Neuroprotective Properties of Carvacrol Against Cadmium-induced Neurotoxicity in Male Rats

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Abstract

Cadmium (Cd), is a heavy metal reported to be associated with oxidative stress and inflammation. In this paper, we investigated the possible protective effects of carvacrol (CRV) against Cd-induced neurotoxicity in rats. Adult male Sprague Dawley rats were treated orally with Cd (25 mg/kg body weight) and with CRV (25 and 50 mg/kg body weight) for 1 week. CRV decreased the levels of malondialdehyde (MDA), glial fibrillary acidic protein (GFAP) and monoamine oxidase (MAO), and significantly increased the levels of glutathione (GSH) and activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in brain tissue. Additionally, CRV alleviated the in levels of inflammation and apoptosis related proteins involving p38 mitogen-activated protein kinase (p38 MAPK), cyclooxygenase-2 (COX-2), nuclear factor kappa B (NF-κB), B-cell lymphoma-3 (Bcl-3), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), myeloperoxidase (MPO), prostaglandin E2 (PGE2), neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), cysteine aspartate specific protease-3 (caspase-3) and Bcl-2 associated X protein (Bax) in the Cd-induced neurotoxicity. CRV also decreased the mRNA expression of matrix metalloproteinases (MMP9 and MMP13), as well as 8-hydroxy-2'-deoxyguanosine (8–OHdG) level, a marker of oxidative DNA damage. Collectively, our findings indicated that CRV has a beneficial effect in ameliorating the Cd-induced neurotoxicity in the brain of rats.

1. Introductıon

Cadmium (Cd) is the seventh most toxic industrial heavy metal found in air, water, soil, food, and cigarette smoke and is classified as a human carcinogenic substance (Govil et al., 2012; Wang and Du, 2013). Commercially, it is used in batteries, lasers, TV screens, paint pigments, copper alloys, galvanizing metallic, cosmetics, fungicides, and in lots of different products (Alnahdi and Sharaf, 2019). Epidemiological and clinical evidence have demonstrated that Cd exposure severely affects nervous system and is related to neurological disorders, including memory disorders, attention deficits, headache, olfactory dysfunction, vertigo, parkinsonism symptoms, and other anomalies (Ciesielski et al., 2013; Li et al., 2016; Wang and Du, 2013). After absorption by the gastrointestinal tract or lungs, Cd enters the bloodstream, crosses the blood-brain barrier, and enters the neurons through voltage-gated calcium channels due to ionic and molecular mimicry, causing neurotoxicity in the central and peripheral nervous system (Al Olayan et al., 2020). Cd has been shown to induce brain injury through inducing oxidative damage in some of crucial biomolecules such as proteins, lipids, thiols and DNA (Ben et al., 2016). In recent years, several studies have focused on new therapeutic agents that may help alleviate heavy metal-induced neurodegeneration (Akinyemi and Adeniyi, 2018). Natural products possess protective effects against damage caused by heavy metals via scavenging free radicals and strengthening the antioxidant defense system (Caglayan et al., 2019b).

Carvacrol (CRV), a phenolic monoterpene, is mainly found in the essential oil fraction of oregano, thyme, and wild bergamot (Kandemir et al., 2021; Taslimi et al., 2017). It has been considered as a food additive and flavoring agent in the food industry for decades (Zare Mehrjerdi et al., 2020). CRV has a broad spectrum of pharmacological properties including antioxidant, anti-inflammatory, antitumor, antimicrobial

and antifungal activities (Cui et al., 2015; Sharifi-Rad et al., 2018). It has also been reported to be a potential antioxidant that reduces the effect of free radicals and prevents neurodegenerative changes (Wang et al., 2017).

Although numerous studies have been carried out to investigate the biochemical roles of CRV, the possible protective effects of CRV against Cd-induced neurotoxicity have yet to be investigated. Therefore, considering the neuroprotective potential of CRV and the importance of natural compounds as a source of novel drugs, we examined the protective effects after treatment with CRV in a model of Cd-induced brain injury in rats.

2. Material And Methods

2.1. Chemicals

Carvacrol, cadmium chloride ($CdCl_2$) and all other chemicals and reagents were obtained from Sigma–Aldrich chemicals (St. Louis, MO, USA). All commercial rat ELISA kits were purchased from YL Biont (Shanghai, China). All commercial rat enzyme-linked immunosorbent assay (ELISA) kits used in this study were purchased from YL Biont, (Shanghai, China).

2.2. Animals

Thirty-five male Sprague Dawley rats, aged 10-12 weeks and weighing 250–280 g were used in this interventional study. The animals were kept under standard conditions (temperature 24 ± 2°C and 12 h light/dark cycle). Feed and water were provided *ad libitum*. All the experiments were designed and conducted according to the ethical norms approved by Atatürk University Animal Experiments Ethics Committee (Approval No: 2018/12/217).

2.3. Experimental design

The animals were randomly divided into five groups of seven animals each. CRV was suspended in distilled water while Cd was dissolved in distilled water. Both chemicals were administered orally to the rats and the total experimental duration was 7 days. Experimental groups are given below.

- Group I (control): Normal rats received physiological saline.
- Group II (CRV): CRV (50 mg/kg, b. w.) (Barnwal et al., 2017).
- Group III (Cd): $CdCl_2$ (25 mg/kg, b. w.) (Kim et al., 2018).
- Group IV (Cd + CRV-25 group): $CdCl_2$ (25 mg/kg, b. w.) + CRV (25 mg/kg, b. w.).
- Group V (Cd + CRV-50 group): $CdCl_2$ (25 mg/kg, b. w.) + CRV (50 mg/kg, b. w.).

At the end of the experimental period (on the 8th day), the animals were decapitated and their brain tissues were isolated and then washed with saline for biochemical analysis.

2.4. Preparation of tissue homogenates

The removed brain tissue was first frozen with liquid nitrogen and then pulverized with a tissue shredder device (TissueLyser II, Qiagen, Netherlands). This tissue was diluted with 1.15% potassium chloride at a ratio of 1:10 (w/v) and then homogenized using the same homogenizer. After homogenization, brain tissue samples were centrifuged and oxidative stress biomarkers were studied in the supernatant. Details of homogenates required for oxidative stress biomarkers and lipid peroxidation analyzes were given in our previous study (Çelik et al., 2020b).

2.5. Assay of lipid peroxidation in brain tissue

Malondialdehyde (MDA) level, the main product of lipid peroxidation was determined as described by Placer et al. (1966 and its levels were expressed as nmol/g of tissue.

2.6. Assay of enzymatic and non-enzymatic antioxidants in brain tissue

The antioxidant markers superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities and glutathione (GSH) levels were analyzed using the methods Sun et al. (1988, Lawrence and Burk (1976, Aebi (1984 and Sedlak and Lindsay (1968 respectively. Total protein content of brain tissue used to calculate above mentioned antioxidant biomarkers were determined according to the method of (Lowry et al., 1951).

2.7. Assay of inflammation markers in brain tissue

Brain tissue was homogenized (3500 rpm for 15 min) in phosphate buffer (pH 7.4, 0.1 M) using a tissue lyser device (TissueLyser II, Qiagen). The tissue homogenization procedures for all commercial ELISA kits were performed according to our previous study (Yardım et al., 2020). The levels of nuclear factor kappa B (NF-κB), B-cell lymphoma-3 (Bcl-3), mitogen-activated protein kinase 14 (MAPK 14), cyclooxygenase-2 (COX -2), myeloperoxidase (MPO), prostaglandin E2 (PGE2), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) in the supernatant separated after centrifugation were analyzed according to the manufacturer's protocols and were reported as ng/g protein.

2.8. Assay of GFAP and MAO levels in brain tissue

The GFAP and MAO levels in the brain homogenates were measured by a rat ELISA kit following the manufacturer's recommendations.

2.9. Assay of apoptotic markers in brain tissue

The levels of the apoptotic markers cysteine aspartate specific protease-3 (caspase-3) and Bcl-2-associated X protein (Bax) in brain homogenates were analyzed using the rat ELISA kit according to the manufacturer's instructions.

2.10. Assay of Oxidative DNA damage marker in brain tissue

Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in brain homogenates were determined using commercial ELISA kits according to manufacturer's instructions.

2.11. Real-Time PCR analysis in brain tissue

Total RNA was isolated from the brain tissue of rats using QIAzol Lysis Reagent (Qiagen, Cat: 79306, Germany) according to the manufacturer's instructions. From the total RNAs obtained, cDNAs were synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ Cat: 4368814, USA). In the final stage, TNF-α (F: CTCGAGTGACAAGCCCGTAG, R: ATCTGCTGGTACCACCAGTT), IL-1β (F: ATGGCAACTGTCCCTGAACT, R: AGTGACACTGCCTTCCTGAA), MMP9 (F: AGCTGGCAGAGGATTACCTG, R: ATGATGGTGCCACTTGAGGT) ve MMP13 (F: ATGGTCCAGGAGATGAAGAC, R: TGCAGACGCCAGAAGAATCT) mRNA transcript levels of genes were determined by ROTOR-GENE Q (Qiagen, Germany) device after reaction with cDNAs, primers and QuantiTect SYBR Green PCR Master Mix (Qiagen, Cat: 204143, Germany). β-actin (F: CAGCCTTCCTTCTTGGGTATG, R: AGCTCAGTAACAGTCCGCCT) was used as internal control. The mRNA transcript levels of the relevant genes were calculated using the CT values obtained from the device and the $2^{-\Delta\Delta CT}$ method developed by Livak and Schmittgen (2001.

2.12. Statistical analysis

Statistical evaluation was performed using one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 5.0. All values were expressed as the mean ± standard error of the mean (SEM). Significance level was set at $p < 0.05$.

3. Results

3.1. Antioxidant parameters and lipid peroxidation in brain tissue

In our study, 7 days after Cd and/or CRV treatments, levels of the antioxidant biomarkers (SOD, CAT, GPx, and GSH) and oxidants such as MDA were investigated in the brain tissues. Orally administrated Cd significantly ($p<0.05$) reduced SOD, CAT and GPx activities and GSH contents in brain tissues as compared with the control group. In the Cd+CRV-25 and Cd+CRV-50 groups, treatment with CRV was found to significantly ($p<0.05$) increase these antioxidant defense biomarkers (Fig. 1A-D). In addition, MDA levels, which are an important indicator of lipid peroxidation were found to ($p<0.05$) increase with Cd administration. However, treatment of Cd-intoxicated rats (groups IV and V) with CRV significantly reduced MDA levels in the brain tissue (Fig. 1E).

3.2. Inflammatory markers in brain tissue

To further understand the ameliorative effects of CRV on Cd-induced neuroinflammation, the protein levels of NF-κB, MAPK-14, Bcl-3, nNOS, iNOS, COX-2, MPO and PGE2, and the mRNA expression levels of TNF-α and IL -1β were investigated. Our ELISA results showed that Cd significantly ($p<0.05$) increased the levels of NF-κB, MAPK-14, Bcl-3, nNOS, iNOS, COX-2, MPO and PGE2. However, co-administration of CRV resulted in a significant reduction in the levels of these inflammatory markers (Fig. 2A-C, 3A-C and 4A-B).

The effects of CRV and/or Cd on mRNA expression levels of TNF-α and IL-1β in brain tissue are presented in Fig. 5A and B. Our RT-PCR results demonstrated that the mRNA expression levels of TNF-α and IL-1β were markedly increased in the brain of the Cd-induced rats. However, treatment with CRV (25 and 50 mg/kg) decreased the levels of these inflammatory parameters.

3.3. Effect of CRV on GFAP and MAO levels in brain tissue

Exposure of the rats to Cd significantly ($p < 0.05$) increased GFAP level and MAO activity in the brain tissue as compared to the control group. However, CRV treatment prevented the increase in GFAP level and MAO activity compared to the Cd-induced group ($p < 0.05$).

3.4. Effect of CRV on MMP9 and MMP13 mRNA transcript levels in brain tissue

In this study, Cd treatment for 7 days significantly ($p < 0.05$) enhanced the mRNA expression levels of MMP9 and MMP13 in the brain tissue of rats. As shown in Fig. 5C and 5D, CRV treatment attenuated MMP 9 and MMP 13 mRNA expression levels respectively.

3.5. Apoptotic markers in brain tissue

The effect of CRV on Cd-induced apoptotic status in the brain tissue was evaluated. There were significant ($p < 0.05$) increases in the caspase-3 and Bax levels in the rats that received Cd. Interestingly, co-administration of CRV with Cd significantly ($p < 0.05$) decreased the levels of these apoptotic parameters (Fig. 6A and B).

3.6. Effect of CRV on 8-OHdG levels in brain tissue

Administration of Cd resulted in a significant increase in level of 8-OHdG as compared to the control group ($p < 0.05$). Co-treatment of CRV significantly decreased the elevation of 8-OHdG level in comparison with the Cd-induced group ($p < 0.05$, Fig. 7).

4. Discussion

Cadmium is a toxic substance of increasing significance due to occupational contaminant, industrialization, smoking, and lack of efficient treatment for Cd poisoning (Afifi and Embaby, 2016). Cd has been documented to have a range of biological toxicities, including reproductive toxicity, neurotoxicity, genotoxicity, and teratogenic effects (Abdel Moneim et al., 2014; Phuagkhaopong et al., 2017). Although this heavy metal toxicity is now documented in almost every organ, the central nervous

system remains highly sensitive to low levels of Cd exposure (Alnahdi and Sharaf, 2019). Cd accumulation in brain tissue by crossing the blood-brain barrier is considered an important mechanism of brain damage caused by this metal (Ben et al., 2016). Accumulating evidence suggests that Cd attacks thiol group-containing proteins, including GSH, and causes disruptions in energy metabolism, membrane integrity, and mitochondrial function. It has been reported that oxidative damage and excessive ROS production are triggered as a result of the resulting deterioration. In addition, Cd can replace some cofactors such as zinc and iron, which are required for SOD and CAT activities, thus reducing the antioxidant capacity of these enzymes (Al Olayan et al., 2020; Martelli et al., 2006). Recent experimental studies have reported that Cd causes oxidative stress by decreasing the levels of non-enzymatic and enzymatic antioxidants biomarkers in the rat brain tissue (Adefegha et al., 2016; Al Olayan et al., 2020). As shown in Fig. 1A-E, Cd significantly increased lipid peroxidation and decreased antioxidant enzyme activities (SOD, CAT and GPx) and GSH level in brain tissue. However, CRV treatment reduced Cd-induced brain damage by increasing antioxidant enzyme activities that had been reduced by Cd.

Exposure to Cd is associated with neuroinflammation due to increased secretion of proinflammatory cytokines (Al Olayan et al., 2020). It has been documented that Cd can induce neuroinflammatory pathology by mechanisms including activation of microglia, blood-brain barrier leakage, and infiltration of immune cells into the brain (Ashok et al., 2015; Rogers et al., 2016). NF-κB and MAPK signaling pathways play an important role in regulating the expression of proinflammatory genes and are considered promising target molecules for the treatment of neuroinflammatory diseases (Cho et al., 2016; Çelik et al., 2020a). Notably, NF-κB is known as a ROS-sensitive nuclear transcription factor that regulates the expression of proinflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2 and iNOS (Yardım et al., 2021). NF-κB can be up-regulated by p38 MAPK because p38 MAPK affects NF-κB levels by promoting phosphorylation of IκB, resulting in dissociation and degradation of IκB and NF-κB complexes (Kandemir et al., 2020). NF-κB leaving the IκB complex enters the nucleus and regulates the transcription of the above-mentioned target genes (Caglayan et al., 2019a; Liu et al., 2018). In a previous study, it was determined that cadmium increased the levels of NF-κB, p38 MAPK, IL-6 and IL-8 in neuron and astrocyte cell culture (Phuagkhaopong et al., 2017). In the present study, Cd increased the levels of inflammation-related parameters such as NF-κB, p38α MAPK, Bcl-3, COX-2, MPO, PGE2, nNOS and iNOS, as well as transcript levels of TNF-α and IL-1β in brain tissue, while CRV showed anti-inflammatory effect by decreasing levels of these biomarkers.

Matrix metalloproteinases (MMPs) are endopeptidases that arrange the cell-matrix composition and are considered major proteases involved in extracellular matrix degradation (Mehana et al., 2019). In pathophysiological conditions, aberrant expression of MMPs induces chronic inflammation and leads to the progression of neurodegenerative diseases (Brkic et al., 2015). In the case of neuroinflammation, MMPs increase the permeability of central nervous system barriers by destabilizing tight junction proteins or disrupting the extracellular matrix, which in turn leads to infiltration of immune cells into the brain and cell death (Rosenberg, 2009). In this study, our RT-PCR results showed that Cd increased mRNA transcript levels of MMP9 and MMP13 in rat brain tissue. It has been found that therapeutically administered CRV reduces MMP9 and MMP13 transcription levels.

Accumulating evidence suggests that Cd severely affects the function of the nervous system by inducing neuronal apoptosis (López et al., 2003). Apoptosis involves the regulation of activation of proteolytic caspase enzymes as a result of interactions between several protein families. Although multiple pathways can induce apoptosis, it has been reported that the mitochondrial pathway is mostly involved in central nervous system apoptosis (Unsal et al., 2013). Caspase-3, Bcl-2 and Bax have an important function in the regulation of cellular apoptosis as they contribute to the apoptotic process (Eldutar et al., 2017; Kandemir et al., 2021). Consistent with a previous study (Al Olayan et al., 2020), our results revealed that Cd initiates the apoptotic event in brain tissue by upregulating proapoptotic proteins such as caspase-3 and Bax. In spite of that, CRV administration remarkably regulated these apoptotic biomarker levels.

8-OHdG, one of the most studied oxidized metabolites, is recognized as a biomarker for oxidative damage of DNA (Caglayan et al., 2018; Graille et al., 2020). A linear relationship between ROS production and 8-OHdG formation has been documented indicating that ROS triggers 8-OHdG formation (Caglayan et al., 2019b; Kucukler et al., 2020). It has been reported that 8-OHdG is formed as a result of the interaction of the hydroxyl radical, which is the most important oxygen-free radical, with the nucleobases of the DNA chain such as guanine (Graille et al., 2020). In a previous study, it was reported that Cd increased 8-OHdG levels by causing oxidative DNA damage in brain cortex tissue (Al omairi et al., 2018). In the present study, we found a significant increase in the level of 8-OHdG in the Cd-intoxicated group, and treatment with CRV significantly modulated the level of this marker.

5. Conclusion

In conclusion, CRV exhibited an ameliorating effect on the Cd-induced neurotoxicity in male rats. This ameliorative effect was mediated by the inhibition of oxidative stress, apoptosis, and neuroinflammation. Due to the antioxidant property of CRV, it could be a very promising compound for the treatment of Cd-related neurodegenerative diseases. However, more research is needed on the profound mechanism of action of CRV.

Declarations

Conflict of interest

The authors have no confict of interest.

Ethical approval

Ethics committee approval of the study was obtained from Atatürk University Experimental Animals Local Ethics Committee (Approval No. 2018/12/217).

Consent to participate

Not applicable.

Consent for publication

The authors give their consent for the publication of this manuscript.

Author contribution

MOY, HÇ and AG designed the research. AG and TD conducted experiments. AG, TD, and ES analyzed data. MOY and CC wrote the manuscript. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Declaration of Competing Interest

The authors report no declarations of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Figures

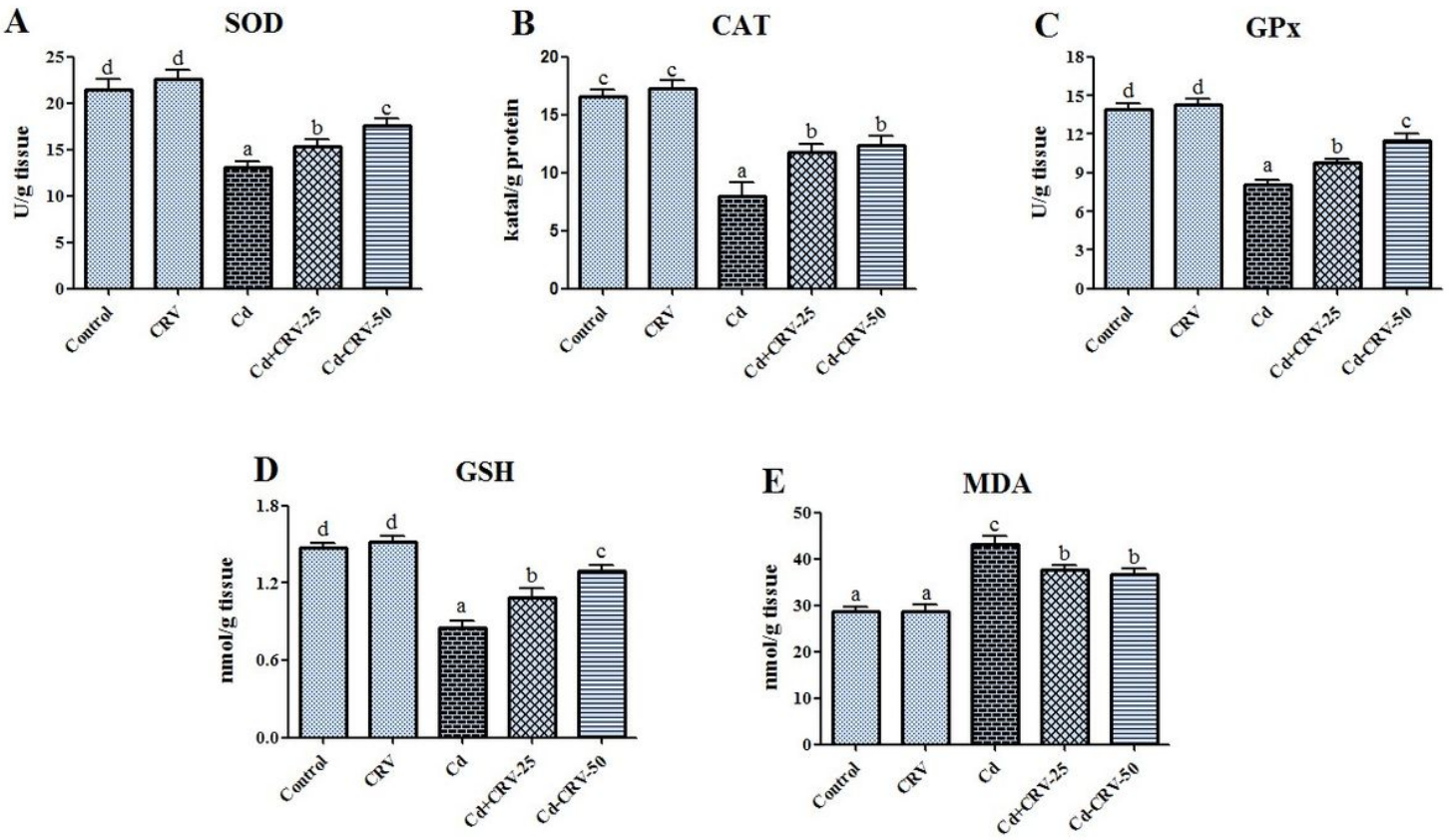

Figure 1

Protective effects of CRV treatment on brain (A) SOD, (B) CAT and (C) GPx enzyme activities and (D) GSH and (E) MDA levels in rats. Values are expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$). The lowest value is marked with “a” and the sign letter is changed gradually when the statistical difference occurs.

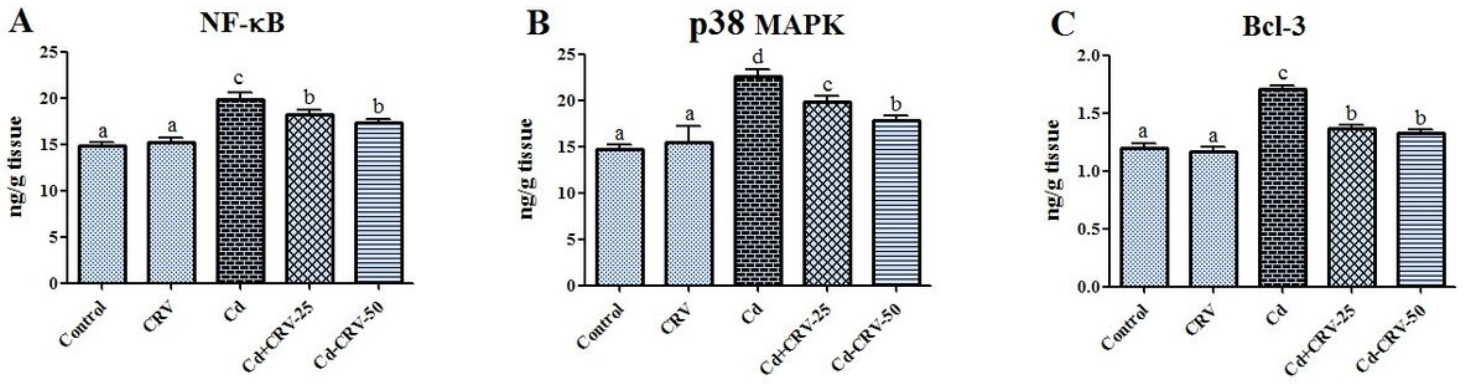

Figure 2

Protective effects of CRV treatment on brain (A) NF-κB, (B) MAPK 14 and (C) Bcl-3 levels in Cd-treated rats. Values are expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$).

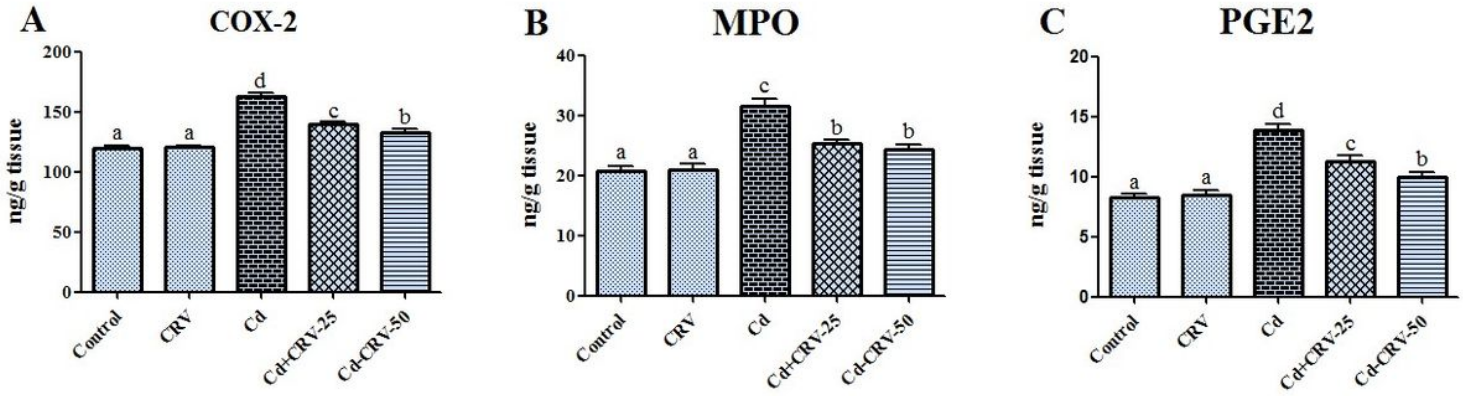

Figure 3

Protective effects of CRV treatment on brain (A) COX-2, (B) MPO and (C) PGE2 levels in Cd-treated rats. Values are expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$).

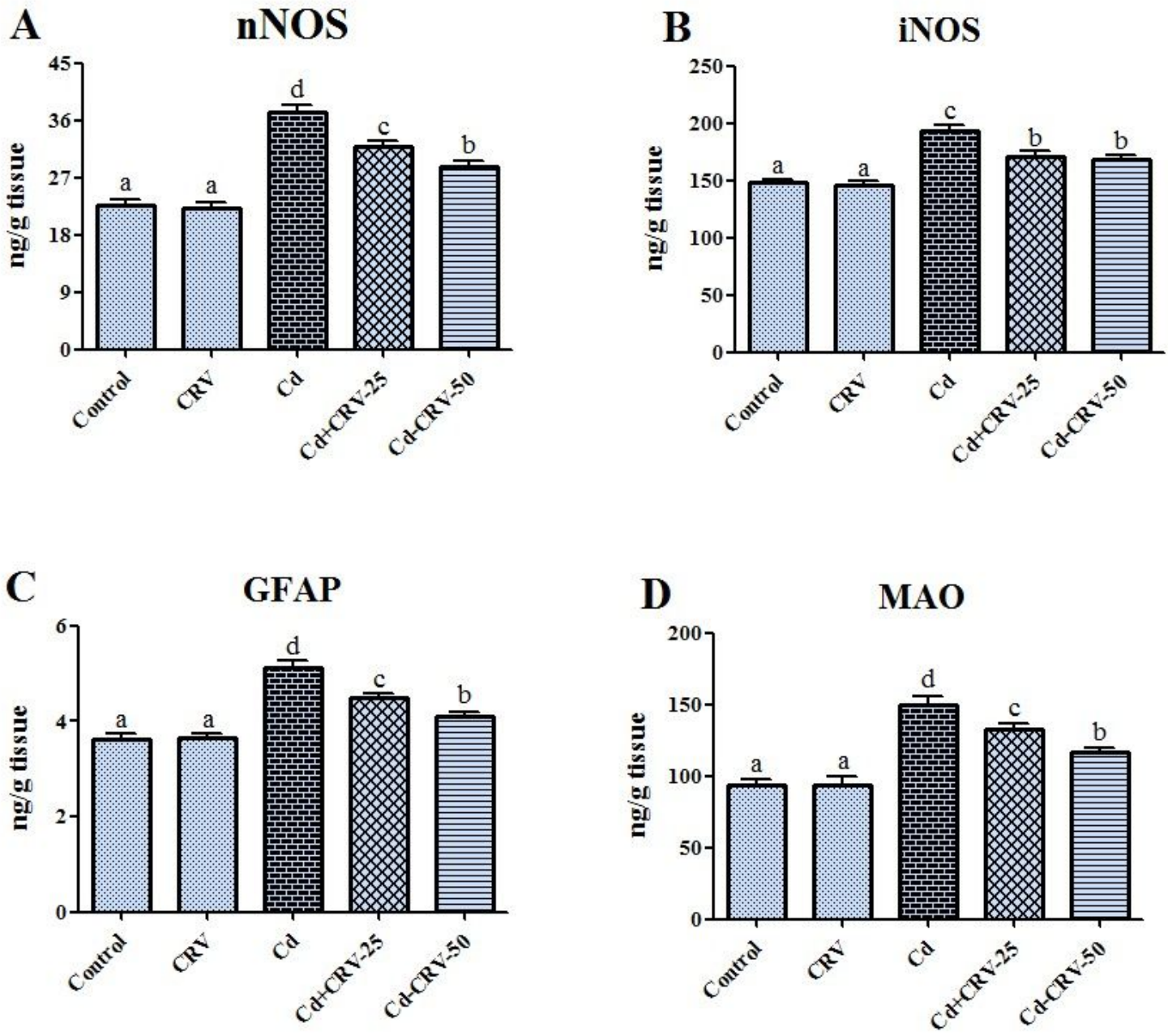

Figure 4

Protective effects of CRV treatment on brain (A) nNOS, (B) iNOS, (C) GFAP and (D) MAO levels in Cd-treated rats. Values are expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$).

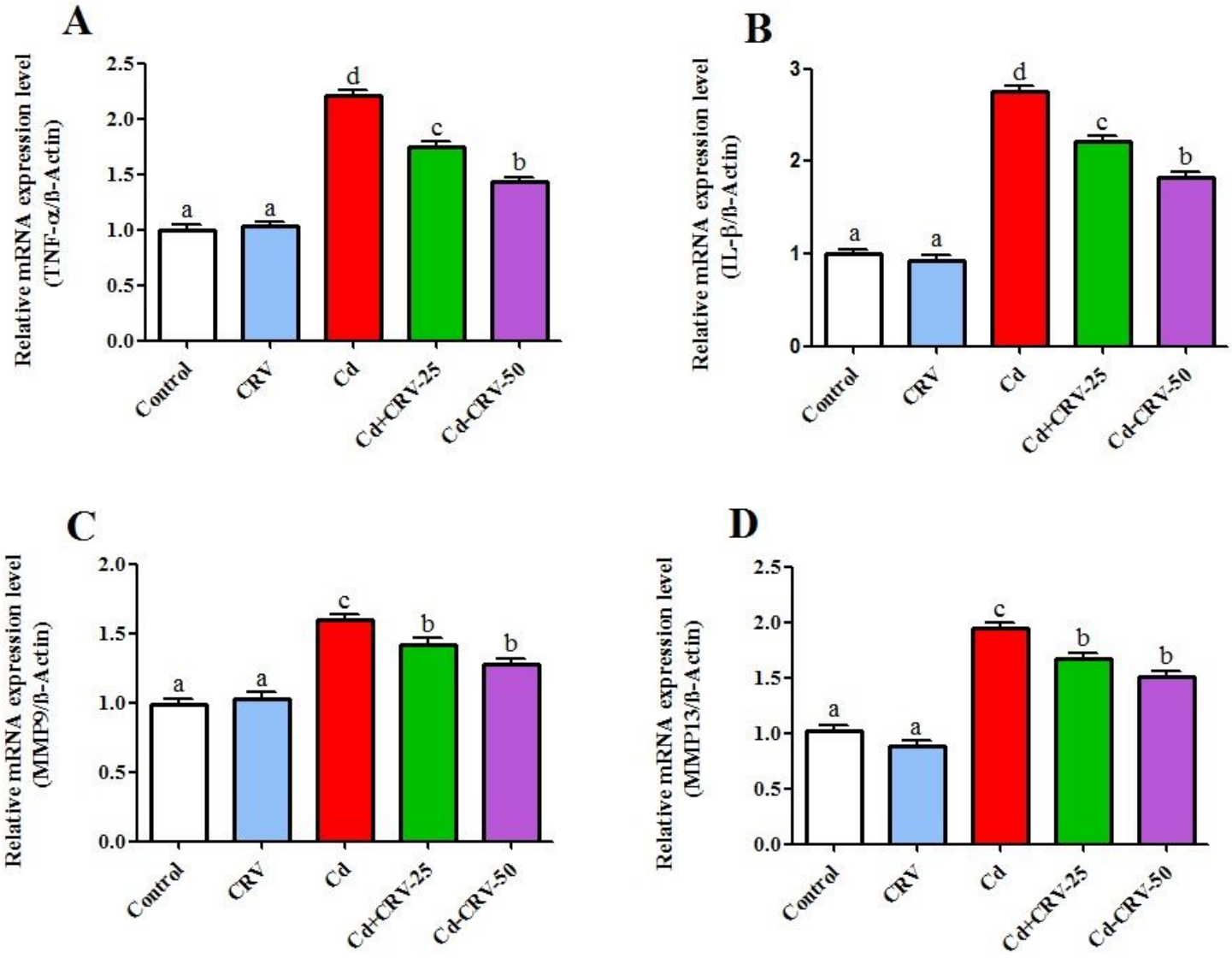

Figure 5

The mRNA transcript level of TNF-α, IL-1β, MMP9 and MMP13 in the brain of rats. (A) Represent the relative mRNA expression levels of TNF-α. (B) Represent the relative mRNA expression levels of IL-1β. (C) Represent the relative mRNA expression levels of MMP9. (D) Represent the relative mRNA expression levels of MMP13. Data were expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$).

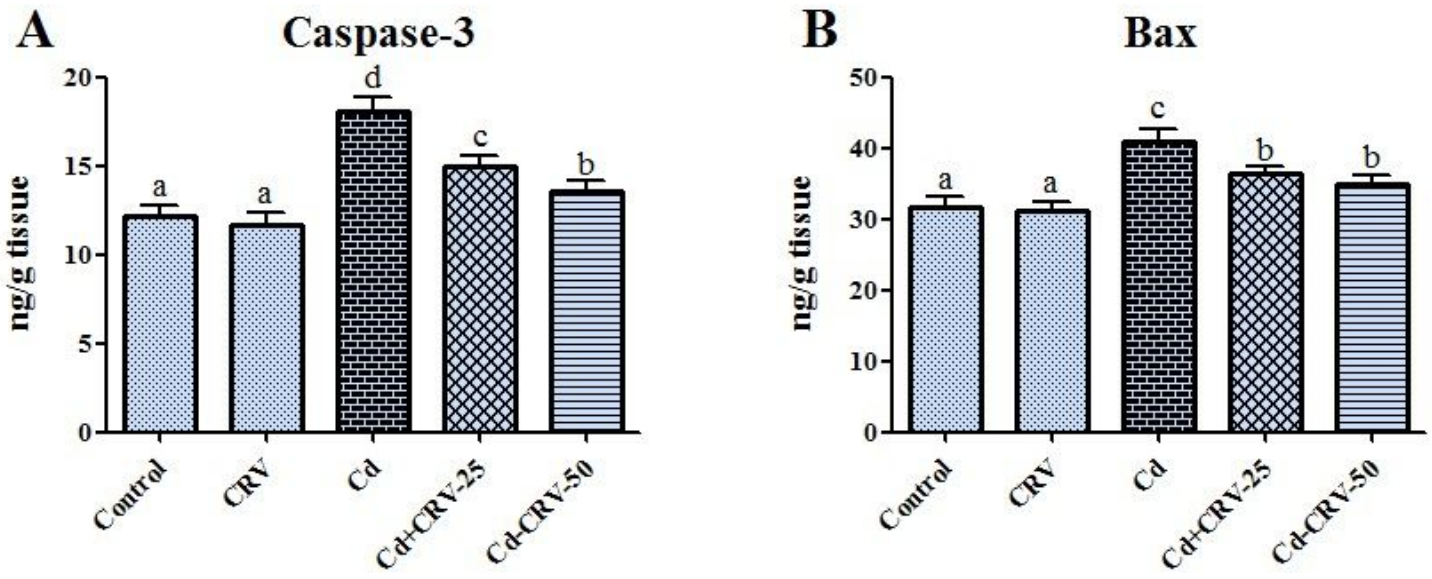

Figure 6

Protective effects of CRV treatment on brain (A) caspase-3 and (B) Bax levels in Cd-treated rats. Values are expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$).

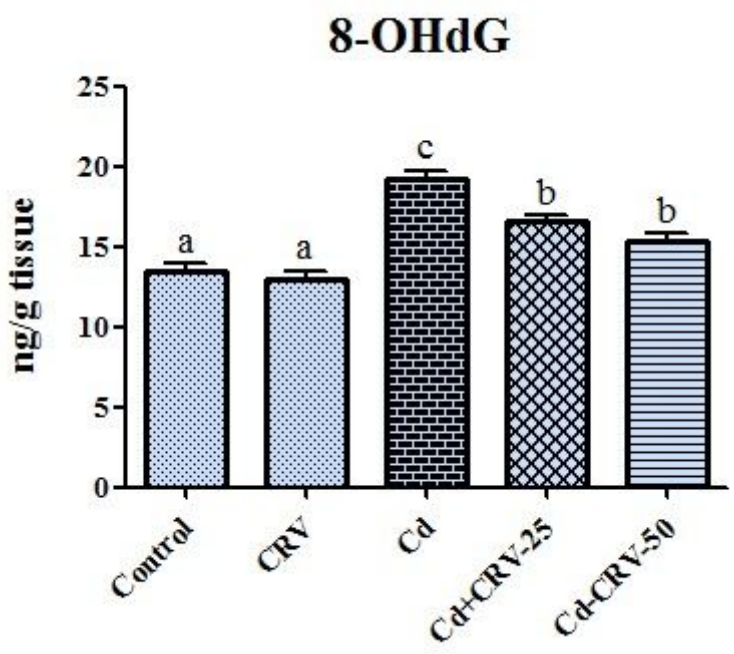

Figure 7

Protective effects of CRV treatment on brain 8-OHdG level in Cd-treated rats. Values are expressed as mean ± SEM. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$).