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PROTOCATECHUIC ACID ATTENUATES 4-VINYLCYCLOHEXENE DIEPOXIDE-INDUCED SPLEEN INJURY IN WISTAR RATS

Maduako Ikenna Chukwuemeka[1*], Sharon Oluchi Osawe[2], Omorede Ikponmwosa-Eweka[3]

[1]Chemoprevention & Toxicology Laboratory, Department of Medical Biochemistry, College of Medical Sciences, Benson Idahosa University, Benin City, Nigeria. imaduako@biu.edu.ng
[2]Biochemistry Option, Department of Biological Sciences, Faculty of Science, Benson Idahosa University, Benin City, Nigeria. sosawe@biu.edu.ng
[3]Department of Medical Biochemistry, University of Benin, Nigeria. omorede.aguebor@uniben.edu

Abstract
Emerging concerns exist with occupational/environmental exposure to 4-vinylcyclohexene diepoxide (VCD), an industrial by-product of daily-use plastic manufacturing process. This is because its biotransformation yields toxic metabolites injurious to essential body organs. This study aims to evaluate the protective potential of protocatechuic acid (PCA) against VCD-induced splenic injury. 35 adults male Wistar rats, randomly divided into 5 groups, were administered the following for 7 days: 2 mL corn oil (Group I), 100 mg/kg of VCD (Group II), 100 mg/kg of PCA (Group III), VCD + 50 mg/kg of PCA (Group IV), VCD + 100 mg/kg of PCA (Group V). Our results showed significantly elevated ($p < 0.05$) MDA, RONS, MPO, NO, TNF-α, and IL-1β levels and reduced GSH levels, SOD, CAT, and GST activities, as well as compromised structural integrity in spleen of rats exposed to VCD alone, indicative of its trigger of oxidative stress and inflammation. However, levels and activities of these biomarkers of oxidative stress and inflammatory mediators were significantly reversed ($p < 0.05$) in the spleen of VCD-exposed rats co-administered with graded doses of PCA. Our data also showed that PCA attenuated the VCD-induced distortion of the morphological pattern in the splenic tissues of exposed rats. Hence, PCA shows promise as a potential therapeutic or prophylactic agent for mitigating VCD-induced splenic injury associated with occupational/environmental exposure

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***Corresponding author email** imaduako@biu.edu.ng +2348037308699

Introduction

One important industrial chemical, 4-vinylcyclohexene diepoxide (VCD), is a metabolic derivative of 4-vinylcyclohexene (VCH). Primarily, VCH emerges as a byproduct during the homodimerization of 1,3-butadiene prevalent in the production of everyday goods, including car tyres, rubber, plastics, electronic housing, and agricultural pesticides. The potential for occupational and environmental exposure to its derivatives (VCD and VCH) attracts researchers interest [1]. Recent studies have indicated that in the liver, VCH undergoes biotransformation mediated by cytochrome P450 (CYP450) enzymes. This metabolic process leads to the production of two more toxic monoepoxides, 4-vinylcyclohexene-1,2-epoxide and 4-vinylcyclohexene-7,8-epoxide [2]. Human exposure to 4-vinylcyclohexene diepoxide remains a concern in public health and industrial toxicology owing to its specific mechanism of organ damage. Several documented evidences have shown that VCD can selectively destroy the ovarian reserve [3 – 6], elicit hepatorenal damage [7], spermatogenic impairment and hormonal disruption [8], and destruction of gut microbiota [9].

Among the diverse antioxidants found in the human diet, polyphenols represent the most abundant group, usually occurring as phenolic acids, flavonoids, lignans, and stilbenes [10]. The phenolic acids are prevalent phytochemicals distinguished by at least one carboxylic acid group. One such example is the protocatechuic acid (PCA), a dietary phytochemical [10], which is abundant in roselle leaves (*Hibiscus sabdariffa*) used in the preparation of zobo (a local Nigerian nutritional tonic drink) [11]. PCA is found in many edible plants, including brown rice, onion scales, plums, berries, olive and white wines, and rosemary [12 – 16]. PCAs have been shown to possess several pharmabiological activities that contribute to their varied health benefits, including antioxidant, anti-inflammatory, anticancer, antidiabetic, and neuroprotective [17 – 20].

In the lymphatic system, the spleen functions in maturation, activation, and modulation of various immune cells and is therefore prone to oxidoinflammatory stress [21]. Although the deleterious effects of VCD on other tissues, including liver, kidney, ovary, testes, and epididymis, have been established, VCD-induced splenic toxicity and the subsequent protective effect of PCA remain unknown. Our study, therefore, investigated the underlying molecular pattern of protection of PCA on the spleen via oxidoinflammatory pathway.

Materials and Methods

For this seven-day study, 35 adult male Wistar rats (180-190 g) were obtained from the University of Benin, Nigeria, Anatomy facility. Before the start of the experiment, the animals were placed on a one-week acclimatization period in a controlled laboratory environment with a 12-hour light/dark cycle. Throughout the study, the rats were housed in hygienic cages and maintained on a standard diet with unrestricted access to water. The study was conducted under the oversight of the Benson Idahosa University Animal Care and Use Research Ethics Committee (Approval No: 0012/med/064), ensuring that all procedures aligned with the NIH Guide for the Care and Use of Laboratory Animals.

Reagents

4-vinylcyclohexene diepoxide (VCD) (96% purity) was acquired from Sigma-Aldrich, while Protocatechuic acid (PCA) was bought from AK Scientific Inc, USA. All additional reagents used in the laboratory procedures were of analytical grade, sourced from British Drug Houses (Dorset). ELISA kits for tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) were procured from CUSABIO Life Science Inc, and used strictly according to the manufacturer's established protocols.

Animal grouping

Group I: Control (orally administered 2 ml/kg of corn oil)
Group II: VCD (administered orally 100 mg/kg, dissolved in corn oil)
Group III: PCA (administered orally 100 mg/kg, dissolved in corn oil)
Group IV: VCD + PCA_L (administered orally 50 mg/kg, dissolved in corn oil)
group V: VCD + PCA_H (administered orally 100 mg/kg, dissolved in corn oil).
The VCD dose used in this study was based on that reported in previous studies [7,8], while the PCA doses were selected following preliminary studies in our laboratories that demonstrated consistent and measurable beneficial effects on tissue structure and biochemical markers. PCA_L, protocatechuic acid low dose; PCA_H, protocatechuic acid high dose

Preparations of splenic homogenates

Following the conclusion of the seven-day treatment, twenty-four hours after the final treatment, rats were weighed and humanely sacrificed using cervical dislocation. The spleen of each rat was carefully harvested and washed in chilled phosphate-buffered saline to remove residual blood. Each spleen was blotted dry and weighed to determine the organ-to-body weight ratio. Succinctly, the tissues were homogenized and centrifuged to isolate the splenic supernatant. Afterwards, the samples were stored in a deep freezer for biochemical testing. To evaluate the splenic index, it is calculated as spleen index = weight of spleen (g) / body weight (g) X 100.

Estimation of splenic oxidative stress indices

To evaluate the splenic biochemical profile, several standardized spectrophotometric techniques were used. Total protein in the spleen samples was determined using the Bradford method [22]. Superoxide dismutase (SOD) activity was measured by its capacity to inhibit the self-oxidation of epinephrine, as

described by Misra and Fridovich [23]. Catalase (CAT) activity, following the protocol of Claiborne [24], was assessed by measuring the rate of hydrogen peroxide decomposition at 240 nm. Reduced Glutathione (GSH) concentrations were quantified by reaction with DTNB (Ellman's reagent), yielding a coloured product detectable by spectrophotometry Jollow *et al.* [25]. Glutathione S-transferase (GST) activity was evaluated using methods established by Habig *et al.* [26], with 1-chloro-2,4-dinitrobenzene (CDNB) as the reactive substrate. The catalytic rate was determined by measuring the formation of the CDNB conjugate, and the final results were divided by total protein content and expressed as mmol/min/mg protein. Lipid peroxidation (LPO) level in cellular lipids was assessed by quantifying malondialdehyde (MDA) levels via the thiobarbituric acid reactive substances (TBARS) formed according to the methodology of Farombi *et al.* [27]. The resulting reaction mixture was divided by the samples total protein content and reported as nmol MDA/mg protein. Reactive oxygen and nitrogen species (RONS) level in the spleen was quantified by measuring the RONS-mediated conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) into the fluorescent compound DCF [28].

Determination of splenic pro-inflammatory markers

Nitric oxide (NO) concentration was determined by mixing equal volumes of tissue sample and Griess reagent. After incubation, absorbance was measured at 540 nm, and the final concentration was determined from the sodium nitrite standard curve and expressed as mmol nitrite/mg protein [29]. Myeloperoxidase (MPO) activity in the spleen was determined using a modified standard analytical procedure. The enzyme's catalytic rate was monitored spectrophotometrically at 630 nm, and final activity was normalized and expressed as units/mg protein [30]. Furthermore, TNF-α and IL-1β concentrations were assayed using the splenic supernatant with commercially available ELISA kits, using the producer's protocol (CUSABIO Life Science Inc, Wuhan, China).

Microscopic assessment of spleen

Splenic samples were fixed in 10% buffered formalin for 72 hours, then processed through graded alcohol dehydration and xylene, and embedded in paraffin. The 5 µm sectioning was performed using a microtome, and the sections were further stained with hematoxylin and eosin (H&E). To ensure an objective outcome, a pathologist blinded to the experimental groups captured and interpreted photomicrographs for each group [31].

Data Analysis

Statistical comparisons were performed using one-way Analysis of Variance followed by the Bonferroni post hoc test to identify differences between groups. All analyses were conducted using GraphPad Prism 8 software. Results were considered statistically significant at $p < 0.05$.

Results

PCA improves splenic index and histopathological damage in VCD-treated rats

To determine the possible chemopreventive effect of PCA on VCD-initiated splenic injury, we first assessed the spleen index and histopathological changes in splenic tissue. Figure 1 shows a significant decrease in the group intoxicated with VCD alone compared with the control group ($p < 0.05$). However, PCA administration (50 and 100 mg/kg) improved the splenic index ($p < 0.05$). Furthermore, in Figure 2, we analyzed the histopathological pattern following VCD exposure alone and VCD and PCA compared to the control. The VCD-treated rats show reduced white pith with no sharply defined boundaries compared to the control.

PCA attenuated splenic oxidative stress induced by VCD exposure

The ameliorative effects of PCA on VCD-induced splenic damage were assessed using oxidative stress indices (Figure 3). Our study results show that VCD exposure decreased markedly CAT, SOD, and GST activities ($p < 0.05$); however, Figure 4 shows that it significantly elevated MDA and RONS levels ($p < 0.05$), with a concomitant decrease in GSH in splenic tissues, comparable with the control group. However, oxidative damage to splenic tissue was significantly improved in rats treated with PCA, as evidenced by upregulation of antioxidant enzyme activities, increased GSH concentration, and reduced RONS and MDA

levels relative to the group treated with VCD alone.

PCA ameliorated pro-inflammatory proteins in the spleen of VCD-treated rats

Furthermore, we investigated PCA therapeutic potential against VCD-associated splenic injury by assessing inflammatory parameters. As illustrated in Figure 5, VCD-treated rats alone showed a significant increase in NO concentration and MPO activity ($p < 0.05$) compared with controls. Furthermore, our study showed that VCD treatment significantly increased splenic TNF-α and IL-1β levels ($p < 0.05$) relative to the control group, indicating VCD-induced inflammation. Moreover, PCA (50 and 100 mg/kg) administration significantly reduced the increases in proinflammatory cytokine levels (NO, MPO, TNF-α, and IL-1β; $p < 0.05$).

Discussion

Several bodies of scientific evidence have shown that polyphenolic-rich compounds can counteract tissue damage, including nephrotoxicity, hepatotoxicity, reproductive toxicity, and neurotoxicity [28,32,33] caused by various environmental contaminants; hence, we hypothesized that PCA can attenuate VCD-induced splenic damage. Results from our study demonstrated that exposure to VCD alone elicited decrease in the splenic index when compared to the control. However, administration of PCA, dose-dependently reversed the observed decrease in the splenic index relative to the VCD-treated rats. Furthermore, the data presented in this study showed that in the control cohort, lymphocytes were neatly patterned, with distinct boundaries of both white and red pith, and normal central artery morphology. Exposure to VCD alone caused aberrant distortion in the lymphocytes arrangement, disorganized boundaries between the white and red pith, and the central artery. The above pathologies observed in the VCD-treated group were significantly restored to near normal by PCA administration (50 and 100 mg/kg). Our results suggest that PCA conferred significant dose-dependent protection against VCD-induced splenic injury. This is corroborated by previously published data [34].

Environmental toxicants exert their harmful effects on organs by disrupting cellular antioxidant defense systems, leading to oxidative cellular damage [35,36]. Earlier investigations revealed that environmental toxin-induced oxidative stress disrupted normal autophagy function while

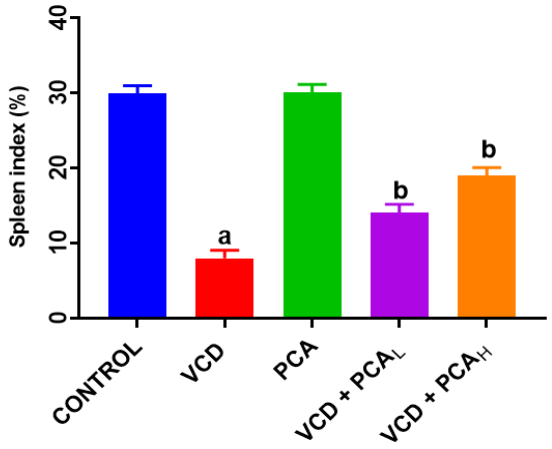

Figure 1. Effect of PCA on the splenic tissue index treated with VCD. Data were expressed in mean ± SD of 7 rats. [a]$P < 0.05$ vs control, [b]$P < 0.05$ vs VCD. Abbreviations: VCD, 4-vinylcyclohexene diepoxide; PCA, Protocatechuic acid; PCA_L, Protocatechuic acid low dose; PCA_H, Protocatechuic acid high dose.

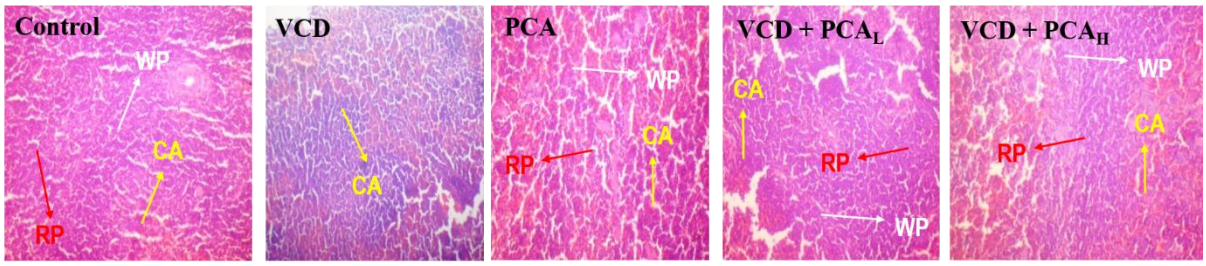

Figure 2. Effect of PCA on the splenic tissue architecture treated with VCD. Hematoxylin & eosin (H&E) staining and light microscopy were used to assess morphological alterations in splenic tissue. Abbreviations: CA, central artery; WP, white pith; RP, red pith.

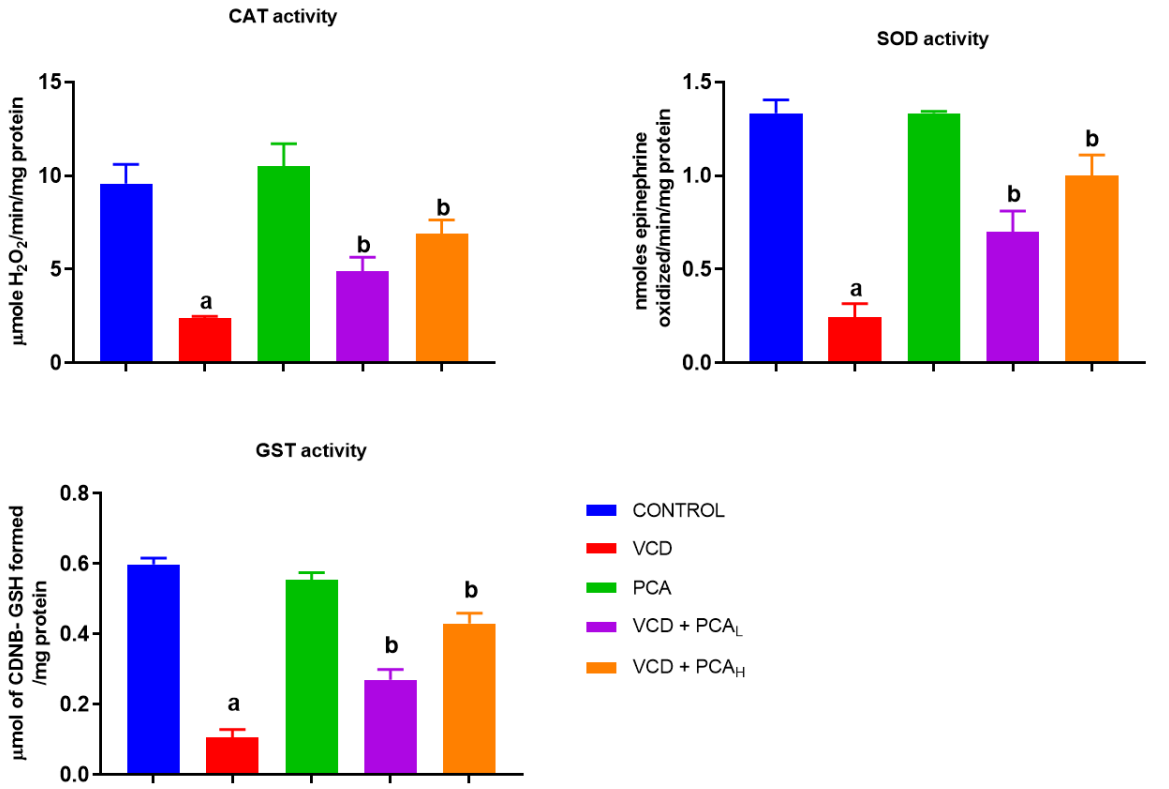

Figure 3. Effect of PCA on the activities of CAT, SOD, and GST in splenic tissues treated with VCD. Data were expressed as mean ± SD of 7 rats. [a]$P < 0.05$ vs control, [b]$P < 0.05$ vs VCD.

promoting apoptotic cell death, ultimately contributing to splenic tissue injury in experimental rats [37]. PCA free radical scavenging ability forms the basis for its protective role against harmful xenobiotics. Recently, PCA has been shown to mitigate oxidative stress and induce cancer chemopreventive activity [11,20]. Firstly, we examined the redox status of splenic tissue following exposure to VCD and/or PCA. Our study data, shows that VCD treatment alone markedly increased the levels of MDA and RONS and decreased the levels of GSH and the activities of CAT, SOD, and GST, suggesting induction of splenic oxidative stress. These findings align with earlier research demonstrating that VCD administration induced oxidative damage in hepatic and renal tissues in rats [38]. However, dose-dependent PCA administration provided antioxidant protection against VCD exposure by ameliorating disrupted oxidative stress indices in the spleen. Oxidative stress was induced in rat splenic tissues following VCD treatment.

Interestingly, our data also showed that graded doses of PCA (50 and 100 mg/kg) significantly increased the activities of CAT, SOD, and GST, significantly reduced the levels of MDA and RONS, and increased the GSH level in spleen tissue following VCD challenge. Our data support the ameliorative effect of PCA against VCD-induced splenic injury.

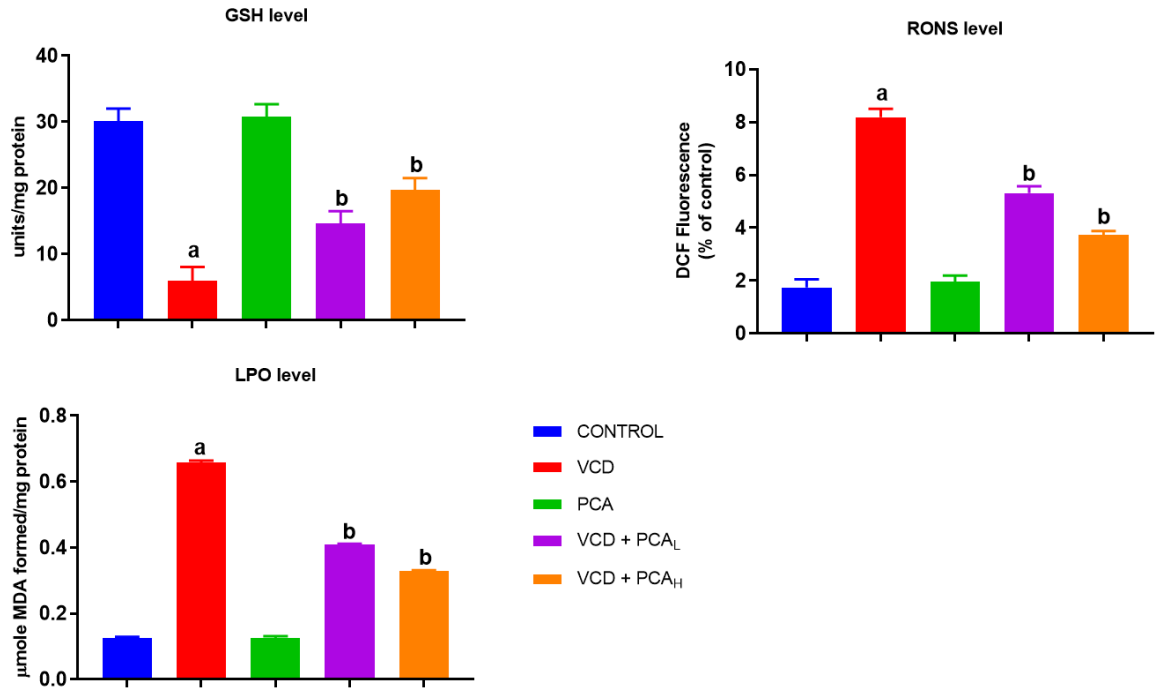

Figure 4. Effect of PCA on the levels of GSH, RONS, and LPO in splenic tissues treated with VCD. Data were expressed as mean ± SD of 7 rats. $^{a}P < 0.05$ vs control, $^{b}P < 0.05$ vs VCD.

Oxidative imbalance acts as a catalyst for inflammatory processes, leading to progressive organ deterioration. Our results show that PCA attenuated the VCD-associated inflammatory response in the spleens of treated rats in a dose-dependent manner. The toxic effects of VCD are not limited to direct cellular damage; they can also initiate a complex immune and inflammatory response [21]. The relationship between inflammation and RONS is a critical component of tissue injury. While oxidative stress is essential for eliminating pathogens and cellular debris, it also intensifies systemic oxidative stress, creating a damaging feedback loop [39]. Splenic tissue is an immune organ central to immune response development and cell regulation; thus, many environmental toxins can cause splenic toxicity, impairment [21], and carcinogenesis [40,41]. Furthermore, certain antioxidant compounds can significantly attenuate inflammatory signaling, hence they can neutralize systemic organ damage initiated by VCD [39,42]. In the present study, our data agreed with earlier reports; our results demonstrated that VCD intoxication elicited the release of splenic proinflammatory factors (NO, MPO, TNF-α, and IL-1β) relative to the control group. However, administration of PCA (50 and 100 mg/kg) suppressed these proinflammatory factors in splenic tissue. Research has shown that PCA, because of its strong antioxidant properties, also exhibits anti-inflammatory effects, a finding supported by earlier studies [17,43].

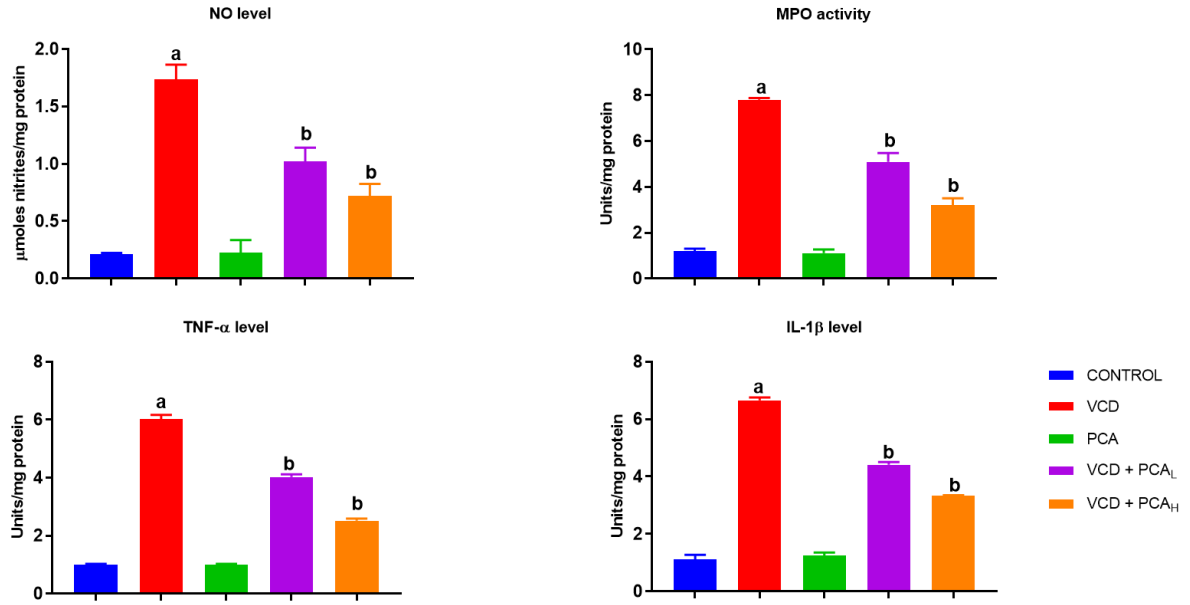

Figure 5. Effect of PCA on NO level, MPO activity, TNF-α, and IL-1β in splenic tissues treated with VCD. Data were expressed as mean ± SD of 7 rats. $^{a}P < 0.05$ vs control, $^{b}P < 0.05$ vs VCD.

Conclusions

Overall, our findings offer a compelling explanation for the pathways underlying VCD-mediated splenic injury. Our research also demonstrated that the therapeutic benefits of PCA are achieved through redox modulation and the suppression of inflammation.

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Conflict of interest

The authors have none to declare.

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