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Original Research Article

Effect of sodium aescinate on methyl parathion-induced myocardial injury in rats

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Abstract

Purpose: *To explore the effects and mechanism of sodium aescinate (SA) on methyl parathion (MP)-induced myocardial injury.*
Methods: *Rats were divided into following groups: In Control group, rats were administered 0.9 % NaCl by intraperitoneal injection. In MP group, rats were administered 20 mg/kg MP by intraperitoneal injection. In MP + SA group, rats were administered 20 mg/kg MP in combination with SA at a concentration of 0.5, 1.0, or 1.5 mg/kg by intraperitoneal injection. Histological changes were assessed by H&E staining. Serum levels of cardiac troponin T (CTnT) and atrial natriuretie peptide (ANP) were measured by automatic biochemical analyzer and real-time polymerase chain reaction (RT-PCR), respectively. The levels of malondiadehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH - Px), and glutathione (GSH) in heart tissue was detected by spectrophotometry. The apoptosis of myocardial cells was measured by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The level of apoptosis-related proteins was assessed by western blot.*
Results: *Superoxide dismutase attenuated MP-induced myocardial injury, and decreased the levels of ANP and cTnT in serum ($p < 0.01$). Superoxide dismutase attenuated the MP-induced decrease in GSH, GSH-px, and SOD expression ($p < 0.05$) but increased MDA level ($p < 0.01$). Moreover, SA inhibited the apoptosis of myocardial cells and regulation of apoptosis-related protein expression (e.g., Bax, Bcl-2, and caspase 3).*
Conclusion: *These results demonstrate that SA attenuates MP-induced myocardial injury by regulating oxidative stress and apoptosis.*

Keywords: *Sodium Aescinate, Methyl Parathion, Acute Organophosphorus Pesticide Poisoning, Myocardial Injury*

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INTRODUCTION

Acute organophosphorus pesticide poisoning (AOPP) is a disease caused by organophosphorus pesticide abuse or accidental poisoning with a high mortality especially in rural regions [1]. It is estimated that AOPP leads to about 3,000,000 deaths worldwide annually [2]. AOPP often results from the accumulation of Ach within synapses of the central and peripheral

nervous systems. Excessive Ach in turn over-stimulates postsynaptic cholinergic receptors and exerts neurotoxicity [3].

Cardiac complications, such as various arrhythmias, dysarteriotony, and myocardial damage, are major causes of death after AOPP [4]. Researchers found that pesticides induce oxidative stress, causing DNA damage and apoptosis in myocardial cells; these effects may constitute the mechanism of MP-induced myocardial injury [5]. However, most research focuses on clinical treatment and lung injury, and only a few studies are aimed at understanding prevention and treatment of myocardial injury.

Sodium aescinate (SA), a sodium salt of escin isolated from *Aesculus hippocastanum*, is used as a dietary supplement or clinical drug for hypertensive intracerebral hemorrhage [6]. Sodium aescinate is widely used as a treatment for tumefaction caused by trauma or surgery, as well as chronic venous insufficiency [7]. Thus, the effects of SA in clinics include anti-inflammation, anti-oxidation, and regulation of microcirculation [8]. Researchers found that SA can attenuate MP-induced lung injury via anti-inflammatory and anti-oxidative effects [9]. However, the effects of SA on MP-induced myocardial injury remains largely unknown. The current study focus on the effects of SA on MP-induced myocardial injury and further investigated the associated mechanism.

EXPERIMENTAL

Reagents

Sodium aescinate (SA) was obtained from LuYe Pharmaceutical Company Limited (Yantai, China). Methyl parathion (36187) was obtained from Sigma-Aldrich (St Louis. MO. USA). The kits for MDA (A003-1), SOD, GSH-px, and GSH were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). All primers were synthesized by Shanghai Sangon Biological Engineering Co. Ltd (Shanghai, China). All antibodies (cleaved caspase 3 (#9661), Bax (#14796), and Bcl-2 (#2764)) were obtained from Cell Signaling Technology (Beverly, MA, USA).

Animals and treatment

Sprague-Dawley rats were obtained from Vital River Laboratory Animal Technology (Beijing, China). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [10] and approved by the animal ethics committee of The People's Hospital of Guang'an City University (approval no. GL201600013). The rats were divided into following groups: control group, MP group, MP + SA group. Control rats were injected with 0.9 % NaCl solution. For the MP group, rats were injected with MP solution (20 mg/kg). For t MP + SA group, rats were injected with an MP solution and SA at a dose of 0.5, 1.0, or 1.5 mg/kg. After 24 h, all rats were anesthetized with pentobarbital (50 mg/kg). Peripheral blood was collected in heparin-treated tubes by and centrifuged to obtain plasma for further analysis. Heart samples were removed for analysis by histological examination [11].

Hematoxylin and eosin (H & E) staining

Heart tissue was fixed in formalin (0.4 %) for H&E staining. Samples were dehydrated in graded alcohol, blocked in paraffin, and cut into 3 ~ 4 μm sections on a sliding microtome (Leica, Buffalo Grove, IL, USA). The sections were stained with H & E solution for 5 min at 37 °C. Finally, the sections were mounted in neutral balsam after dehydration and examined under an optical microscope (Nicon microscope ECLIPSE E600W, Tokyo, Japan) and evaluated blindly by pathologists.

Tissue processing

Blood samples were collected in tubes pretreated with anticoagulant (aprotinin for ANP, heparin for cTnT) and centrifuged (3000 g at 4 °C for 10 min) to obtain plasma for further analysis. Plasma ANP mRNA expression was determined by RT-PCR while plasma cTnT concentrations were analyzed by automatic biochemical analyzer (Roche Diagnostics, Germany). Heart tissue was isolated, washed, and homogenized for 10 min in 0.9 % NaCl solution. After centrifugation, the supernatant was collected for further analysis of MDA, SOD, GSH-px, and GSH using assay kits purchased from Nanjing Jincheng Corp, China.

Determination of oxidative stress markers

The levels of SOD and MDA were detected by WST-1 assay and thiobarbituric acid (TBA) assay, respectively. GSH level and GSH-px activity were determined by spectrophotometry according to the manufacturer's protocol.

Real-time polymerase chain reaction (RT-PCR)

Total RNA from a serum sample was extracted with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reversed

transcribed to cDNA using a Reverse Transcription Kit. Real-time-PCR was analyzed by SYBR Green reagent. All PCR kits were purchased from Promega Corporation (Madison, WI, USA). Primer sequences are listed in Table 1.

Table 1: Primer sequences

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
ANP	TGGATACACTGGCATCTACT	TCTTCACCCTCCTCCTCCTCAA
GAPDH	TGCCCAGAACATCATCCCT	CCTCCTCAGTCTAGCCCAAC

Western blotting

Heart tissue was homogenized and lysed in RIPA lysis buffer and then protein content was quantified using the BCA method (ThermoFisher Scientific, Rockford, IL, USA).After separation on 10 % SDS-PAGE, the proteins were transferred onto PVDF membrane (Millipore, Burlington, MA). After blocking with 5 % non-fat milk, the membranes were incubated with the respective primary antibodies (1:1000 dilution). The proteins were detected using a chemiluminescence imaging machine and analyzed with the NIH Image J software.

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (Cat# TB235, Promega, Madison, WI, USA) was used for the specific detection and quantitation of apoptotic cells. Tissue slides were washed with xylene to remove paraffin, and then washed with decreasing concentrations of ethanol (100, 95, 85, 70, and 50 %) for 5 min at each concentration. The slides were washed with 0.85 % NaCl and PBS, and then fixed with 4 % formaldehyde for 1 min. After washing with PBS twice, cells were permeabilized with proteinase K for approximately 10 min. After another PBS wash, the cells were fixed again with 4 % formaldehyde and then equilibrated with equilibration buffer before labeling with TdT solution for 1 h at 37 °C. Then cells were incubated with Hoechst 33342 solution (ThermoFisher Scientific, Pierce, Rockford, IL, USA) for the detection of cell nuclei. Co-localization of green and blue fluorescence was observed by light microscopy.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical significance was assessed by one-way ANOVA using SPSS19.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Histopathological features of myocardial injury

The structure of myocardial tissues was examined by H&E staining. As shown in Figure 1, the cardiac longitudinal section of the control group had no apparent myocardial injury and showed well-organized myofibrils. In contrast, MP-induced rats exhibited extensive myocardial injury, including disorganized fibres and myocardial cell lysis. Sodium aescinate treatment clearly attenuated these histopathological changes. In fact, myocardial injury in animals which received a high dose of SA (1.5 mg/kg) was less severe than those received a low (0.5 mg/kg) or medium (1.0 mg/kg)dose of SA.

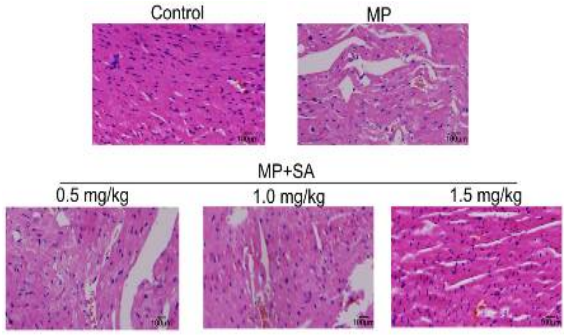

Figure 1: Histopathology of the myocardial tissues as visualized by H&E staining

Serum ANP and cTnT expression

Compared with control group, the levels of serum ANP and cTnT were increased about five-fold and eight-fold, respectively (Figure 2, $p < 0.05$). On the other hand, co-treatment of SA significantly reduced the MP-upregulated levels of ANP and cTNT in a dose-dependent manner (Figure 2, $p < 0.05$).

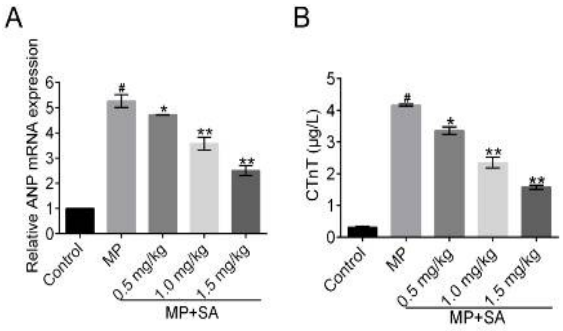

Figure 2: Serum ANP mRNA (A) and cTnT levels (B) in serum of MP-induced rats. $^{*}P < 0.05$ and $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. MP group

Tissue oxidative stress markers

To investigate oxidative stress in the heart, SOD, MDA, GSH-px, and GSH levels or activity were analyzed. As shown in Figure 3 A, the concentration of MDA significantly increased from 8 to 15 nmol/mg ($p < 0.05$) while SA reduced the expression of MDA ($p < 0.01$ for 1.5 mg/kg dosage group) in a dose-dependent manner. The SOD activity in the MP group decreased from 55 (control) to 50 U/mg protein (MP group) (Figure 3 B). However, SA treatment enhanced SOD activity. Furthermore, GSH-px activity (Figure 3 C) and GSH level (Figure 3 D) decreased approximately 40 % following MP treatment, while SA attenuated GSH expression and GSH-px activity in a dose-dependent manner.

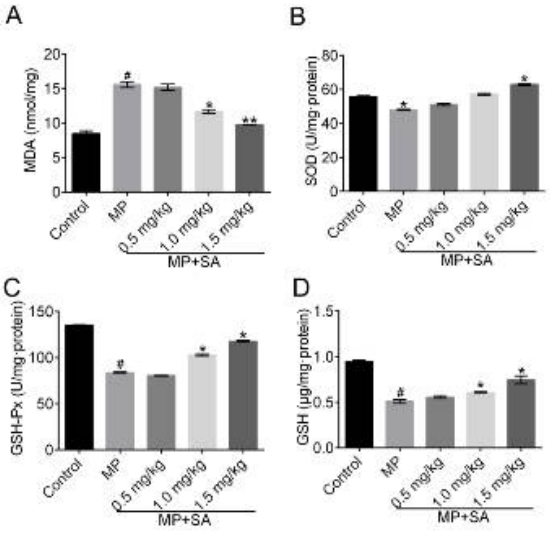

Figure 3: Oxidative stress markers in myocardial tissue. (A) MDA (B) SOD (C) GSH-px (D) GSH; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. MP group

Apoptosis of myocardial cells

To investigate apoptosis, the TUNEL assay was performed alongside western blot analysis of the expression of apoptosis-associated proteins in heart tissues. As shown in Figure 4A, the TUNEL assay results showed that MP significantly increased the percentage of apoptotic cells, which was approximately three times that of the control group. Sodium aescinate (SA) treatment significantly decreased apoptosis of myocardial cells in a dose-dependent manner. The western blotting results revealed that in MP group, the expression of cleaved caspase 3 and Bax increased while Bcl-2 decreased (Figure 4B).Sodium aescinate treatment decreased the expression of cleaved caspase 3 and Bax but increased Bcl-2 in a dose-dependent manner.

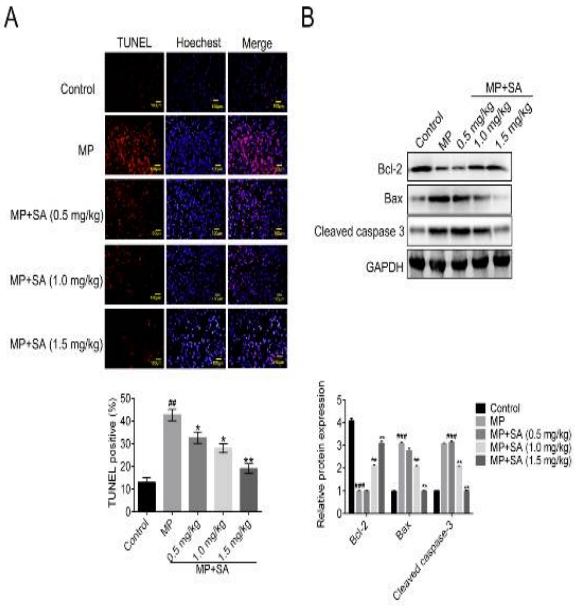

Figure 4: Apoptosis of myocardial cells was analyzed by the TUNEL assay (A) and the expression of apoptosis-related proteins was assessed by western blot (B); $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. MP group

DISCUSSION

Many research studies have demonstrated that SA exerts anti-inflammatory and antioxidant effects, suggesting a mechanism for its myocardial protective effects [12]. AOPP induced many complications, including myocardial injury, partly due to oxidative damage and apoptosis of myocardial cells [5]. The results indicated that SA can attenuate MP-induced myocardial injury and decrease the levels of ANP and cTnT in serum. Furthermore, SA attenuated the oxidative damage in myocardial tissue induced by MP. The apoptosis of myocardial cells and expression of apoptosis-related proteins also decreased after SA treatment. Taken together, these results demonstrate that SA could attenuate MP-induced myocardial injury.

Atrial natriuretie peptide and cTnT have been reported to be the main diagnostic markers for heart failure. The interaction of combined increases in ANP, copeptin, and cTnT is the most potent predictor of increased risk of heart failure [13]. Clinical research demonstrated that serum levels of cTnT and CK-MB were increased in AOPP patients, and that the degrees of myocardial injury can be serious [14]. Few studies have demonstrated the effects of SA on MP-induced myocardial injury. The results for the first time indicated that SA could attenuate MP-induced myocardial injury and decreased the level of serum ANP and cTnT, which may reduce the risks of heart failure.

Many studies have confirmed that MP can induce the over production of reactive oxygen species, leading to oxidative damage [15]. Superoxide dismutase and GSH-px, two endogenous antioxidant enzymes, inhibit the generation of hydroxyl radicals [16]. The activity of SOD and GSH-px decrease when oxidative damage occurs. Malondialdehyde, a product of lipid peroxidation, is often utilized as a biomarker of oxidative damage [17]. Moreover, the expression of glutathione is decreased during oxidative damage [18]. Escin mixture, which is isolated from *Aesculus hippocastanum,* has been reported to possess anti-oxidative effects and exhibit a protective effect on the liver architecture [19].

Escin decreased MDA in the blood, liver, kidney, and heart, SOD of liver, and increased GSH of blood and liver in high-fat diet treated mice [19]. The results of this present research demonstrate that MP treatment decreased the enzymatic activity of GSH-px and SOD while increasing MOD and decreasing GSH. Sodium aescinate, a sodium salt of escin, decreased the level of MDA and increased the activity of SOD and GSH-px along with the content of GSH. The protective effects of SA on myocardial tissue were associated with antioxidant effects, which is in accordance with previous research.

Myocardial apoptosis is also a common implication in myocardial injury [20]. Thus, the effects of SA on myocardial apoptosis induced by MP have also been investigated.. Sodium aescinate could also protect against lung injury induced by intestinal ischemia/reperfusion (I/R) by regulating the expression of Bcl-2 and Bax proteins and inhibiting the apoptosis of lung cells [9]. This research demonstrate that SA can exert cardio-protective effect by inhibiting MP-induced apoptosis of myocardial cells.

This study indicates that SA exerts a protective effect on MP-induced myocardial injury due to its anti-inflammatory and anti-oxidative abilities. Moreover, the results indicate that SA can be a promising agent for treating myocardial injury induced by AOPP.

CONCLUSION

SA affords cardio-protective effects in MP-induced myocardial injury. The effects may be due partly to its anti-oxidative and anti-apoptotic activities. This study indicates the potential value of sodium aescinate in the SA treatment of myocardial injury in AOPP.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Zhan Su designed all the experiments and revised the paper. Suxin Luo and Tian Tuo formed the experiments, Junjie Wen wrote the paper.

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