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and produce a red formazon dye. The SOD activity is measured by the degree of inhibition of this reaction. One unit of SOD activity was defined as the amount of enzyme that inhibits the rate of INT reduction by 50%. The results are expressed as unit/mg of protein (Pourkhalili et al., 2011).

2.2.12. Measurement of lipid peroxidation (LPO)

Malondialdehyde (MDA) is an end product of the cellular lipid peroxidation that reacts with thiobarbituric acid (TBA) to generate a complex that can be measured spectrophotometrically, named as TBA reactive substances (TBARS). The supernatant was mixed with TBA (0.67% w/v) and incubated for 30 min in boiling water bath. The resulting TBARS adducts were extracted with n-butanol by vigorous shaking. Then the solution was centrifuged and cooled, and absorbance was recorded at 532 nm. The method was calibrated with tetraethoxypropane standard solutions (Kei, 1978; Ranjbar et al., 2010).

2.2.13. Measurement of reactive oxygen species (ROS)

The measurement of ROS production was carried by the use of 2′,7′-dichlorofluorescin diacetate (DCF-DA), which is converted into highly fluorescent DCF by cellular peroxides. The supernatants were incubated with 5 μM DCF-DA at 37 °C for 30 min in the dark. Then fluorescence was recorded with 488 nm excitation and 525 nm emission using a fluorometer (Momtaz et al., 2010).

2.2.14. Determination of total thiol molecules

Total thiol levels of supernatants were determined as described previously (Hu, 1994; Mohammadi et al., 2011a). Briefly, a volume of supernatant (10 μL) was mixed with 200 μL of Tris–EDTA buffer (Tris base [0.25 M], EDTA [20 mM], pH 8.2) and then mixed with 4 μL of DTNB (5,5-dithiobis-2-nitrobenzoic acid) (10 mM) in methanol. After incubation at 37 °C for 30 min, the color appeared. The absorbance of the supernatant was measured against a blank at 412 nm.

2.2.15. Caspase-3 and -9 activity assay in heart tissue

Caspase-3 and -9 activities were measured in heart samples by colorimetric assays. The principle of these assays is based on the ability of caspase 3 and -9 to hydrolyze respectively Ac-DEVD-pNA and Ac-LEHD-pNA and release pNA (p-nitroaniline). Cleavage of the pNA from these specific substrates produces a yellow color which is monitored by an ELISA reader at 405 nm. The amount of yellow color produced upon cleavage is proportional to the amount of caspase activity present in the heart sample. Briefly, an appropriate amount of the heart tissue was weighed out and rinsed in ice-cold saline to remove the blood. Then the tissue minced and homogenized in lysis buffer containing MgCl2 (2 mM), KCl (50 mM), EDTA (2 mM), Triton X100 (%1), HEPES (50 mM; pH = 7.4) and centrifuged at 12,000 *g* for 20 min. A 50-ml volume of the supernatant was incubated in caspase buffer (100 mM HEPES, pH 7.4, 20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol) containing 100 mM of caspase-3 and -9 specific substrate ((Ac-DEVD-pNA and Ac-LEHD-pNA), respectively) for 4 hours at 37 °C. Then, absorbance was measured at 405 nm (Hosseini et al., 2013). Results were calibrated with known concentrations of pNA and expressed in nanomoles per pNA released per hour per milligram of protein (nanomoles per hour milligrams of protein).

2.2.16. Flow cytometry detection of apoptosis and necrosis

At first, cardiomyocytes were isolated from heart tissue by the method of Schlüter and Schreiber (2005). Then cells were stained with annexin V-FITC and propidium iodide (PI) according to the kit protocols and cardiomyocytes were analyzed by flow cytometry (Apogee Flow System, UK) (Krifka et al., 2012).

2.3. Statistical analysis

All values are expressed a mean ± standard error of the mean (SEM). Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test for multiple comparisons. A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. ECG, HR and BP

In general, administration of AVP and milrinone alone and especially co-administration of both drugs had a beneficial effect on hemodynamic parameters after AlP exposure. BP drastically dropped in AlP group through 60–180 min interval and HR initially increased followed by progressive bradycardia (Tables 1 and 2). The ECG analysis showed noticeable abnormalities, including QRS widening, ST elevation, QTc and PR interval prolongation in AlP group through 30–180 sections compared with control group (Table 3 and Fig. 1). Administration of AVP alone and in combination

Table 1
Changes in blood pressure in various groups.

Time (min)	0–30	30–60	60–90	90–120	120–150	150–180
Control	86.97 ± 1.08	82.715 ± 2.42	94.52 ± 3.97	99.33 ± 1.21	102.19 ± 2.53	101.8 ± 0.48
ALP	90.38 ± 7.15	75.92 ± 6.23	61.25 ± 9.42[a]	48.12 ± 0.39[a]	53.72 ± 5.11[a]	54.25 ± 0.75[a]
AVP + AlP	79.9 ± 6.28	114.6 ± 7.76[a,b]	121.5 ± 6.41[a,b]	119.6 ± 12.07[b]	111.5 ± 6.62[b]	98.1 ± 4.88[b]
Milrinone + AlP	82.0 ± 9.87	74.6 ± 4.85	73.7 ± 4.67[a]	77.5 ± 3.89[b]	74.44 ± 2.26[a,b]	79.62 ± 1.04[a,b]
Milrinone + AVP + AlP	82.33 ± 6.96	107.2 ± 9.11[b]	96.55 ± 8.97[b]	89.33 ± 2.16[b]	91.4 ± 2.7[b]	86.25 ± 3.95[a,b]

Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).

[a] Significantly different from the control group at $p < 0.01$.

[b] Significantly different from the AlP group at $p < 0.01$.

Table 2
Changes in heart rate in various groups.

Time (min)	0–30	30–60	60–90	90–120	120–150	150–180
Control	335.22 ± 1.79	367.3 ± 4.21	382.7 ± 1.99	391.4 ± 4.21	389.8 ± 1.68	376.33 ± 7.84
AlP	292.7 ± 11.21	308.3 ± 6.61	269.21 ± 4.15[a]	260.66 ± 5.74[a]	188.83 ± 21.3[a]	171.51 ± 9.46[a]
AVP + AlP	320.71 ± 15.12	253.08 ± 13.91[a]	249.3 ± 6.16[a,b]	250.98 ± 1.96[a,b]	261.05 ± 2.44[a,b]	285.86 ± 13.06[a,b]
Milrinone + AlP	302.51 ± 23.52	355.55 ± 11.76[b]	321.81 ± 5.93[a,b]	316.83 ± 6.84[a,b]	341.93 ± 2.69[a,b]	361.08 ± 8.72[b]
Milrinone + AVP + AlP	319.65 ± 14.1	290.58 ± 20.34[a]	358.56 ± 1.91[a,b]	347.95 ± 8.01[a,b]	335.1 ± 5.49[a,b]	351.93 ± 9.42[b]

Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).
[a] Significantly different from the control group at $p < 0.01$.
[b] Significantly different from the AlP group at $p < 0.01$.

with milrinone showed a dramatic increase in BP as compared to AlP group while milrinone alone could not improve BP. Administration of milrinone and in combination with AVP led to a significant increase in HR in comparison with the AlP group while AVP initially decreased HR followed by progressive increase. The prolongation of QTc and PR interval and broadening of QRS were reduced in all AVP and milrinone treated groups, as compared to AlP group. Administration of AVP and milrinone markedly stabilized ST segment changes after intragastric administration of AlP.

3.2. Activity of mitochondrial respiratory complexes

In order to analyze the cardiac mitochondrial function, the activity of each mitochondrial complex was separately evaluated. As illustrated in Table 4, no significant changes were observed in complex I and II activities in all treated groups. Complex IV activity noticeably decreased in AlP group in comparison with control group ($p < 0.01$). Except milrinone (milrinone + AlP) treated group, all treated groups significantly increased complex IV activity compared to AlP group. However, AVP treated group still differed significantly with control group in terms of complex IV activity (Table 4).

3.3. Measurement of cardiac energy as ADP/ATP ratio

ADP/ATP ratio noticeably increased in AlP treated group in comparison with the control group ($p < 0.01$). Administration of AVP alone and in combination with milrinone significantly decreased the ADP/ATP ratio compared to AlP group, whereas milrinone alone could not significantly reduce the ADP/ATP ratio in rats poisoned with AlP (Table 4).

3.4. Antioxidant enzyme (CAT and SOD) activity in heart tissue

The activity of the CAT enzyme in cardiac tissue significantly decreased in AlP group when compared to control group ($p < 0.01$). Administration of AVP and milrinone alone had no significant effect on CAT activity after AlP exposure, but co-administration of both drugs showed significant increase in CAT activity when compared

Table 3
Changes in ECG parameters of various groups.

Time	Variable	Control	AlP	AVP + AlP	Milrinone + AlP	Milrinone + AVP + AlP
0–30	QRS (ms)	13.9 ± 0.19	16.43 ± 0.94[a]	16.96 ± 0.75[a]	17.5 ± 1.76[a]	17 ± 0.63[a]
	QTc (ms)	94.38 ± 2.69	108.83 ± 6.96	93.44 ± 2.31	117.9 ± 3.66[a]	110.31 ± 2.12
	PR (ms)	41.97 ± 0.23	48.98 ± 2.09	53.43 ± 1.86[a]	52.36 ± 3.51[a]	50.86 ± 1.81
	ST (μv)	39.61 ± 2.09	73.14 ± 20.76	63.64 ± 7.97	94.81 ± 5.53[a]	78.66 ± 3.67
30–60 min	QRS (ms)	14.4 ± 0.23	20.31 ± 0.32[a]	17.3 ± 0.45[a,b]	19.16 ± 0.41[a]	18.8 ± 0.54[a]
	QTc (ms)	86.09 ± 3.01	179.05 ± 11.03[a]	150.94 ± 6.97[a,b]	146.97 ± 1.51[a,b]	143.78 ± 3.51[a,b]
	PR (ms)	43.17 ± 0.98	53.97 ± 2.19[a]	55.93 ± 1.48[a]	56.7 ± 0.9[a]	55.23 ± 2.61[a]
	ST (μv)	34.78 ± 2.59	172.61 ± 6.25[a]	93.86 ± 5.48[a,b]	110.46 ± 6.75[a,b]	97.07 ± 8.23[a,b]
60–90 min	QRS (ms)	14.2 ± 0.21	20.75 ± 0.46[a]	16.5 ± 0.55[b]	19.5 ± 0.54[a]	18.2 ± 0.95[a,b]
	QTc (ms)	99.62 ± 2.12	177.36 ± 3.82[a]	148.84 ± 1.29[a,b]	155.71 ± 1.61[a,b]	141.63 ± 1.33[a,b]
	PR (ms)	42.89 ± 0.45	55.92 ± 1.75[a]	52.34 ± 1.12[a]	55.98 ± 1.21[a]	55.59 ± 2.54[a]
	ST (μv)	41.24 ± 3.46	173.94 ± 5.18[a]	74.98 ± 3.47[a,b]	122.32 ± 4.22[a,b]	66.87 ± 3.74[a,b]
90–120 min	QRS (ms)	14.8 ± 0.18	22.33 ± 1.78[a]	17 ± 0.73[b]	19 ± 0.63[a]	17.16 ± 0.41[b]
	QTc (ms)	111.98 ± 1.94	179.51 ± 2.27[a]	139.76 ± 1.99[a,b]	165.98 ± 0.85[a,b]	132.96 ± 0.91[a,b]
	PR (ms)	41.28 ± 0.53	60.54 ± 2.52[a]	49.2 ± 3.47[b]	56.41 ± 1.26[a]	52.58 ± 3.21[a]
	ST (μv)	34.75 ± 3.37	167.21 ± 4.02[a]	68.61 ± 2.03[a,b]	98.26 ± 6.29[a,b]	46.86 ± 5.18[a,b]
120–150 min	QRS (ms)	15.7 ± 0.24	24.13 ± 0.79[a]	17.66 ± 1.94[b]	19.66 ± 0.52[b]	17.33 ± 0.51[b]
	QTc (ms)	99.15 ± 2.92	189.61 ± 6.18[a]	152.4 ± 2.55[a,b]	166.16 ± 1.45[a,b]	129.06 ± 1.29[a,b]
	PR (ms)	43.92 ± 0.88	59.84 ± 2.0[a]	48.35 ± 2.53[b]	55.29 ± 1.31[a]	49.79 ± 2.38[b]
	ST (μv)	25.37 ± 3.12	163.45 ± 3.57[a]	73.63 ± 2.11[a,b]	82.05 ± 5.06[a,b]	41.99 ± 1.93[a,b]
150–180 min	QRS (ms)	15.1 ± 0.23	24.36 ± 1.41[a]	17.3 ± 0.57[b]	20.63 ± 0.51[a,b]	17.5 ± 0.54[b]
	QTc (ms)	90.73 ± 2.65	227.46 ± 3.81[a]	149.31 ± 1.46[a,b]	163.66 ± 1.76[a,b]	126.31 ± 0.42[a,b]
	PR (ms)	41.75 ± 0.49	61.92 ± 1.18[a]	48.12 ± 3.3[b]	56.03 ± 0.51[a]	46.77 ± 3.96[b]
	ST (μv)	27.71 ± 2.78	164.65 ± 5.18[a]	61.43 ± 4.4[a,b]	76.85 ± 2.64[a,b]	47.83 ± 3.11[a,b]

Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).
[a] Significantly different from the control group at $p < 0.01$.
[b] Significantly different from the AlP group at $p < 0.01$.

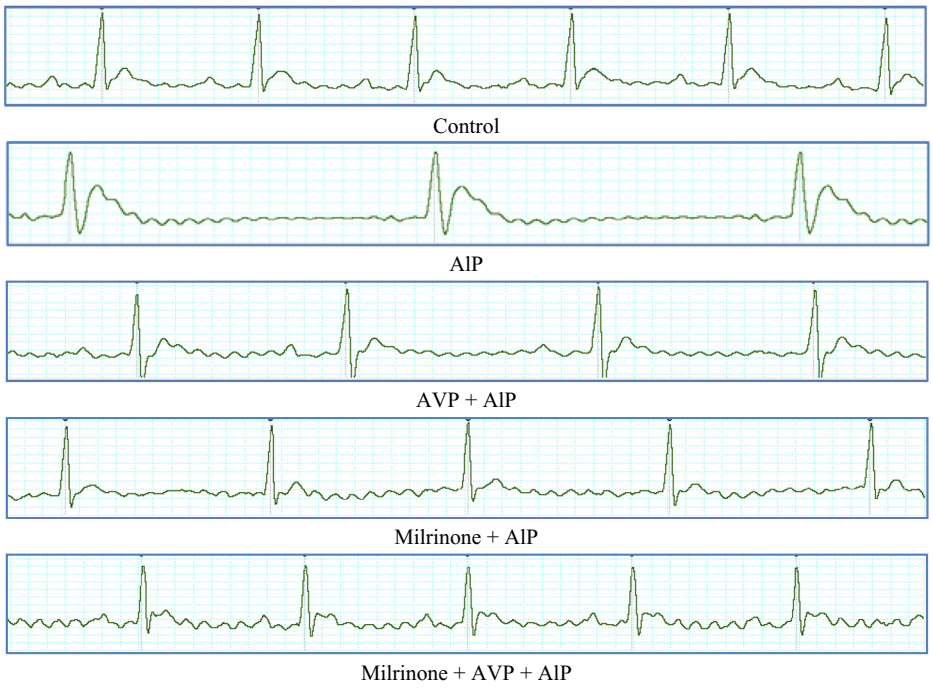

Fig. 1. Changes in ECG parameters in various groups. The control group received almond oil alone; AlP group (0.25 LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + AVP group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).

to AlP group ($p < 0.01$). However, insignificant changes were noted in SOD activity when all groups were compared (Table 5).

3.5. Oxidative stress biomarkers

Lipid peroxidation (tissue MDA levels) in animals treated with AlP was significantly higher than controls ($p < 0.01$). Co-administration of both the drugs significantly reduced the MDA levels in heart tissue; however, none of AVP and milrinone alone showed significant reduction in MDA level as compared to AlP ($p < 0.01$). AlP exposure decreased the antioxidant power in heart tissue, compared to control ($p < 0.01$). The levels of total thiol molecules in cardiac tissue were significantly reduced in AlP group in comparison with control group ($p < 0.01$). In groups where AVP and milrinone were administrated alone, total thiol molecules did not change significantly. However, co-administration of both drugs caused a significant increase in total thiol levels when compared to AlP group ($p < 0.01$). AlP exposure led to a remarkable increase in cardiac ROS compared to control group ($p < 0.01$). Administration of AVP alone and in combination with milrinone significantly reduced cardiac ROS as compared to AlP group ($p < 0.01$, Table 6).

3.6. Caspase-3 and 9 activities

Caspase-3 and 9 activities in heart tissue significantly increased following AlP poisoning compared to control group ($p < 0.01$). Administration of AVP alone and in combination with milrinone demonstrated a significant reduction in the activity of caspase 9 in comparison to AlP group, but milrinone alone could not significantly change caspase 9 activity in rats poisoned with AlP. AVP and milrinone significantly decreased caspase-3 activity in all treated groups compared to AlP group; however, these drugs could not reduce the activity of this caspase to control group level (Fig. 2).

3.7. Apoptosis and necrosis detection analysis by flow cytometry

Cardiomyocyte apoptosis and necrosis were assessed by annexin V-FITC/PI staining. Necrotic (annexin V-/PI+) and late apoptotic cells (annexin V+/PI+) appeared in quadrant A and B, respectively; and quadrant C and D were indicative of viable (annexin V–/PI–) and early apoptotic cells (annexin V+/PI–), respectively. As shown in Figs. 3 and 4, there was a high level of viable cells in the control condition. Employment of AlP induced a significant decrease in

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

	Control	ALP	AVP + AlP	Milrinone + AlP	Milrinone + AVP + AlP
Complex IV (K/min/mg)	412.33 ± 4.87	293.19 ± 10.79[a]	341.43 ± 5.41[a,b]	309.82 ± 11.87[a]	383.98 ± 9.08[b]
Complex I (nmol/min/mg)	232.65 ± 4.34	206.51 ± 11.97	214.19 ± 7.84	213.17 ± 3.66	235.08 ± 11.12
Complex II (nmol/min/mg)	79.21 ± 3.95	63.65 ± 8.93	70.27 ± 7.38	66.27 ± 5.46	74.29 ± 4.52
ADP/ATP ratio	2.14	4.92[a]	3.65[a,b]	4.11[a]	3.22[b]

Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (0.25 LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP (+vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).

[a] Significantly different from the control group at $p < 0.01$.

[b] Significantly different from AlP group at $p < 0.01$.

Table 5
Effects of various treatments on heart catalase (CAT) and superoxide dismutase (SOD).

Group	CAT activity (U/mg protein)	SOD activity (U/mg protein)
Control	23.19 ± 1.19	0.193 ± 0.01
ALP	18.08 ± 2.83[a]	0.211 ± 0.02
AVP + AlP	19.17 ± 2.03[a]	0.205 ± 0.012
Milrinone + AlP	20.85 ± 2.15	0.207 ± 0.024
Milrinone + AVP + AlP	21.83 ± 1.81[b]	0.214 ± 0.011

Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (0.25LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).
[a] Significantly different from the control group at $p < 0.01$.
[b] Significantly different from the AlP group at $p < 0.01$.

Table 6
Effects of various treatments on oxidative stress biomarkers in heart tissue.

Group	*LPO (μm/mg protein)	**TTM (nmol/mg protein)	#ROS (U/mg protein)
Control	0.328 ± 0.04	0.137 ± 0.006	24.04 ± 4.88
ALP	0.850 ± 0.05[a]	0.095 ± 0.003[a]	74.58 ± 5.78[a]
AVP + AlP	0.667 ± 0.01[a,b]	0.109 ± 0.009[a]	48.17 ± 7.43[b]
Milrinone + AlP	0.722 ± 0.02[a]	0.104 ± 0.002[a]	57.35 ± 5.95[a]
Milrinone + AVP + AlP	0.640 ± 0.02[a,b]	0.118 ± 0.003[a,b]	42.83 ± 0.61[b]

Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (0.25 LD50) received only aluminium phosphide; AlP + AVP group received AlP (LD50) + vasopressin (2.0 U/kg); AlP + milrinone group received AlP (LD50) + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg).
* LPO: Lipid peroxidation.
** TTM: Total thiol molecules.
# ROS: Reactive oxygen species.
[a] Significantly different from the control group at $p < 0.01$.
[b] Significantly different from the AlP group at $p < 0.01$.

cardiomyocyte viability of more than 30%. Administration of AVP and milrinone in treated group increased cell viability. There was no significant difference among groups in terms of necrotic cells. But early and late apoptosis decreased in the treated group in proportion to the cell viability. Although administration of AVP and milrinone caused significant increase in cell viability and decrease in early and late apoptosis compared to AlP group, there was still a significant difference between treated groups and control group.

4. Discussion

The present study was conducted to evaluate the positive role of AVP and milrinone in acute AlP toxicity through protection of cardiovascular system, prevention of oxidative stress and apoptosis, restoration of mitochondrial complex activities and cellular ATP reserve.

In this study, AlP exposure produced significant changes in cardiovascular functions, such as severe drop in BP and HR. Severe hypotension, which is unresponsive to conventional treatments, is one of the main causes of death following AlP poisoning (Anand et al., 2011; Bayazıt et al., 2000; Chugh et al., 1991; Karami-Mohajeri et al., 2013b; Ragone et al., 2002; Singh et al., 1989, 1996). To solve this problem, AVP was chosen and administrated to rats poisoned by AlP. AVP is a unique and strong vasoactive hormone which has an important role in the control of vascular tone (Holmes et al., 2004). This hormone can restore vascular tone in refractory vasodilatory states, such as septic shock, hemorrhagic shock, and shock after cardiac surgery or even overdose and poisoning (Holmes et al., 2004; Russell, 2007; Treschan and Peters, 2006). As expected, AVP noticeably increased BP, whereas milrinone could not overwhelm hypotension that might be due to its vasodilator effects. But the beneficial effect of milrinone on heart rate was much better than AVP. This may be due to improvement in myocardial contractility by inotropic effect of milrinone. There are many evidences and reports that indicate milrinone not only increases cardiac output, but also elevates HR and cardiac index without significant change in BP (Alousi et al., 1983; Feneck, 1991, 1992). As mentioned above, in addition to reducing HR, phosphine causes a severe drop in blood pressure that is usually unresponsive to volume replacement and conventional vasopressors (e.g. catecholamines). However, administration of AVP dramatically elevated BP even superior to normal levels (60–90 min). Two main reasons have been suggested to this response (Feneck, 1991; Morales et al., 1999). First, patients with vasodilatory shock usually

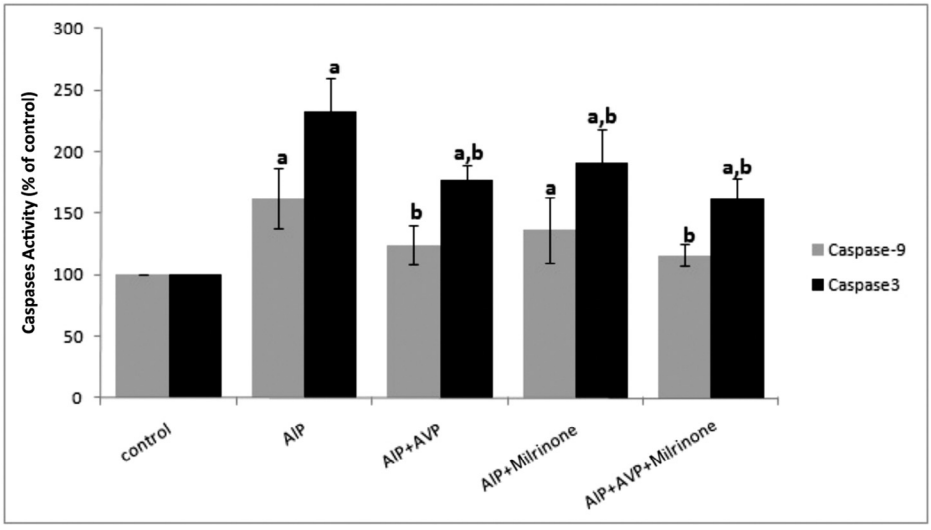

Fig. 2. Effects of various treatments on caspase-3 and -9 activities in rat heart tissue. Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (0.25 LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg). [a]Significantly different from the control group at $p < 0.01$. [b]Significantly different from the AlP group at $p < 0.01$.

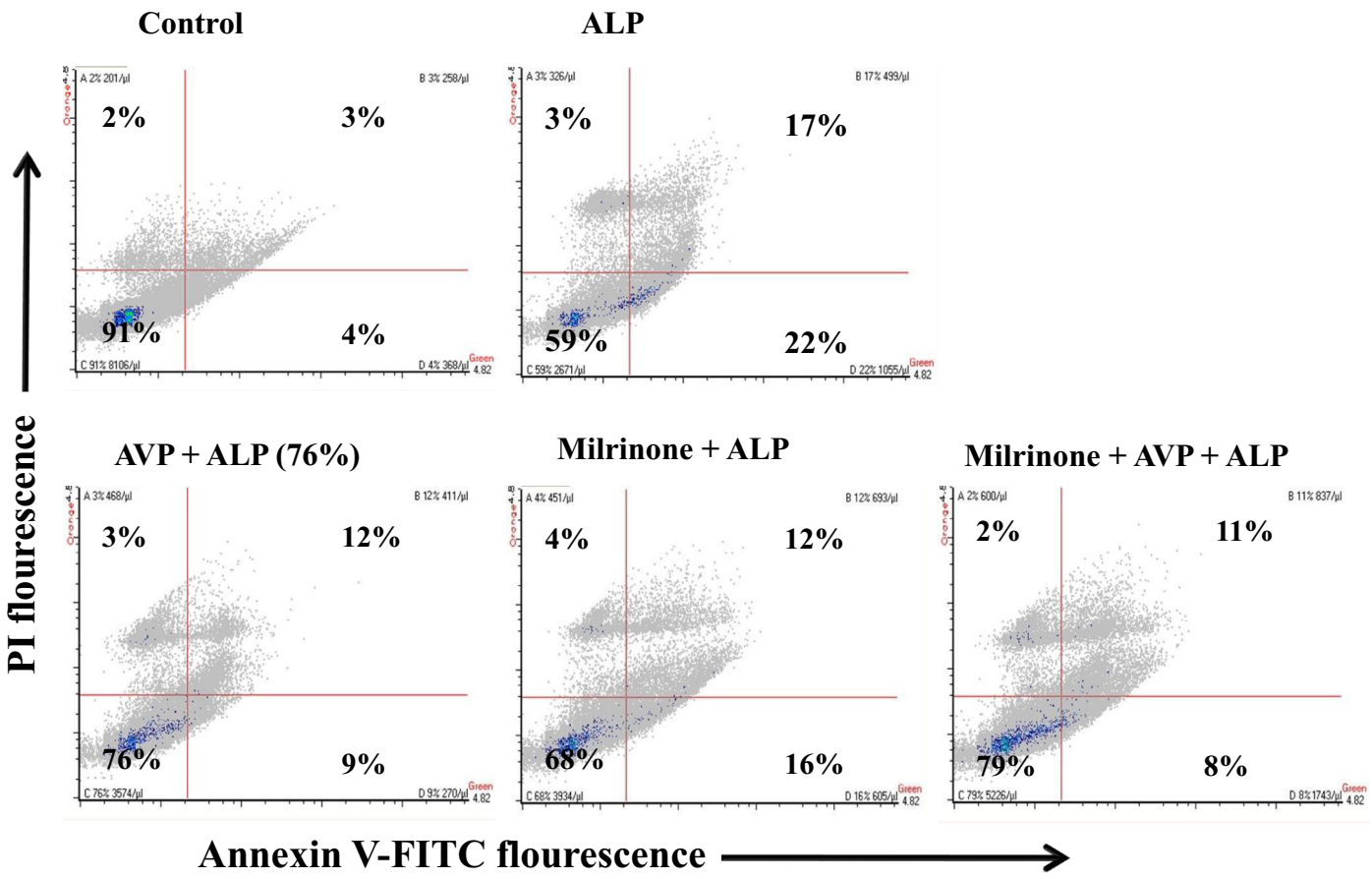

Fig. 3. Flow cytometric analysis of the cardiac cells in various groups. The numbers in the corner of each square present the percentage of cells for annexin V^-/PI^--viable cells (the number in left low corner); annexin V^+/PI^--early apoptotic cells (the number in right lower corner); annexin V^+/PI^+-lated apoptotic cells (the number in right high corner) and V^-/PI^+-necrotic cells (the number in left high corner). All experiments were performed on three separate occasions, and a representative experiment is presented.

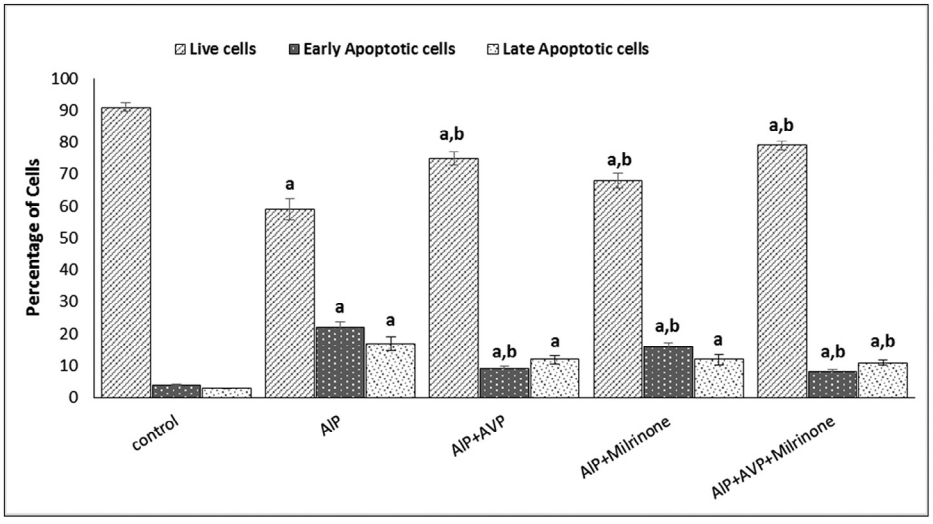

Fig. 4. Effects of various treatments on the percentage of viable, early and late apoptotic cells. Cardiomyocytes were submitted to annexin V/PI double staining and analyzed by flow cytometry to determine cell viability. Data are mean + SEM of six animals in each group. The control group received almond oil alone; AlP group (0.25 LD50) received only aluminium phosphide; AlP + AVP group received AlP + vasopressin (2.0 U/kg); AlP + milrinone group received AlP + milrinone (0.25 mg/kg); AlP + AVP + milrinone group received AlP + vasopressin (2.0 U/kg) + milrinone (0.25 mg/kg). [a]Significantly different from the control group at $p < 0.01$. [b]Significantly different from the AlP group at $p < 0.01$.

demonstrate a deficiency of AVP that show hypersensitivity to exogenous AVP. Second, this response may be due to impairment in the release of vasopressin and upregulation of AVP receptors. AlP administration also produced some electrocardiographic abnormalities including ST changes, QRS widening, QTc and PR prolagation. ST segment in the ECG indicates the end of ventricular depolarization and the beginning of repolarization (Baghaei et al., 2014). According to previous findings, AlP can induce both ST elevation and depression that indicate myocardial and pericardial damage (Shah et al., 2009; Soltaninejad et al., 2012). In this study, ST elevation was mostly observed following AlP poisoning and administration of both AVP and milrinone had beneficial effect on ST changes. It has been reported that there is a significant correlation between ST segment elevation and mortality rate and therefore this parameter can help us to predict the severity and usefulness of therapeutic strategies in acute AlP poisoning (Karami-Mohajeri et al., 2013b; Soltaninejad et al., 2012). QRS complex, which tracks depolarization of ventricles (Baghaei et al., 2014), was widen in AlP groups and administration of AVP and milrinone significantly decreased QRS widening produced by AlP toxicity. PR and QTc intervals, indicators of electrical conduction in heart tissue (Baghaei et al., 2014), were significantly prolonged following AlP poisoning. These conduction delays probably indicate the heart block or ischemic injuries in animals poisoned with AlP. Administration AVP and milrinone could successfully lessen these abnormalities. It should be noted that administration of inappropriate dose of these drugs (AVP and milrinone) can also deteriorate AlP toxicity and increase electrocardiographic abnormalities.

AlP caused considerable changes in oxidative stress biomarkers. MDA and ROS levels severely increased after AlP exposure. In addition, concurrent decrease on catalase activity and total thiol molecules were observed in AlP group. According to previous human and animal studies, AlP induces oxidative stress which is usually assessed by measuring MDA content in the different samples. It is produced via disruption of the flow of electrons across the electron transfer chain (ETC), leading to excessive production of free radicals along with alteration in antioxidant defense system such as a decrease in the catalase activity (Anand et al., 2012, 2013; Dua and Gill, 2004; Dua et al., 2010; Kariman et al., 2012; Tehrani et al., 2013). But the activity of SOD, an enzyme that dismutates superoxide radicals to H_2O_2, did not change in all treated groups, even in AlP group. Contradictory results regarding the effect of AlP on SOD activity have been reported. Some investigators believe that phosphine, the active ingredient of AlP tablets, diminishes cellular antioxidant defense through inhibition of SOD and induces cellular toxicity (Ayobola, 2012; Mehrpour et al., 2012). Some others claim that phosphine stimulates SOD activity and then increase the H_2O_2 levels (Anand et al., 2011; Gurjar et al., 2011). The excessive H_2O_2 load, which is more stable and invasive than superoxide, produces protein denaturation and lipid peroxidation in cell membranes leading to raised MDA levels (Anand et al., 2011; Yim et al., 1998). Administration of milrinone alone in rats poisoned with AlP did not cause any significant effect on oxidative stress biomarkers and it did not have any antioxidant activity on AlP toxicity. However, milrinone potentiated the cardioprotective effects of AVP. For instance, AVP alone could not increase the catalase activity and the level of total thiol molecules but co-administration of milrinone and AVP significantly improved the level of these two biochemical parameters. According to some in vitro and in vivo studies, milrinone is able to improve oxidative damage in some pathological conditions. It has been reported that milrinone through its antioxidant property not only can increase the secretion of insulin in response to glucose but also decrease the level of ROS and increase total thiol levels leading to the increase of islet viabilities (Milani et al., 2005; Mohammadi et al., 2011b). Also, the results of human studies demonstrated that milrinone can reduce MDA levels and cell membrane lipid peroxidation in patients with congestive heart failure or hemorrhagic shock (Karakozis et al., 1999; White et al., 2006). Administration of AVP alone and in combination with milrinone improved oxidative stress biomarkers which may be due to their beneficial effect on cardiac function and vascular tone. In support of these findings, it has been reported that AVP exerts its cardioprotective effect against ischemia-reperfusion injuries in heart tissue by reducing the MDA levels and other important markers of cardiac injuries (Nazari et al., 2011).

Although the exact mechanism of action of phosphine is still unknown, the results of several studies indicate that complex IV (cytochrome c oxidase) is the primary site of interaction with phosphine in the ETC (Dua and Gill, 2004; Dua et al., 2010; Mehrpour et al., 2012; Nath et al., 2011; Singh et al., 2006). From our results we observed a significant reduction in the level of the cytochrome c oxidase in the heart tissue of rats poisoned with AlP but in contrast to some previous reports did not observe significant changes in complex I and II activities which may be due to the low dose of AlP used in our biochemical studies (Anand et al., 2012). It seems that phosphine is a nonspecific cytochrome inhibitor and interacts with any enzyme and macromolecule containing heme groups. Phosphine induces methemoglobinemia through reduction of heme structure in hemoglobin (Anand et al., 2012; Lall et al., 2000; Shadnia et al., 2010). This poison also inhibits catalase, which contains a heme group in its structure (Anand et al., 2012; Bolter and Chefurka, 1990). ETC dysfunction induced by phosphine causes problems in cellular energy demands and ATP levels (rise in the ADP/ATP ratio). Administration of AVP alone and especially in combination with milrinone in rats intoxicated with AlP prevented the decrease in complex IV activity and the depletion of ATP reserve so that there was no significant difference between control and AlP + AVP + milrinone groups. In support of our results, it has been reported that AVP causes the activation of key enzymes of mitochondrial oxidative metabolism through the elevation of intramitochondrial calcium (Assimacopoulos-Jeannet et al., 1986). Also, another report has demonstrated that addition of vasopressin to hepatocytes can increase the respiratory rate by up to 35% (Korzeniewski et al., 1995). Although administration of milrinone alone caused no significant change in complex IV activity and ADP/ATP ratio, it potentiated beneficial effects of AVP in AlP + AVP + milrinone group. This may be due to the preventive effect of milrinone on the opening of mitochondrial permeability transition (MPT) pore (Lescan et al., 2013). MPT pores represent a process which can induce cell death, either by ATP depletion or by releasing apoptosis-inducing factor (e.g. cytochrome c) (Armstrong, 2006).

Based on histopathological findings, phosphine can also induce apoptosis and necrosis in several tissues (Anand et al., 2012; Shah et al., 2009). The findings of electron microscopy on the heart, kidney and liver tissues have shown that mitochondria were swollen, dysmorphic with enlarged and disrupted cristae after acute AlP exposure (Anand et al., 2012). Mitochondrial swelling and outer membrane rupture are associated with the release of proapoptotic factors such as cytochrome c from the intermembrane space. Release of cytochrome c activates caspase-9, which in turn activates caspase-3 (Heusch et al., 2010). In this study the activities of caspase-9 and -3 were assessed and the results showed that AlP exposure increased the activity of caspase-3 more than caspase-9 which probably means other mechanism/s, rather than involvement of mitochondria, play an imperative role in AlP induced toxicity. Administration of AVP alone and in combination with milrinone in rats poisoned with AlP caused a significant decline in the activity of caspase-9 and -3, so that there were no significant difference between these treated groups and control group in terms of caspase-9 activity. Milrinone and AVP affect apoptosis pathways via different mechanisms. AVP inhibits apoptosis in different cells via V1 receptor and protein kinase C signaling pathways. Activation of

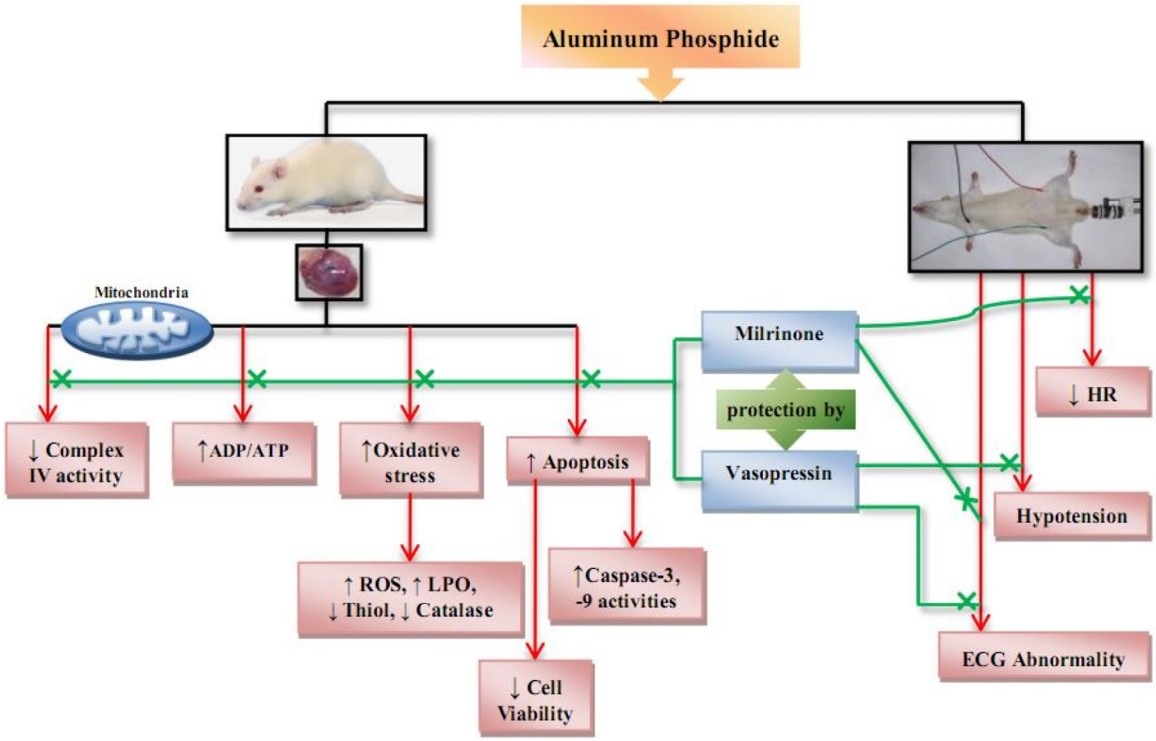

Fig. 5. Summary of the mechanisms involved in toxicity of AlP and protection ways. AVP and milrinone have potential application in the treatment of cardiovascular complications induced by AlP poisoning for which there is currently no specific antidote.

V1 receptors by AVP induces phosphorylation-inactivation of the pro-apoptotic protein and consequently decreases in cytosolic cytochrome c and caspase-3 activation (Chen et al., 2008; Higashiyama et al., 2001). It should be noted that AVP induces its antiapoptotic effect only when the apoptotic stimulation exists and under normal condition does not affect the basal level of the apoptosis (Higashiyama et al., 2001). In addition to vasoactive effect, AVP has mitotic effect on some cells and exerts its effects by activating the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) cell signaling pathways (Ghosh et al., 2001). Milrinone exerts its cardioprotective effects via cAMP and protein kinase A (PKA) dependent, but PKC-independent mechanisms in the heart (Huang et al., 2011; Sanada et al., 2001).

We also evaluated cardiomyocytes of various groups by flow cytometry to analyze viable, apoptotic and necrotic cells. The results showed AlP exposure caused the significant decrease in the percentage of viable cardiomyocytes along with decrease in apoptotic cells. There is no report about the effect of phosphine on caspase enzyme activity and the percentage of viable, apoptotic and necrotic cells; however, our results are consistent with histopathological findings which were performed on various human and animal tissues (Anand et al., 2012). Surprisingly, administration of both milrinone and AVP, alone and in combination, prevented the decrease of viable cells, but there was still significant differences between treated groups and control group. It seems that milrinone exerts its beneficial effects with other mechanisms that are not addressed in this study.

In conclusion, our results demonstrated that poisoning with AlP causes electrocardiographic abnormalities and mitochondrial dysfunction leading to oxidative stress, ATP depletion and apoptosis in the heart tissue of rat. As illustrated in Fig. 5, administration of AVP and milrinone can improve most of these unfavorable changes produced by AIP. It should be noted that under certain conditions (such as the use of higher and inappropriate doses), these drugs can even induce some arrhythmias and worsen the AlP toxicity; therefore, the dosage should be carefully considered and monitoring of cardiovascular system and other vital organs should be performed very closely.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Authors' contributions

MA gave the idea, SNO, and MS were consulted; AJ did the study as a PhD student; AB, RS, MB, SH, and MG helped in performing the experimental part of the study. All authors were involved in data analysis and interpretation. AJ and MC drafted the paper and MA edited the manuscript. All authors read and approved the final version.

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