

**Effect of Tetrahydrocurcumin against Hypertension and Oxidative Stress in Rats with Combined Exposure to Lead and Cadmium**

**ผลของเทตราไฮโดรเคอร์คูมินต่อการต้านภาวะความดันเลือดสูงและภาวะเครียดออกซิเดชันในหนูแรทที่ได้รับตะกั่วและแคดเมียมร่วมกัน**

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**ABSTRACT**

Tetrahydrocurcumin (THU) is a strong antioxidant hydrogenated from curcumin. The present study aimed to investigate the therapeutic effect of THU (50 and 100 mg/kg) in rats with combined exposure to lead (Pb, 100 mg/L) and cadmium (Cd, 10 mg/L) via drinking water for 16 weeks. Rats treated with THU at weeks 12<sup>th</sup>-16<sup>th</sup> of Pb plus Cd exposure showed a significant reduction in blood pressure, hemodynamic disturbance, vascular dysfunction and oxidative stress when compared with Pb plus Cd treated controls (p<0.05). These ameliorative effects of THU were associated with down-regulation of NADPH oxidase (p47phox and gp91phox subunit) expression, a major source of vascular superoxide production, and up-regulation of eNOS expression. Data of this study suggest that THU is a promising antioxidant and chelating agent for the treatment of heavy metals poisoning.

**บทคัดย่อ**

เทตราไฮโดรเคอร์คูมิน (THU) เป็นสารต้านออกซิเดนต์ที่จากปฏิกิริยาไฮโดรจิเนชันของเคอร์คูมิน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลการรักษาของ THU (ขนาด 50 และ 100 มิลลิกรัม/กิโลกรัม) ในหนูแรทที่ได้รับน้ำดื่มที่ผสมตะกั่ว (100 มิลลิกรัม/ลิตร) และแคดเมียม (10 มิลลิกรัม/ลิตร) เป็นระยะเวลา 16 สัปดาห์ หนูแรทที่ได้รับ THU ระหว่างสัปดาห์ที่ 12-16 ของการได้รับตะกั่วผสมแคดเมียมพบว่ามีการลดลงของค่าความดันเลือด พลศาสตร์การไหลเวียนเลือดที่ผิดปกติ หลอดเลือดเสียหายที่ และภาวะเครียดออกซิเดชันเมื่อเทียบกับกลุ่มที่ได้รับตะกั่วผสมแคดเมียม (p<0.05) ผลบรรเทาความผิดปกติของ THU สอดคล้องกับการลดลงของระดับโปรตีน NADPH oxidase (p47phox and gp91phox subunit) ซึ่งเป็นแหล่งสำคัญในการสร้างซูเปอร์ออกไซด์ของหลอดเลือดรวมทั้งเพิ่มระดับโปรตีน eNOS การศึกษานี้ชี้ว่า THU เป็นสารต้านออกซิเดนต์และจับโลหะหนักซึ่งสามารถนำไปรักษาภาวะพิษจากการได้รับสารโลหะหนักได้

**Key Words:** Tetrahydrocurcumin, Cadmium, Lead, Hypertension, Vascular Dysfunction, Oxidative Stress

**คำสำคัญ:** เทตราไฮโดรเคอร์คูมิน แคดเมียม ตะกั่ว ความดันเลือดสูง หลอดเลือดเสียหายที่ ภาวะเครียดออกซิเดชัน

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## Introduction

Lead (Pb) and cadmium (Cd) are classified as toxic and non-essential elements. They pose a major public health concern because of their ubiquitous presence and global contamination in the environment as well as their established toxicity. Several studies suggest that exposure to Pb or Cd separately contributes to the etiology of chronic diseases, such as cardiovascular disease (CVD) (Navas-Acien., et al. 2007; Tellez-Plaza et al., 2012).

Cd exposure is associated with hypertension by promoting the proliferation of vascular smooth muscle cells (VSMCs) and collagen deposition (Kukongviriyapan et al., 2014) resulting in increased vascular resistance (Martynowicz et al., 2004). Meanwhile, Pb impairs vasomotor tone and increases VSMCs proliferation (Bhatnagar, 2006). Moreover, a marked increase in oxidative stress during Cd or Pb exposure affects the vascular cells by reducing the availability of the vasodilator nitric oxide (NO), a key regulator of vascular homeostasis. It is suggested that a direct oxidized endothelial nitric oxide synthase (eNOS), may lead to vascular damage and dysfunction (Navas-Acien et al., 2004).

Generally, people have been continuously exposed to more than one metal at a time. Since Pb and Cd are widely dispersed in the environment and considered to be the most toxic metals to humans and animals, therefore, the present study was designed to evaluate the toxic effects of chronic exposure to low levels of Cd and Pb in experimental animals.

It has been demonstrated that heavy metals-induced oxidative stress are attenuated after antioxidant treatment (Donpunha et al., 2011). Therefore, one can postulate that antioxidant may be

able to restore the vascular function and attenuate hypertension occurred after exposure to toxic metals. Tetrahydrocurcumin (THU) is an antioxidative substance which is derived from curcumin of *Curcuma Longa* Linn. (Turmeric) by hydrogenation. THU effectively protects against endothelial dysfunction, oxidative stress and hypertension (Nakmareong et al., 2012). However, the effect of THU on alleviation of the toxicities of heavy metals exposures is still lacking.

## Objective of the study

To investigate the effect of THU on hemodynamic status, vascular function and oxidative stress in rats with chronically exposed to low levels of lead and cadmium.

## Methodology

### Animals and treatments

Adult male Sprague-Dawley rats, weighing 160-180 g. were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The experimental protocol was approved by the committee on the Ethics of Animal Experiments of the Khon Kaen University (AEKKU 4/2555). After an adaptation periods of seven days, rats were randomly allocated to four groups (n=6/group): **(1) control group;** received deionized water, **(2); Pb+Cd group;** received a drinking water contained lead acetate ( $\text{PbAc}_2$ , 100 mg/L.) plus cadmium chloride ( $\text{CdCl}_2$ , 10 mg/L.), **(3) and (4) Pb+Cd+THU50 and ; Pb+Cd+THU100;** received a drinking water contained Pb plus Cd and orally administered with THU at doses of 50 and 100 mg/kg/day, respectively.

PbAc<sub>2</sub> and CdCl<sub>2</sub> were dispersed in deionized water as drinking water for sixteen weeks. THU (50 or 100 mg/kg BW) was dissolved in propylene glycol (PG) and orally administered once daily to rats starting from week 12<sup>th</sup> to 16<sup>th</sup> of Pb plus Cd exposure. PG was also administered to group (2).

#### **Hemodynamic status and vascular responsiveness**

At the end of experiments, rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Systolic blood pressure (SP), diastolic blood pressure (DP), mean arterial pressure (MAP), heart rate (HR) and hindlimb blood flow (HBF) were measured as a previous described (Boonla et al., 2014). Hindlimb vascular resistance (HVR) was calculated from MAP divided by HBF. Thereafter, vascular responsiveness to an endothelium-dependent vasodilator, acetylcholine (ACh) at doses of 3, 10 and 30 nmol/kg was determined.

#### **Assay of oxidative stress markers and antioxidant glutathione**

The production of superoxide anion (O<sub>2</sub><sup>•-</sup>) in rat aortas was detected by lucigenin-enhanced chemiluminescence method as previously described (Nakmareong et al., 2012). The malondialdehyde (MDA), a lipid peroxidation marker, was assessed in the plasma using the TBA assay (Kukongviriyapan et al., 2014). Glutathione (GSH) in the blood was determined spectrophotometrically following a previously described method (Boonla et al., 2014).

#### **Western Blot Analysis**

The expression levels of eNOS and NADPH oxidase (gp91phox and p47phox subunits) proteins were determined in the aortic homogenates by Western blotting as previous described (Kukongviriyapan et al., 2014). Immunoblotting with β-actin was used as an internal control of protein loading. The intensity value was normalized to β-actin

and then expressed as percentage changes from the normal controls.

#### **Statistical analysis**

Data obtained were expressed as mean ± S.E., and n refers to the number of animals used. The significance of differences between means was analyzed by one-way analysis of variance (ANOVA) and followed by post-hoc Duncan's multiple range test. Statistical significance was assigned at a p value of less than 0.05.

### **Results**

#### **THU improves hemodynamics and vascular function**

Administration of THU at a dose of 50 or 100 mg/kg did not alter arterial blood pressure or heart rate in normal control rats (data not shown). A combined exposure to low levels of Pb and Cd caused a significant increase in systolic, diastolic, mean arterial blood pressure and heart rate when compared with the normal controls (p<0.05, Table 1). THU at a dose-dependent manner significantly reduced arterial blood pressure and HR of rats with combined exposure to Pb and Cd (p<0.05, Table 1). It is noted that THU at high dose (100 mg/kg) was able to maintain blood pressure to near normal values. Increased arterial blood pressure in rats exposed to Pb plus Cd was associated with decreased HBF and increased HVR. A significant increase in HBF was found after THU treatment, thereby HVR was reduced in Pb+Cd exposed rats (p<0.05, Table 1).

Impairment of vascular responses to ACh was found in rats exposed to Pb and Cd (Figure 1). Suggesting that Pb and Cd disturb the endothelial function. Interestingly, THU at high dose significantly restored endothelial responsiveness when compared with Pb+Cd-treated controls.

**Table 1** Effect of THU on hemodynamics in all experimental groups

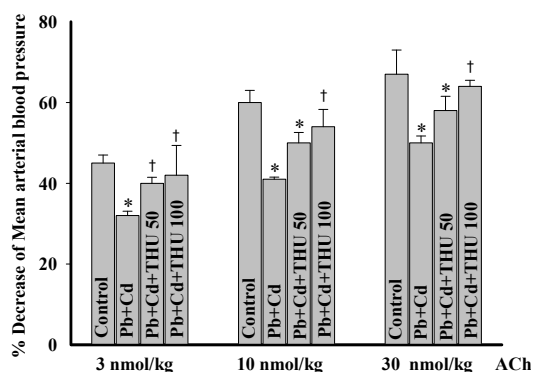
Parameters	Control	Pb+Cd		
		Vehicle	THU 50	THU 100
Systolic pressure (mmHg)	118.5±2.1	168.7±1.9 <sup>*</sup>	141.4±3.8 <sup>*†</sup>	132.5±2.7 <sup>*†‡</sup>
Diastolic pressure (mmHg)	79.3±1.7	108.1±1.4 <sup>*</sup>	95.7±2.8 <sup>*</sup>	84.5±2.2 <sup>†</sup>
MAP (mmHg)	92.2±2.0	128.9±3.7 <sup>*</sup>	110.4±2.7 <sup>†</sup>	100.4±2.1 <sup>†</sup>
Heart rate (beats/min)	340.6±3.8	428.4±6.3 <sup>*</sup>	388.2±5.9 <sup>*</sup>	369.7±4.1 <sup>*†‡</sup>
HBF (ml/min/100 g tissue)	7.8±0.9	4.6±0.2 <sup>*</sup>	5.9±0.8 <sup>*</sup>	6.3±0.7 <sup>‡</sup>
HVR (mmHg/ml/min/100 g tissue)	12.0±1.2	27.7±2.8 <sup>*</sup>	18.6±1.5 <sup>*</sup>	15.8±1.8 <sup>‡</sup>

Results are expressed as mean ± SEM., n=6/group. MAP, mean arterial pressure; HBP, hindlimb blood flow; HVR, hindlimb vascular resistance. <sup>\*</sup>p<0.05 vs. controls; <sup>†</sup>p<0.05 vs. Pb-Cd treated group; <sup>‡</sup>p<0.05 vs. Pb-Cd+THU 50-treated group.

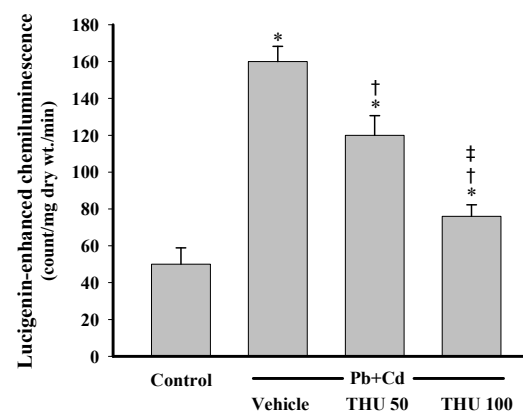
**THU alleviates oxidative stress and improves antioxidant status**

There was a marked increase in rate of vascular O<sub>2</sub><sup>•-</sup> production in Pb+Cd-treated rats as compared to normal controls (p<0.05, Figure 2).

The increase of O<sub>2</sub><sup>•-</sup> level was associated with an increase in plasma MDA and a reduction of blood GSH (p<0.05, Figure 3, 4). These results confirmed that increased oxidative stress was found after Pb and Cd exposure. THU dose-dependently

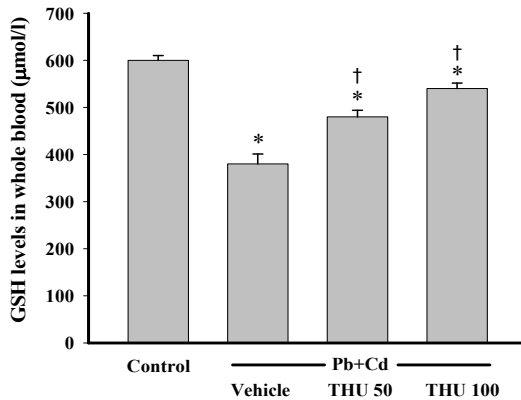


**Figure 1** Effect of THU on vascular responsiveness to acetylcholine (ACh) in all experimental groups. Results are expressed as mean ± SEM., n=6/group. <sup>\*</sup>p<0.05 vs. controls; <sup>†</sup>p<0.05 vs. Pb-Cd treated group.

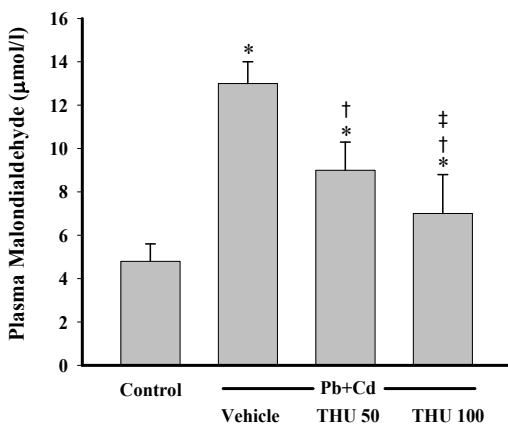


reduced both vascular O<sub>2</sub><sup>•-</sup> and plasma MDA levels. **Figure 2** Effect of THU on vascular O<sub>2</sub><sup>•-</sup> production in all experimental groups. Results are expressed as mean ± SEM., n=6/group. <sup>\*</sup>p<0.05 vs. controls; <sup>†</sup>p<0.05 vs. Pb-Cd treated group, <sup>‡</sup>p<0.05 vs. Pb-Cd+THU 50-treated group.

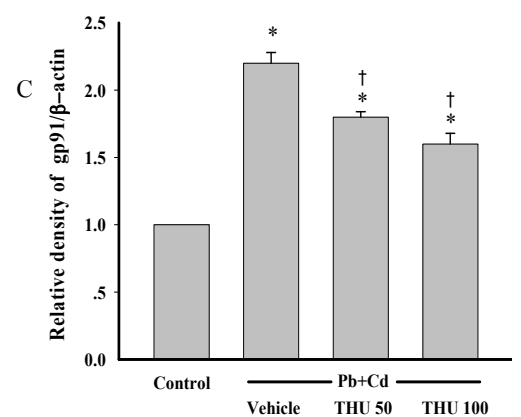
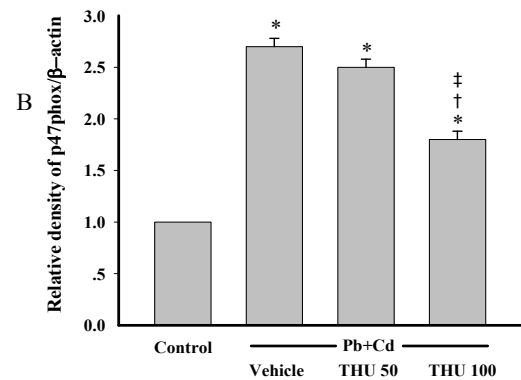
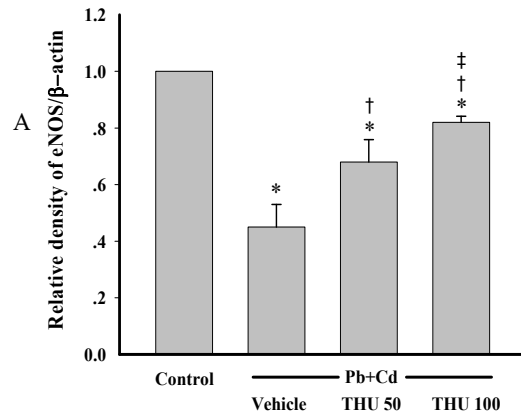
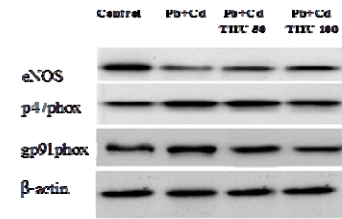
( $p < 0.05$ , Figure 2-3). Moreover, THU also recovered the blood GSH ( $p < 0.05$ , Figure 4). Interestingly, it is found that the antioxidant activity of THU was correlated with decrease in blood pressure and improvement of vascular function in Pb+Cd-treated



**Figure 3** Effect of THU on plasma malondialdehyde in all experimental groups. Results are expressed as mean  $\pm$  SEM.,  $n=6$ /group. \* $p < 0.05$  vs. controls; † $p < 0.05$  vs. Pb-Cd treated group,



**Figure 4** Effect of THU on GSH levels in whole blood in all experimental groups. Results are expressed as mean  $\pm$  SEM.,  $n=6$ /group. \* $p < 0.05$  vs. controls; † $p < 0.05$  vs. Pb-Cd treated group, ‡ $p < 0.05$  vs. Pb-Cd+THU 50- treated group.



**Figure 5** Effect of THU on eNOS (A), p47phox (B) and gp91phox (C) protein expression. Results are expressed as mean  $\pm$  SEM.,  $n=6$ /group. \* $p < 0.05$  vs. controls; † $p < 0.05$  vs. Pb-Cd treated group.

### THU induces up-regulation of eNOS and down-regulation of NADPH oxidase

Endothelial nitric oxide synthase (eNOS) is responsible for most of vascular NO production. In this study, down-regulation of eNOS protein was found in the aortic tissues of Pb+Cd-treated rats when compared with normal controls ( $p < 0.05$ , Figure 4). Administration with THU significantly increased the eNOS protein expression when compared with the Pb+Cd treated group ( $p < 0.05$ , Figure 4). These results suggest that the restoration of hemodynamic status and endothelial function by THU is associated with up-regulation of eNOS.

In vasculature, the enzymatic source of  $O_2^{\cdot-}$  is NADPH oxidase. Over-activity of NADPH oxidase can cause vascular oxidative stress from excessive  $O_2^{\cdot-}$  production. In this study, we examined gp91phox and p47phox, two regulatory subunits of NADPH oxidase in aortic homogenates. Pb plus Cd increases NADPH oxidase activity as demonstrated by the increase in gp91phox and p47phox expressions in Pb+Cd exposed rats (Figure 5). This confirms that NADPH oxidases, especially p47phox and gp91phox, are the dominant enzymatic source of vascular  $O_2^{\cdot-}$  production. THU supplementation was able to suppress these protein expressions. These results were consistent with a reduction in  $O_2^{\cdot-}$  levels in the aortas of rats treated with THU.

### Discussion and Conclusions

The important findings of this study are that chronic exposure to Pb and Cd induced hypertension, hemodynamic disturbance, endothelial dysfunction and oxidative stress. Supplementation with THU during Pb and Cd exposure preserves vascular function and mitigates oxidative stress.

It has been suggested that heavy metals-induced oxidative stress play an important role in the pathogenesis of hypertension by enhancing vascular dysfunction and decreasing NO bioactivity (Fioresi et al., 2013; Kukongviriyapan et al., 2014). Cd, itself can increase (Patra et al., 2011). The present study demonstrated that Pb+Cd-induced high blood pressure is associated with decreased NO bioavailability, since a large amount of  $O_2^{\cdot-}$  rapidly reacted with NO to form peroxynitrite (ONOO<sup>-</sup>) (Zielonka et al., 2010). ONOO<sup>-</sup> potentially switches eNOS from a NO-generating to a superoxide-generating enzyme or eNOS uncoupling (Zielonka et al., 2010). Reduced aortic eNOS protein expression in Pb+Cd-treated rats as found in this study may be due to reduced functional eNOS homodimer and the down-regulation of eNOS gene expression.

The suppression of the vascular response to ACh after Pb+Cd exposure indicated that Pb+Cd exposure induced endothelial dysfunction. This might be due to a down-regulation of eNOS expression, as found in this study and other studies on heavy metals-induced endothelial damage (Prozialeck et al., 2008). Moreover, reduced endothelium-dependent vasodilation also related to increased vascular tone which contributes to the increased vascular resistance as found in this study.

Pb and Cd co-exposure caused an increase in  $O_2^{\cdot-}$  production. Further exploration of the enzymatic source of  $O_2^{\cdot-}$  generation in this study revealed that gp91phox (NOX2), a catalytic subunit and p47phox, a cytosolic subunit of NADPH oxidase were over-expressed in Pb+Cd exposed animals. This suggests that the vascular  $O_2^{\cdot-}$  was generated from this enzyme.

In the present study, we found that THU dose-dependently decreased  $O_2^{\cdot -}$  production and reduced oxidative stress in rats exposed to Pb+Cd. The plausible mechanisms may act via the suppression of NADPH oxidase activity by inhibiting the gp91phox subunit with p47phox (Drummond et al., 2011). Moreover, it has been demonstrated that the beta-diketone group of THU suppressed NADPH oxidase as an outcome from suppression of NF-KB activation (Rahman et al., 2006).

In conclusion, results obtained in this study provide the experimental evidence concerning the ameliorative effects of THU on reduction of hypertension, improvement of hemodynamics and endothelial function, and alleviation of oxidative stress in rats with combined exposure to low levels of Pb and Cd. Collectively, data of this study suggest the beneficial effect of THU as a dietary supplement following heavy metals-induced hypertension and vascular dysfunction.

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