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ผลต้านภาวะความดันเลือดสูงและภาวะเครียดออกซิเดชันของกรดอะเซียติกในหนูแรทที่ได้รับแคดเมียมเป็นระยะเวลานาน

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Antihypertensive and Antioxidative Effects of Asiatic Acid in Rats with Long-Term Exposure to Cadmium

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หลักการและวัตถุประสงค์: แคดเมียม (Cd) เป็นโลหะหนักที่มีพิษชนิดหนึ่งที่ก่อให้เกิดการทำลายเนื้อเยื่อต่างๆที่เกิดจากออกซิเดชันและเกี่ยวข้องกับภาวะความดันเลือดสูง สารต้านออกซิเดชันถูกนำมาใช้เพื่อต่อต้านพิษที่เกิดขึ้นจาก Cd กรดอะเซียติก (AA) คือสารเพนทะไซคลิกไทรเทอร์พีนอยด์ที่สกัดได้จากต้นบัวบก (*Centella asiatica*) และมีฤทธิ์ต้านออกซิเดชัน การศึกษานี้มีวัตถุประสงค์เพื่อสำรวจว่า AA สามารถลดภาวะความดันเลือดสูงและภาวะเครียดออกซิเดชันในหนูแรทที่ได้รับ Cd เป็นเวลานาน

วิธีการศึกษา: หนูแรท เพศผู้ พันธุ์ Sprague-Dawley ได้รับ Cd ทุกวัน ผ่านทางน้ำดื่มที่ประกอบด้วยแคดเมียมคลอไรด์ 10 พีพีเอ็ม เป็นเวลา 16 สัปดาห์ หนูแรทในกลุ่มควบคุมจะได้รับน้ำกรองเป็นน้ำดื่ม หนูแรทถูกป้อนด้วย AA (15 หรือ 30 มก./กก.) วันละครั้งในช่วง 4 สัปดาห์สุดท้ายของการทดลอง

ผลการศึกษา: Cd เหนี่ยวนำให้เกิดภาวะความดันเลือดสูงในหนูแรทโดยการเพิ่มความดันเลือดของหลอดเลือดแดงและเพิ่มความต้านทานการไหลเวียนเลือดส่วนปลาย Cd ลดการขยายตัวของหลอดเลือดที่ตอบสนองต่อยาขยายหลอดเลือดอะซิติลโคลีน ($p < 0.05$) ในขณะที่การตอบสนองต่อยาขยายหลอดเลือดโซเดียมไนโตรปรัสไซด์ไม่เปลี่ยนแปลง AA ให้ผลตามระดับความเข้มข้นในการปรับพลศาสตร์การไหลเวียนเลือดให้ดีขึ้นและเพิ่มการตอบสนองของเซลล์เอนโดทีเลียมอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) AA ลดภาวะเครียดออกซิเดชัน เพิ่มกลูตาไธโอนในเลือด และเพิ่มระดับไนเตรท/ไนไตรท์ในพลาสมา นอกจากนี้ AA ยังช่วยลดการสะสม Cd ในเลือดและในเนื้อเยื่อหลอดเลือดด้วย

สรุป: ผลการศึกษาเหล่านี้บ่งชี้ว่า AA อาจเป็นสารต้านออกซิเดชันที่ช่วยป้องกันภาวะความดันเลือดสูง ภาวะเอนโดที

Background and objective: Cadmium (Cd) is a toxic heavy metal which causes oxidative damage to various tissues and associated with hypertension. Antioxidants have been used to protect against Cd intoxication. Asiatic acid (AA) is a pentacyclic triterpenoid extracted from Bua-Bok (*Centella asiatica*) and possesses strong antioxidant activities. This study aimed to investigate whether AA could attenuate hypertension and oxidative stress in rats chronically exposed to Cd.

Methods: Male Sprague-Dawley rats were exposed to Cd daily via consumption of water containing 10 ppm cadmium chloride for 16 weeks. Rats in the control groups received deionized water as drinking water. AA (15 or 30 mg/kg b.w.) was orally administered once daily for the last 4 weeks of the experiment.

Results: Cd induced hypertension in rats by increasing arterial blood pressure and peripheral vascular resistance. Cd significantly attenuated the vasodilating responses to acetylcholine ($p < 0.05$) while the vasodilating responses to sodium nitroprusside were unchanged. AA in a dose-dependent manner significantly improved hemodynamics and increased endothelial-dependent responses ($p < 0.05$). AA alleviated oxidative stress, enhanced blood glutathione and increased plasma nitrate/nitrite level. Moreover, AA also reduced the accumulation of Cd in blood and vascular tissues.

Conclusions: These results suggest that AA is a pro-

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เลียมทำงานผิดปกติและภาวะเครียดออกซิเดชันที่เหนี่ยวนำโดย Cd

tective antioxidant against Cd-induced hypertension, endothelial dysfunction and oxidative stress.

คำสำคัญ: กรดอะเซียติก; แคดเมียม; ความดันเลือดสูง; ไนตริกออกไซด์; ภาวะเครียดออกซิเดชัน

Keywords: Asiatic acid; Cadmium; Hypertension; Nitric oxide; Oxidative stress

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Introduction

Cadmium (Cd) is one of the common contaminant metals listed by The Agency for Toxic Substance and Disease Registry (ATSDR)[1]. Cd is frequently used in various industrial activities, including the production of alloys, pigments and batteries. There has been an increasing global public health concern associated with environmental contamination by Cd. The main routes of exposure to Cd are via eating contaminated food, smoking cigarettes and working in Cd-contaminated places[2]. Chronic exposure to Cd causes toxic effects to various tissues and organs, including cardiovascular, renal, liver, musculoskeletal, reproductive, endocrine, central nervous and peripheral nervous systems[3]. It has been reported that vascular endothelium is a critical target of Cd toxicity which leads to many cardiovascular diseases such as hypertension, coronary heart disease and myocardial infarction[4, 5]. Cd increases oxidative stress and attacks endothelium and vascular smooth muscle cells, thereby leads to vascular damage and dysfunction[5, 6]. Since natural antioxidants play an important role in alleviating oxidative stress, supplementation of antioxidants may be an effective treatment of Cd poisoning.

Bua-Bok (*Centella asiatica*) is a medicinal plant widely used in Southeast Asian countries.

Asiatic acid (AA; a pentacyclic triterpenoid) is an active compound isolated from *Centella asiatica*. It has been demonstrated that AA is a potent antioxidant and prevented the neuronal impairment caused by quinolinic acid in rats[7]. AA also reduced oxidative stress and inflammation in 2K-1C renovascular hypertensive rats[8], and reduced blood pressure of L-NAME hypertensive rats[9]. Since there is lack of information about the blood lowering property of AA in the Cd-intoxicated condition. The present study was designed to elucidate whether supplementation of AA could reduce blood pressure, alleviate oxidative stress and improve endothelial function in rats with long-term exposure to Cd.

Methods

Chemicals

Asiatic acid (AA; ≥98% purity by HPLC analysis) was purchased from Leap Labchem Co., Ltd. (Hangzhou, China). Cadmium chloride ($CdCl_2$), glutathione, ethylenediamine tetraacetic acid (EDTA), thiobarbituric acid (TBA), sodium dodecylsulfate (SDS), 1, 1, 3, 3-tetraethoxypropane, metaphosphoric acid (MPA), 2,4-dinitrophenylhydrazine (DNPH), bovine serum albumin fraction V (BSA), N-(1-nepthyl) ethylenediamine dihydrochloride (NED), sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) were from Sigma-Aldrich Pte. Ltd. (Singapore). Acetylcholine chloride and sodium nitropusside were obtained from Fluka Chemika Co. Ltd. (Buchs, Switzerland). All other chemicals used were of analytical grade quality.

Animals

Male Sprague-Dawley rats weighing 180–200 g were obtained from the National Laboratory Animal Center, Nakhonpathom, Thailand. The rats were housed in the Heating, Ventilation and Air-Conditioning (HVAC) System with 12 h dark/light cycle in the Northeast Laboratory Animal Center, Khon Kaen University, Thailand, and were fed with a standard chow diet (Chareon Pokapan Co. Ltd., Samutprakarn, Thailand). The study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University (AEKKU-NELAC 17/2558). All surgical procedures were performed under standard anesthesia, and all efforts were made to minimize suffering. After one week of acclimatization, rats were randomly divided into five groups (n = 8/group): group I-control, group II-control + AA 30 mg/kg b.w., group III-Cd alone, group IV-Cd + AA 15 mg/kg b.w., and group V-Cd + AA 30 mg/kg b.w. The control groups received deionized water (DI) as drinking water whereas Cd-treated groups received drinking water containing $CdCl_2$ (10 ppm or 10 mg/L) continuously for 16 weeks. AA (15 or 30 mg/kg) was orally administered once daily for the last 4 weeks of the experiment.

Indirect blood pressure measurement

To monitor the changes in arterial blood pressure of rats during exposure to Cd, blood pressure was assessed by the indirect tail cuff method. Briefly, a conscious rat was placed in restrainer and allowed to rest inside the cage for 15 min prior to blood pressure measurement. The rat tail was placed inside the tail cuff, an automatically inflated and released. Systolic blood pressure (SBP) values of 3-4 consecutive readings were recorded by using a rat tail sphygmomanometer (IITC model 179 blood pressure analyzer, Woodland Hills, CA, USA).

Measurement of hemodynamic status and vascular responsiveness

After 16 weeks, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). A tracheotomy was performed for spontaneous breathing, and left femoral artery was cannulated with polyethylene catheter connected to a pressure transducer for continuous monitoring of blood pressure (BP) using the Acqknowledge data acquisition analysis software (BIOPAC Systems Inc., California, USA). The left femoral vein was cannulated with polyethylene tubing for infusion of vasoactive drugs. Baseline BP values were monitored in animals for 10 min. After obtaining stable baseline measurements, an endothelium-dependent vasodilator, acetylcholine (ACh; 3, 10, 30 nmol/kg) and an endothelium-independent vasodilator, sodium nitroprusside (SNP; 1, 3, 10 nmol/kg), were randomly infused intravenously, while blood pressure was continuously monitored. Thereafter, hindlimb blood flow (HBF) was measured by placing electromagnetic flow probe around the abdominal aorta connected to an electromagnetic flowmeter (Carolina Medical Electronics, North Carolina, USA). Hindlimb vascular resistance (HVR) was calculated from the mean arterial pressure (MAP) divided by HBF. At the end of experiment, rats were sacrificed by overdose of the anesthetic drug. Blood samples were withdrawn from abdominal aorta for assays of oxidative stress markers. The aortas were rapidly excised from the animals and used for analysis of superoxide anion ($O2^{\bullet-}$) production and Cd content.

Assay of oxidative stress markers and NO metabolites

$O2^{\bullet-}$ production in vascular tissues was measured using lucigenin-enhanced chemiluminescence method[10]. Lipid peroxidation, as measured by malondialdehyde (MDA) level in plasma, was estimated using thiobarbituric acid (TBA) as described previously[10]. The antioxidant glutathione (GSH) in whole blood was assayed by a previously described method[11]. Nitrite and nitrate, the end products of nitric oxide (NO) metabolism, were used as indicator of NO production. Plasma nitrate/nitrite was determined by an enzymatic conversion method with the Griess reaction as previously described[12].

Assay of Cd contents

Cd content in whole blood and aortic tissues were determined by using inductively coupled plasma mass spectrometry (ICP-MS) method (Agilent 7500 ICP-MS model, Santa Clara, CA, USA) according to the manufacturer's recommendation as previously described [12]. The Cd contents were expressed in μg/L and μg/g of tissue wet weight.

Statistical analysis

Data are presented as means ± S.E.M. Statistical differences were evaluated by one-way analysis of variance (ANOVA) and followed by Student Newman–Keul's test to show specific group differences. All analysis was performed using SigmaStat software version 3.1. Statistical significance was determined at a level of $p < 0.05$.

Results

Effect of AA on hemodynamics and vascular responsiveness

The baseline data of systolic blood pressure (SBP) in all studied groups obtained from indirect blood pressure measured by using a rat tail sphygmomanometer are displayed in Figure 1. There were no differences in SBP among all groups at the beginning of experiments. SBP increased progressively in Cd-treated rats during experimental periods for 16 weeks ($p < 0.05$, Fig. 1), indicating the development of hypertension in rats exposed to Cd. A significant reduction in SBP was found in rats exposed to Cd treated with AA (15 or 30 mg/kg) for 4 weeks when compared with those treated with Cd alone ($p < 0.05$, Fig. 1). The effect of AA was found in a dose-dependent manner.

A significant increase in SBP, diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) was found in Cd-treated rats ($p < 0.05$, Table 1), which is consistent with the data obtained from indirect blood pressure measurement. The elevation

of arterial blood pressure was accompanied by decreasing HBF and increasing HVR ($p < 0.05$, Table 1). Rats supplemented with AA during Cd exposure showed a significant decrease in blood pressure and HVR whereas HBF increased ($p < 0.05$, Table 1). The improvement of hemodynamic status in Cd-exposed rats treated with AA was found in a dose-dependent manner. The data indicate that AA supplementation reduced arterial blood pressure and total peripheral resistance, which leads to prevent the development of hypertension during Cd exposure. There was no alterations in all hemodynamic parameters in control rats-treated with AA, indicating that AA does not change the normal physiological condition (Table 1).

A marked blunting of response to ACh was found in rats with long-term exposure to Cd ($p < 0.05$, Fig. 2A), suggesting the endothelial dysfunction was present in animals exposed to Cd. AA, especially at high dose improved endothelial function by increasing the vasodilating responses to ACh (Fig. 2A). It is found that the vasodilating responses to SNP were not different in all groups (Fig. 2B).

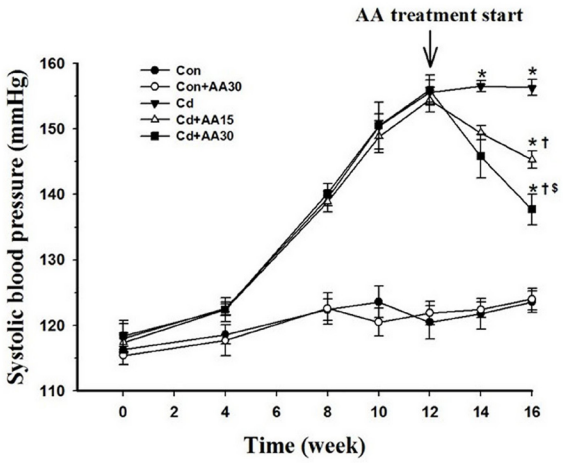

Figure 1 Effect of asiatic acid on systolic blood pressures monitoring throughout 16 weeks of experiments.
Data are expressed as mean ± S.E.M. (n = 6-8/group). *p<0.05 compared to control group, †p<0.05 compared to Cd-treated group, $^{\$}$p<0.05 compared to Cd+AA15 mg/kg –treated group.

Effect of AA on oxidative stress

Increased oxidative stress was found in rats-exposed to Cd, as shown by increasing vascular $O_2^{\bullet-}$ production and elevating plasma MDA ($p < 0.05$, Fig. 3A and B), whereas the blood GSH was markedly decreased ($p < 0.05$, Fig. 3C). A decrease in plasma nitrate/nitrite was found in Cd-treated rats compared to untreated controls ($p < 0.05$, Fig. 3D). Treatment with AA, particularly at high dose significantly alleviated oxidative stress, increased antioxidant GSH and increased plasma nitrate/nitrite in Cd-exposed rats ($p < 0.05$, Fig. 3). It appeared that alleviation of oxidative stress after AA treatment was associated with a restoration of hemodynamics.

Effect of AA on Cd contents

Data in Figure 4 demonstrate the levels of Cd contents in blood and vascular tissues of all experimental groups. A marked increase in Cd concentrations was found in the blood and vascular tissues of rats after Cd exposure for 16 weeks ($p < 0.05$, Fig. 4A and B). Meanwhile, the level of Cd

Table 1 Effect of asiatic acid on hemodynamics of rats in all experimental groups

Parameters	Control	Control+AA30	Cd	Cd+AA15	Cd+AA30
SBP (mmHg)	118 ± 4	119 ± 3	158 ± 4*	139 ± 4*†	131 ± 4$^{*\dagger\$}$
DBP (mmHg)	84 ± 4	85 ± 3	102 ± 4*	98 ± 4*†	92 ± 3$^{*\dagger\$}$
MAP (mmHg)	95 ± 4	96 ± 3	121 ± 4*	111 ± 4*†	105 ± 4$^{*\dagger\$}$
HR (beats/min)	354 ± 41	359 ± 44	421 ± 30*	410 ± 25*†	388 ± 31$^{*\dagger\$}$
HBF (ml/min/ 100 g tissue)	8.7 ± 0.5	8.8 ± 0.6	5.5 ± 0.6*	6.8 ± 0.3*†	7.4 ± 0.4$^{*\dagger\$}$
HVR (mmHg/ml/ min/100 g tissue)	10.9 ± 1.1	11.0± 1.2	21.9 ± 3.2*	16.4 ± 1.3*†	14.2 ± 1.3 $^{*\dagger\$}$

SBP, systolic blood pressure; DBP, diastolic blood pressure, MAP, mean arterial pressure; HR, heart rate; HBF, hindlimb blood flow; HVR, hindlimb vascular resistance.
Data are expressed as mean ± S.E.M. (n = 6-8/group). *p<0.05 compared to control group, †p<0.05 compared to Cd-treated group, $^{\$}$p<0.05 compared to Cd+AA15 mg/kg –treated group.

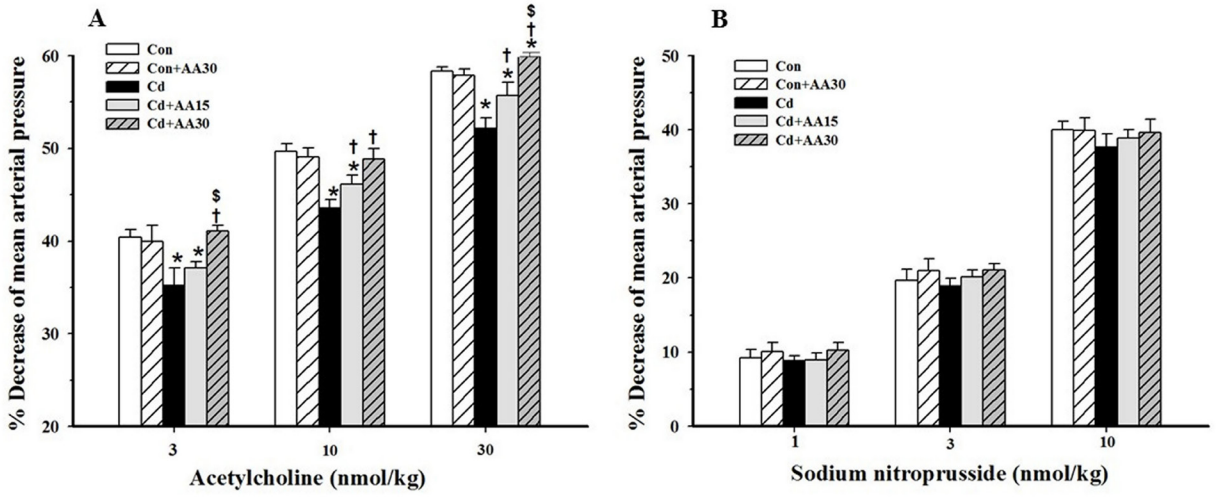

Figure 2 Effect of asiatic acid on mean arterial pressure in responses to acetylcholine and sodium nitroprusside at various concentrations. Data are expressed as mean ± S.E.M. (n = 6-8/group). *p<0.05 compared to control group, †p<0.05 compared to Cd-treated group, $p<0.05 compared to Cd+AA15 mg/kg –treated group.

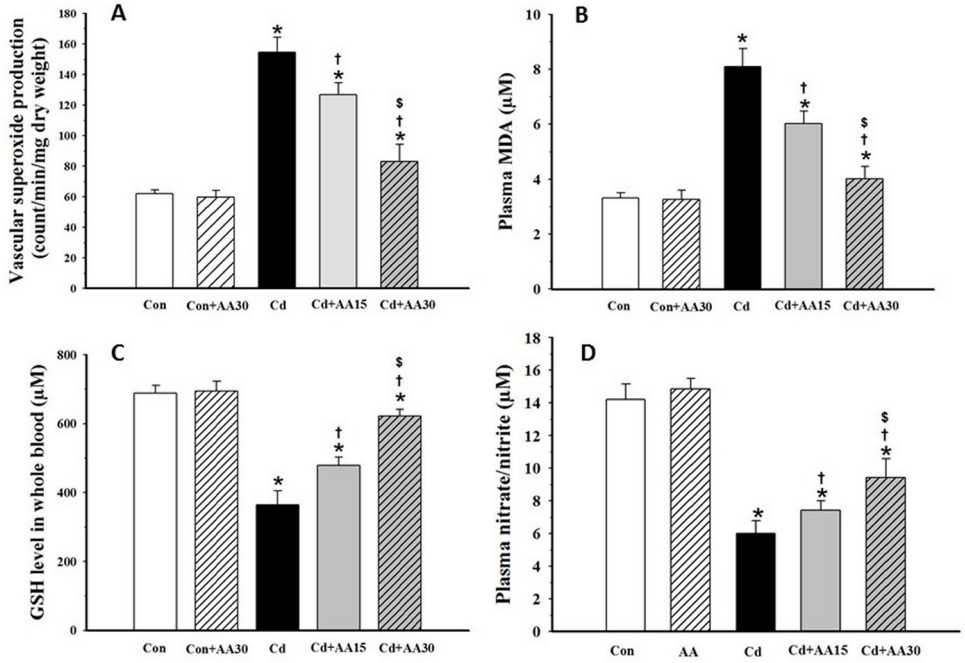

Figure 3 Effect of asiatic acid on vascular superoxide production (A), plasma malondialdehyde (MDA) (B), glutathione (GSH) level in whole blood (C) and plasma nitrate/nitrite (D) in all experimental groups. Data are expressed as mean ± S.E.M. (n = 6-8/group). *p<0.05 compared to control group, †p<0.05 compared to Cd-treated group, $p<0.05 compared to Cd+AA15 mg/kg –treated group.

contents in blood and tissues were very low in controls (Fig. 4). AA supplementation in a dose-dependent manner significantly reduced Cd contents in both blood and tissues ($p < 0.05$, Fig. 4).

Discussion

In this study, AA can restore the hemodynamics of rats exposed to Cd by lowering blood pressure, increasing hindlimb blood flow, reducing peripheral vascular resistance and decreasing heart rate. The improvement of hemodynamic status are associated with the increase in endothelial function, decrease in oxidative stress and reduction in Cd accumulations in the blood and tissues. It has been demonstrated that Cd decreased the functional availability of the potent vasodilator NO, which leads to increase blood pressure [13]. In consistent with our results, the increase in blood pressure of Cd exposed rats was accompanied by a

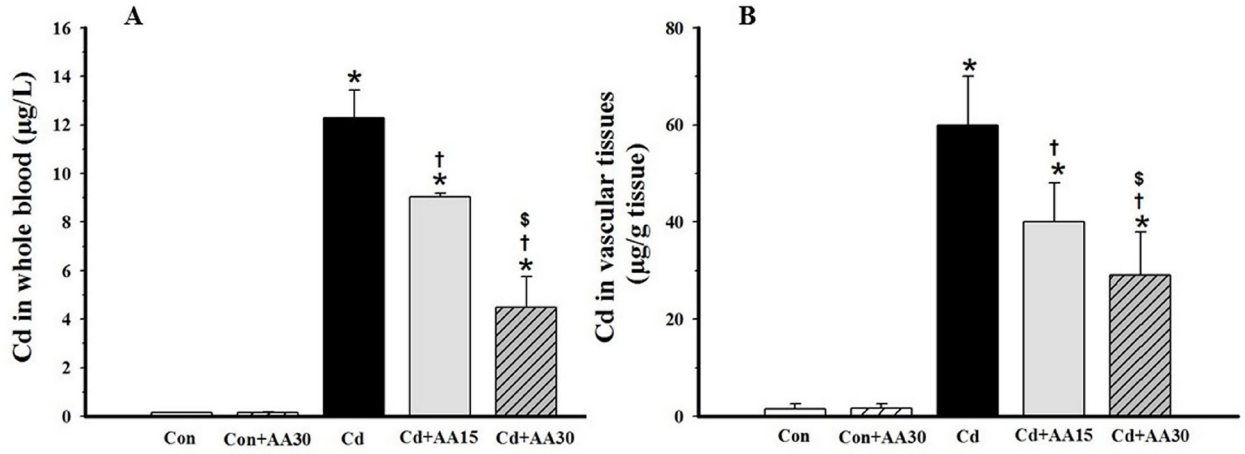

Figure 4 Effect of asiatic acid on cadmium content in blood (A) and vascular tissues (B) in all experimental groups. Data are expressed as mean ± S.E.M. (n = 6-8/group). *p<0.05 compared to control group, †p<0.05 compared to Cd-treated group, $p<0.05 compared to Cd+AA15 mg/kg –treated group.

blunted responses to endothelial-dependent vasodilator, ACh. Yoopan et al. suggested that the impairment of endothelial function after Cd exposure might be due to a dysfunction of muscarinic cholinergic and NO-dependent pathway in the vascular endothelium[14]. Moreover, L-arginine-NO pathway has been proposed to play an important role in Cd-induced endothelial dysfunction and vascular inflammation[15]. In agreement with this, we have found that endothelial NO synthase (eNOS) was decreased while inducible NO synthase (iNOS) was increased in the vascular tissues after Cd exposure[11], and tetrahydrocurcumin could modulate the eNOS/iNOS pathway. However, the effect of AA on eNOS/iNOS pathway is needed for further exploration.

It is evident that oxidative stress plays a key role in endothelial dysfunction and also exerts its effects in various pathophysiological conditions[16]. It is found that Cd interacts with reactive thiols in the mitochondrial membrane and causes mitochondrial permeability transition, which inhibits the respiratory chain reaction, thereby generates reactive oxygen species (ROS)[17]. $O_2^{\bullet-}$ is one of the major species of ROS that react with NO to form peroxynitrite, which causes NOS uncoupling in endothelial cells and leads to low NO bioavailability. As shown in our results, there was increased in $O_2^{\bullet-}$ production and reduced plasma nitrate/nitrite in Cd exposed rats, and these alterations were ameliorated after treatment with AA. The plausible mechanism involved with this effect is the antioxidant property of AA, which might be through the L-arginine-NO pathway since AA significantly stimulates the NO production. Moreover, AA also alleviated oxidative stress, increased antioxidant GSH and reduced Cd accumulation in the animal body after Cd exposure. Therefore, AA may be used as an antioxidant and metal chelator for detoxification.

Conclusion

The present study provides evidence about the beneficial effects of AA on reduced hypertension, improved endothelial function and alleviated oxidative stress in rats after exposure to Cd for 16 weeks. The ameliorating effects of AA against Cd toxicity may be mediated by its antioxidant and chelating properties. The overall findings support the idea of using Bua-Bok which contains high amount of asiatic acid as a food supplement to prevent hypertension and reduce oxidative stress in Cd-intoxication.

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