



## Effect of caffeic acid derivatives on polychlorinated biphenyls induced hepatotoxicity in male mice

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### Abstract

Chronic exposure to coplanar polychlorinated biphenyls (PCBs), a potent inducer of toxic reactive oxygen species (ROS), in the environment and food can cause liver diseases. It remains unknown whether caffeic acid derivatives (CADs) exerted protective effect on PCB-induced hepatotoxicity. We sought to evaluate the activities of 3 CADs on PCB169-induced oxidative stress and DNA damage in the liver. Male ICR mice were administered with 1  $\mu$ mol/mL PCB169 at 5 mL/kg body weight for 2 weeks. The mice were given CADs by gastric gavage for 3 weeks. We found that PCB169 decreased the growth rate and reduced the levels of superoxide dismutase (SOD), glutathione (GSH) and GSH peroxidase (GPx). It increased the liver weight, malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels and CYP1A1 activity in the liver tissues and plasma of mice ( $P < 0.05$ ). Pretreatment of mice with CADs restored the above parameters to normal levels. There was a synergistic protective effect between CADs in preventing MDA and 8-OHdG formation and inducing CYP1A1 and phase II metabolism enzyme (SOD, GPx) activities ( $P < 0.05$ ). In conclusion, PCB169 induced hepatotoxicity and pretreatment with CADs had synergistic protective effects on liver damage.

**Keywords:** caffeic acid derivatives (CADs), PCB169, 8-OHdG, synergy, hepatotoxicity

### INTRODUCTION

Polychlorinated biphenyls (PCBs), a family of 209 different congeners, were produced and widely used in industrial settings due to their stability under a broad range of chemical, thermal and electrical conditions<sup>[1]</sup>. Although the production of these chemicals was halted in the 1970s, their resistance to breakdown and their lipophilicity allow them to be biologically magnified in the food chain and persist in the environment<sup>[2]</sup>. Humans are exposed to PCBs primarily from dietary intake of contaminated foods. As PCBs are lipid solu-

ble, humans can accumulate them in tissues<sup>[3]</sup> and such bioaccumulation causes human health concern<sup>[4]</sup>. It has demonstrated previously that PCBs, especially coplanar PCBs, can lead to multiple negative effects on health, such as liver damage<sup>[5-8]</sup>.

In the family of PCBs, the most toxic of these PCBs is 3, 3', 4, 4', 5, 5'-hexachlorobiphenyl (PCB169). Previous studies have shown that PCB169 has led to changes in many parameters associated with liver damage, such as superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione (GSH)<sup>[9,10]</sup>. Whether food ingredients can affect the above factors

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and have protective effect on PCB-induced hepatotoxicity remain to be fully elucidated.

Polyphenol compounds contained in plant derived foods, such as coffee beans, fruits, vegetables, olive oils and wines, have been reported to exert antitumor, anti-inflammatory and antioxidant properties<sup>[11,12]</sup>. Caffeic acid derivatives (CADs) such as chlorogenic acid (ChA), ferulic acid (FeA), and rosmarinic acid (RoA) are common polyphenol compounds in traditional Chinese diet. A previous study reported that such phytochemicals protect the liver from oxidative damage by enhancing the anti-oxidative defense system<sup>[13]</sup>. However, whether these CADs have resistance to oxidative damage induced by PCB169 has not been well documented. A growing number of in vitro and in vivo studies indicated that combinations of dietary chemo-preventive agents can sometimes result in significant activities at concentrations where any single agent is inactive<sup>[14-16]</sup>. In natural plant foods, combinations of phytochemicals are likely to influence the effects by affecting overlapping and complementary mechanisms<sup>[16]</sup>. On the other hand, isolated pure compounds may lose their biological activity or may not behave in the same way as in the complex matrix of the original food item. Therefore, in addition to the characterization of chemo-preventive effects of individual compounds, evaluation of synergistically acting phytochemicals is of particular interest. In the present investigation, we hypothesized that a variety of CADs have synergistic protective effect on PCB196-induced hepatotoxicity by adjusting phase II metabolic enzymes and oxidative stress index in male ICR mice.

## MATERIALS AND METHODS

### Chemicals

ChA, FeA, and RoA with purity of more than 99% were purchased from Zelang Medical Technology Co., LTD, Nanjing, China. 3, 3', 4, 4', 5, 5'-hexachlorobiphenyl (PCB169, > 99% purity) was obtained from Sigma-Aldrich (St. Louis, MO). Kits for testing oxidative parameters were all purchased from Jiancheng Bioengineering Institute, Nanjing, China.

### Animals

The study protocol was approved by the authors' affiliated institutions. Animal welfare and the experimental procedures were carried out strictly in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council of USA, 1996). Male ICR mice (5 weeks old, weighing 25–30 g at the beginning of the experiments) were purchased from

Nanjing Medical University Animal Center (Nanjing, China) and acclimatized to the environment for 3 weeks prior to use. They were given food and distilled water *ad libitum* and were housed in individual wire cages under controlled conditions with a temperature of  $22 \pm 1^\circ\text{C}$ , a humidity of  $50 \pm 10\%$  and a 12-hour light-dark cycle.

One  $\mu\text{mol/mL}$  PCB169 was dissolved in corn oil. The animals received 1  $\mu\text{mol/mL}$  PCB169 with 5 mL/kg body weight (BW) for 2 weeks. The animals were randomly divided into 9 groups (G1 ~ G9) with 10 animals per group and the following agents were given by gastric gavage for 3 weeks: Group 1, physiological saline (blank); Group 2, corn oil (control); Group 3, ChA; G4, FeA; Group 5, RoA; Group 6, ChA + FeA (1:1); Group 7, ChA + RoA (1:1); Group 8, FeA + RoA (1:1); Group 9, ChA + FeA + RoA (1:1:1). The final concentration of CADs was 150  $\mu\text{g/mL}$ , and animals were given a single gavage dose of CADs of 10 mL/kg BW. Two weeks after the gavage, the eyeballs were removed and each liver was excised, weighed and stored at  $-80^\circ\text{C}$  until further analysis. Metabolic cages were used to collect urine of mice 24 hours the day before the mice were sacrificed.

### Measurement of CYP1A1 activity

CYP1A1 activity in hepatic microsomal fractions was determined according to a slightly modified method of Martin<sup>[17]</sup> by fast determination of the ethoxyresorufin-*O*-deethylase activity with a fluorescence plate-reader which was expressed as nmol/min/mg pro. The monooxygenase reaction was detected by spectrophotometry at excitation wavelength of 550 nm and emission wavelength of 585 nm.

### Measurement of lipid peroxidation and hepatic GSH contents

The steady-state level of MDA was analyzed by measuring the level of thiobarbituric acid reactive substances (TBARS) spectrophotometrically at 532 nm by using 1, 1, 3, 3-tetraethoxypropane (Sigma) as the standard. The level of total GSH was measured spectrophotometrically at 412 nm with yeast GSH reductase, 5, 5'-dithio-bis(2-nitrobenzoic acid) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)<sup>[18]</sup>. The GSH peroxidase (GSH-Px) activities in the liver tissue homogenate were determined by using the total GSH peroxidase assay kit. Briefly, total GSH-Px activities were determined by using cumene hydro-peroxide as the substrates. Absorbance change at 340 nm was followed in a Beckman DU-800 for 5 minutes. One unit of enzymatic activity was defined as the amount of protein that oxi-

dizes 1  $\mu\text{m}$  of NADPH per minute, expressed as units per mg protein.

### Measurement of hepatic SOD activities and the 8-OHdG levels in urinary

Hepatic SOD activities and urinary 8-OHdG levels were determined according to the manufacturer's instructions ((Jiancheng Bioengineering Institute). SOD activities were expressed as units of SOD activity per milligram protein. 8-OHdG was expressed in ng/L. Metabolic cages were performed to collect urine of mice 24 hours a day.

### Statistics

The effect of PCB169 treatment and CADs on various responses was studied by using ANOVA and factorial analysis. The overall significance of the results was examined by using SAS 9.0. The differences between groups were considered statistically significant at  $P < 0.05$  with the appropriate Bonferroni correction made for multiple comparisons. The results were presented as mean  $\pm$  SEM.

## RESULTS

### Growth rate and organ weights

The growth rate of mice treated with PCB169 significantly decreased in a dose dependent manner. Co-administration of 2 or 3 CADs significantly attenuated the decrease in the growth rate than the administration of ChA, FeA, RoA, corn oil and saline alone at the same dosage of PCB169 induction. Interestingly, Group 9 of ChA + FeA + RoA (1:1:1) had a better inhibitory effect on the growth rate compared to Group 6, 7 and 8 with 2 kinds of CADs, indicating a

synergistic protective action of CADs on animal growth inhibition induced by PCB169.

Furthermore, the relative liver weight (as percentage of liver/BW) was significantly increased along with the increase in the dosage of PCB169 except for Group 9 treated with 1  $\mu\text{mol/mL}$  PCB. The combined use of 2 or 3 kinds of CADs had a significant inhibitory effect on liver tissue swelling induced by PCB169 ( $P < 0.05$ ), but CADs alone did not have such effect (**Table 1**).

### Effects on CYP1A1 activity

CYP1A1 induction was examined in the liver (**Table 1**). PCB169 significantly increased hepatic microsomal cytochrome P-450 enzyme activities ( $P < 0.05$ ). The CYP1A1 activity in the combination groups of 2 (Group 6 to 8) or 3 (Group 9) types of CADs was significantly lower than that of the control group ( $P < 0.05$ ), but it was not observed in the single CAD group (Group 3 to 5). PCB169-induced CYP1A1 expression was significantly reduced when the animals were pretreated with 2 or 3 CADs in combination at 1.5 mg/kg body weight ( $P < 0.05$ ).

### Malondialdehyde (MDA) analysis

MDA levels increased with increasing doses of PCB169. FeA had synergistic action with RoA (Group 8) and ChA plus RoA (Group 9) in significantly attenuating increase in MDA levels ( $P < 0.05$ ), while ChA combined with FeA (Group 6) and RoA (Group 7) reduced MDA changes (**Table 2**). The combination of ChA + FeA + RoA (1:1:1) reduced the increased levels of MDA by 50.8% compared to normal saline (Group 1). Single CADs (Group 3 to 5) exhibited no effect on changes in MDA levels induced by PCB169. Administration of 2 or 3 CADs combination significantly attenuated the PCB-induced

**Table 1** Effect of CADs on growth rate and relative liver weight and liver CYP1A1 activities in PCB169-treated mice

Group	Growth rate (%)	Relative liver weight (%)	Liver CYP1A1 activities (nmol/minute/mg Pro)
G1 (saline)	2.41 $\pm$ 0.25	12.31 $\pm$ 0.16	2.59 $\pm$ 0.13
G2 (corn oil)	2.42 $\pm$ 0.36	13.01 $\pm$ 0.17	2.52 $\pm$ 0.15
G3 (ChA)	2.11 $\pm$ 0.44	11.33 $\pm$ 0.11	2.51 $\pm$ 0.13
G4 (FeA)	2.20 $\pm$ 0.15	11.31 $\pm$ 0.23	2.07 $\pm$ 0.15
G5 (RoA)	2.33 $\pm$ 0.27	11.32 $\pm$ 0.19	2.63 $\pm$ 0.21
G6 (ChA+ FeA)	2.11 $\pm$ 0.26	10.31 $\pm$ 0.22*	2.03 $\pm$ 0.18*
G7 (ChA+RoA)	2.01 $\pm$ 0.37	10.21 $\pm$ 0.24*	2.09 $\pm$ 0.22*
G8 (FeA+RoA)	2.12 $\pm$ 0.13	10.12 $\pm$ 0.12*	2.02 $\pm$ 0.10*
G9 (ChA+ FeA+RoA)	1.91 $\pm$ 0.24	9.33 $\pm$ 0.11*	1.16 $\pm$ 0.23*

Data are expressed as mean  $\pm$  SEM ( $n=10$ ). One-way ANOVA was performed to examine statistically significant differences ( $P < 0.05$ ) between each different combinations of CADs level and the corn oil treatment (indicated by "\*" if significant). CAD: caffeic acid derivatives.

**Table 2** Effect of CADs on serum MDA and GSH and GSH-Px levels in treated mice

Group	MDA (mmol/mg Pro)	GSH (mg/g Pro)	GSH-Px (U/mg Pro)
G1 (saline)	2.97 ± 0.49	1.71 ± 0.24	71.1 ± 1.3
G2 (corn oil)	2.93 ± 0.55	2.01 ± 0.20	77.3 ± 1.2
G3 (ChA)	2.71 ± 0.93	2.89 ± 0.14*	110.1 ± 2.3*
G4 (FeA)	2.67 ± 0.55	2.62 ± 0.12*	113.4 ± 2.0*
G5 (RoA)	2.63 ± 0.91	2.43 ± 0.41*	110.7 ± 2.1*
G6 (ChA+ FeA)	2.17 ± 0.18*	4.03 ± 0.25*	136.3 ± 2.4*
G7 (ChA+RoA)	2.09 ± 0.57*	3.93 ± 0.15*	130.2 ± 2.3*
G8 (FeA+RoA)	2.11 ± 0.20*	3.67 ± 0.35*	110.7 ± 1.7*
G9 (ChA+ FeA+RoA)	1.46 ± 0.23*	4.19 ± 0.19*	143.6 ± 3.3*

Data are expressed as mean ± SEM ( $n=10$ ). One-way ANOVA was performed to examine statistically significant differences ( $P<0.05$ ) between each different combinations of CADs level and the corn oil treatment (indicated by “\*” if significant).

changes in CYP1A1 and MDA ( $P<0.05$ ), but the administration of CADs alone had no effect.

### Effects on glutathione (GSH) and GSH peroxidase (GSH-Px) activities

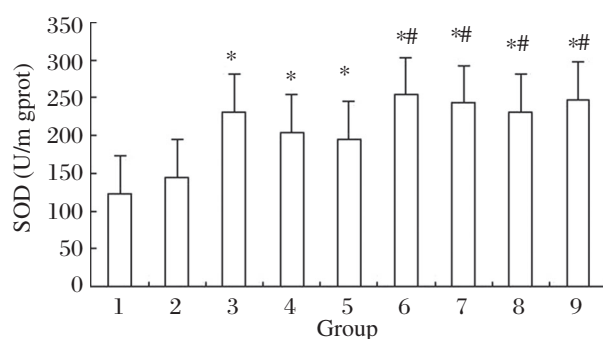
GSH levels in the liver were determined as indicators of oxidative stress. Hepatic levels of both oxidized (GSSG) and reduced GSH were decreased by 3.4% (Group 2) ~ 20.4% (Group 1), indicating that PCB169 caused an overall decrease in hepatic total GSH in mice. Meanwhile, we found similar changes in GSH-Px activity in PCB169 treated mice. Therefore, we concluded that PCB 169 decreased GSH and GSH-Px levels. As shown in **Table 2**, the single or combination use of CADs significantly increased GSH and GSH-Px levels compared to corn oil (Group 2) or normal saline (Group 1) at the same PCB169 concentration ( $P<0.05$ ). The administration of 3 kinds of CADs with equal proportion (Group 9) significantly increased GSH level by 2.45 fold and GSH-Px activity by 2.02 fold, respectively, compared to normal saline alone (Group 1) with the same dosage of PCB169. The CADs-mediated increase in phase II antioxidant enzyme GSH-Px was in part maintained even in the presence of PCB169.

### Effects on hepatic SOD activities

SOD activity was decreased significantly with increasing doses of PCB169 (**Fig. 1**). However, SOD activity was increased significantly ( $P<0.05$ ) by CADs compared to that of mice treated with normal saline (Group 1) or corn oil (Group 2). ChA alone and the combination of 2 CADs attenuated decreases in SOD activities in all groups.

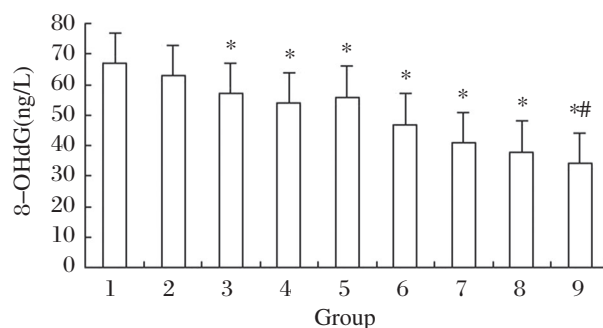
### Effects on urinary levels of 8-OHdG

PCB168 increased the urinary levels of 8-OHdG (**Fig. 2**). CADs alone or in combination exerted sig-



**Fig. 1** Effect of CADs on SOD activities in the PCB169-treated mice. One-way ANOVA was performed to examine statistically significant differences ( $P<0.05$ ) between each different combination of CADs level and the corn oil treatment (indicated by “\*” if significant, “#” indicates significant difference of physiological saline treatment.  $P<0.05$ ). CAD: caffeic acid derivatives.

nificant inhibitory effects on PCB169-induced increase of urinary 8-OHdG levels compared to controls ( $P<0.05$ ). Furthermore, the mixed CADs of ChA + FeA + RoA (1:1:1) had the best synergistic inhibitory effects on 8-OHdG.



**Fig. 2** Effect of CADs on 8-OHdG level in the PCB169-treated mice. One-way ANOVA was used to examine statistically significant differences ( $P<0.05$ ) between each different combinations of CADs level and the corn oil treatment (indicated by “\*” if significant, “#” indicates significant difference of physiological saline treatment.  $P<0.05$ ).

## DISCUSSION

Previous studies report significant liver damage after exposure of mice to high doses of PCB169<sup>[19,20]</sup>. We showed that PCB169 inhibited the growth and reduced the body weight of mice, and it also induced DNA damage as indicated by increased 8-OHdG levels. 8-OHdG was significantly increased by PCB169 in a dose-dependent manner, which is in agreement with the previous findings<sup>[21]</sup>, which immediately oxidizes proteins and cellular DNA<sup>[22]</sup>. This was similarly due to the effects of acute TCDD toxicity<sup>[23]</sup>.

PCB169 is known to induce CYPs, particularly CYP1A. CYP1A1 is not constitutively expressed, but its synthesis can be greatly enhanced by aryl hydrocarbon receptor (AhR) - driven up regulation of gene expression. Dioxin-like PCBs exert their toxicities by activating the AhR, a ligand dependent transcription factor, in a similar manner to that of the most toxic dioxin, TCDD. However, previous study showed that PCB169-liganded AhR behaves differently from TCDD-liganded AhR in immune cells<sup>[5]</sup>. PCBs induce liver cancer in rodents and possibly in humans<sup>[25]</sup>. Although their mechanism of carcinogenicity is unknown, it is assumed that AhR activation may be involved at least in the promoting activity of certain PCBs<sup>[26]</sup>. PCB169 is the most potent AhR agonist of all 209 PCB congeners. Increase in liver weight is a well known consequence of AhR-mediated increase in the endoplasmic reticulum. Histological analysis confirmed this effect in all PCB doses (data not shown). Consistent with the higher liver weight, PCB169 led to an increase in CYP1A1 activity. The higher CYP1A1 activity in the high PCB169 dosage groups may be due to oxidative activation of the enzyme by ROS or diminished cellular resources available to support protein synthesis<sup>[27]</sup>.

It has been proposed that the consumption of phenolic compounds contributes to the maintenance of human health through modulation of a variety of molecular mechanisms<sup>[29]</sup>. CADs are important for health and antioxidant defense<sup>[30]</sup>. CADs were shown to inhibit methamphetamine induced oxidative stress and DNA damage<sup>[11]</sup>, and to be promising in protecting against cancer in animals and humans<sup>[31]</sup>. In this study, oxidative DNA damage induced by PCB169 was inhibited by CADs whether singly or in combination. Thus, moderate intake of CADs is beneficial to the body and may decrease the risk of oxidative stress. Mechanisms of protective properties of plant-derived polyphenols such as CADs are not simple but may involve induction of phase II antioxidant enzymes. We showed here that CAD treatment markedly induced

both SOD and GSH-Px in a dose-dependent manner. The CAD-mediated increase in these phase II antioxidant enzymes was in part maintained even in the presence of PCB169. Our results showed that increased liver and serum of SOD, GSH-Px and CYP1A1 activities. This increase in protective antioxidant enzyme activity may ameliorate oxidative stress induced by dioxin-like PCB congeners.

A growing number of in vitro and in vivo studies indicated that combinations of dietary chemo-preventive agents may result in significant activities at concentrations where any single agent is inactive. There is accumulating evidence that the actions of phytochemicals administered as dietary supplements alone do not explain the observed health benefits of diets rich in fruits, vegetables and whole grains. The interaction between the CADs in this study showed efficacy in reducing oxidative stress and improving the protective effects on DNA damage. These results suggest that CADs are synergistic in reducing the effects of PCB on the liver and could prove to be useful in alleviating liver toxicity of PCB.

In summary, exposure to PCB169 induced hepatotoxicity and oxidative DNA damage, which can be alleviated by CADs.

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