Biomedicine & Pharmacotherapy 153 (2022) 113360

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Acetyl-L-carnitine and/or liposomal co-enzyme Q10 prevent propionic acid-induced neurotoxicity by modulating oxidative tissue injury, inflammation, and ALDH1A1-RA-RARα signaling in rats

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ARTICLE INFO

Keywords:
Carnitine
Autism
Co-enzyme Q10
Retinoic acid
Oxidative stress
Inflammation

ABSTRACT

Propionic acid (PPA) is a short-chain fatty acid produced endogenously by gut microbiota and found in foodstuffs and pharmaceutical products as an additive. Exposure to PPA has been associated with the development of autism spectrum disorder (ASD). The purpose of this study was to investigate the protective effect of acetyl-L-carnitine (ALCAR) and liposomal Co-enzyme Q10 (CoQ10) against cerebral and cerebellar oxidative injury, inflammation, and cell death, and alterations in ALDH1A1-RA-RARα signaling in an autism-like rat model induced by PPA. The rats were treated with PPA and concurrently received ALCAR and/or CoQ10 for 5 days. The animals were sacrificed, and the cerebral cortex and cerebellum were collected for analysis. PPA caused histopathological alterations along with increased malondialdehyde (MDA), NF-κB p65, TNF-α, and IL-6 in the cerebrum and cerebellum of rats. Reduced glutathione (GSH) and antioxidant enzymes were declined in the brain of rats that received PPA. Concurrent treatment with ALCAR and/or CoQ10 prevented tissue injury, decreased MDA, NF-κB p65, and pro-inflammatory cytokines, and enhanced cellular antioxidants in PPA-administered rats. ALCAR and/or CoQ10 upregulated Bcl-2 and decreased Bax and caspase-3 in the brain of rats. In addition, ALCAR and/or CoQ10 upregulated cerebral and cerebellar ALDH1A1 and RARα in PPA-treated rats. The combination of ALCAR and CoQ10 showed more potent effects when compared with the individual treatments. In conclusion, ALCAR and/or CoQ10 prevented tissue injury, ameliorated oxidative stress, inflammatory response, and apoptosis, and upregulated ALDH1A1-RA-RARα signaling in the brain of autistic rats.

1. Introduction

Autism spectrum disorder (ASD) is a "blanket" term used to describe individuals characterized by impairments and delays in communication and social interactions. In addition to the restricted or repetitive behaviors, social and communication impairments are characteristic features of ASD [1]. ASD comprises a handful of disorders that were previously thought to be separate, namely, Autistic Disorder, Rett's Syndrome, Childhood Disintegrative Disorder, Asperger's Disorder, and Pervasive Development Disorder [1,2]. It was realized that a clear line can't be drawn between these disorders to reach a definitive diagnosis, leading to their eventual merge into one single classification that could be further clarified using various clinical specifiers and modifiers. Numerous genetic and non-genetic factors interact to play a role in the

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https://doi.org/10.1016/j.biopha.2022.113360
Received 17 April 2022; Received in revised form 15 June 2022; Accepted 28 June 2022
Available online 1 July 2022

development of ASD which explains the heterogeneous presentations of this disorder [2]. Heritability was proven to be a common contributor to autism development. Autism risk genes vary, making single gene studies difficult. Therefore, a commonly established pursuit in recent approaches is to look for common pathological and molecular mechanisms to understand the pathogenesis of ASD [2]. It has been shown that autism risk genes mostly encode synaptic integrity and gene expression modulating proteins [3]. Several theories about the natural development of ASD were proposed, and the most validated of all is the neural connectivity and impaired synaptogenesis theory [4]. During the early stages of neural development, redundant neurons are typically discarded to fine-tune the neural circuits in normal individuals versus ASD children where impairment of neural wiring, defective synaptogenesis, and an increase in neuronal cell count occur [4]. Dysregulation between autophagy and apoptosis and disruption of dendritic morphology provoke flawed synaptogenesis [4].

The intestine is a home to millions of commensal bacteria, known as the microbiome, that have a bidirectional relationship with the central nervous system (CNS). The gut microbiome contributes to the normal functioning, development, and homeostasis of the immune system [5]. Therefore, there has been increasing interest in understanding the influence of microbiome-CNS communication on neuropsychiatric health [5,6]. In this context, several findings reported that the exposure to propionic acid (PPA) may be associated with the development of ASD [7,8]. PPA, a short-chain fatty acid, is an intermediate of metabolism produced endogenously by gut microbiota through the fermentation of insoluble dietary carbohydrates and fibers. Exogenous ingestion of PPA, typically found in dairy, and wheat products, processed foodstuffs and numerous pharmaceutical products, can aggravate the symptoms of ASD [9]. However, the exact mechanism through which its action is produced still to be understood completely [10]. PPA is a weak acid exists in lipid-soluble and aqueous forms and can cross the blood-brain barrier (BBB) through specific monocarboxylate transporters both passively and actively [11]. Despite its involvement in ASD development, PPA has an important role in the host's overall bodily performance and as a principal mediator in the gut-brain axis [12]. It is involved in controlling the intestinal neuroendocrine system thereby contributing to apoptosis and functioning as a tumor suppressor [13]. Nevertheless, the neurotoxic properties of PPA become apparent with increased concentration [12] through altering immune responses, cytokine production, and neurotransmitter release [10].

There is growing recent evidence supporting the implication of redox imbalance and oxidative stress in the pathophysiology of ASD [14–16]. In this context, children with ASD showed low plasma reduced glutathione (GSH) whereas the oxidized form was increased, denoting oxidative stress [17]. Besides oxidative stress, neuroinflammation has been implicated in the development of ASD [18]. Therefore, agents with dual antioxidant and anti-inflammatory activity would be effective in ameliorating cerebral oxidative stress and neuroinflammation in ASD. In this study, we evaluated the ameliorative effect of acetyl-L-carnitine (ALCAR) and/or liposomal-Coenzyme Q10 (CoQ10) in a rat model of PPA-induced ASD. ALCAR is an acetyl ester derivative of L-carnitine endogenously synthesized in the human brain, kidney, and liver [19]. During fatty acid oxidation, ALCAR facilitates the uptake of acetyl-CoA and promotes the production of acetylcholine and the synthesis of membrane phospholipids and proteins [20]. ALCAR possesses antioxidant and anti-inflammatory activities evidenced by the reversal of alterations in cardiolipin levels, fatty acid profiles, and metabolic rate when supplemented in the diet of aged rats [21]. The antioxidant properties of ALCAR have been demonstrated in human lymphocytes [22], aged rat brain [23], rat astrocytes [24], and atherosclerotic rats [25]. CoQ10 is the most common form of coenzyme Q found in humans. It is an endogenously produced lipophilic molecule located in numerous eukaryotic cells and lipid membranes [26]. Due to its abundant crucial functions, CoQ10 plays a vital role in the normal performance of the cells, and any imbalance, owing to any dwindling levels or complete absence, wrecks the established equilibrium and may lead to many undesirable consequences [26]. The antioxidant and anti-inflammatory effects of CoQ10 have been reported in disease models of acute pancreatitis [27], cognitive dysfunction [28], and Parkinson's disease [29]. CoQ10 supplementation faces several obstacles, most notably its reduced bioavailability after oral ingestion, a phenomenon that can be explained by its large molecular weight, poor chemical stability as it has unsaturated double bond, thermolability, hydrophobicity, and volatility in light [30]. To improve its properties, considerable research has been carried out, including the use of liposomes that can encapsulate hydrophobic drugs easily and improve their bioavailability [30,31].

2. Materials and methods

2.1. Experimental animals and treatments

Thirty male Wistar rats (160–180 g), supplied by the Animals Care Center, King Saud University, were included in this investigation. The rats were housed in standard cages under standard conditions and a 12-h light/dark cycle and received food and water ad libitum. After one week of acclimatization, the animals were divided into 5 groups ($n = 6$) as follows:

Group I (Control): received 1% carboxymethyl cellulose (CMC) orally for 5 days.

Group II (PPA): received 250 mg/kg PPA (Sigma, USA) [32] suspended in 1% CMC orally for 5 days.

Group III (PPA + ALCAR): received 250 mg/kg PPA and 100 mg/kg ALCAR (Sigma, USA) [33] in 1% CMC orally for 5 days.

Group IV (PPA + CoQ10): received 250 mg/kg PPA and 10 mg/kg liposomal CoQ10 [34] in 1% CMC orally for 5 days. Liposomal CoQ10 (Lipolife, UK) is a formulation of CoQ10 encapsulated in liposomes with nano-sized phospholipid vesicles (less than 200 nm) to ensure maximum absorption.

Group V (PPA + ALCAR/CoQ10): received 250 mg/kg PPA, 100 mg/kg ALCAR and 10 mg/kg liposomal CoQ10 in 1% CMC orally for 5 days.

At day 6, the rats were sacrificed under ketamine/xylazine anesthesia, and the cerebrum and cerebellum were removed. Pieces of the tissues were kept in liquid nitrogen while others were fixed in 10% neutral buffered formalin (NBF). Other samples were homogenized in 10 mM ice-cold Tris–HCl buffer (pH 7.4). The homogenate was centrifuged, and the clear supernatant was separated for the determination of malondialdehyde (MDA), GSH, superoxide dismutase (SOD), catalase (CAT), nuclear factor-kappaB (NF-κB) p65, tumor necrosis factor (TNF)-α, and interleukin (IL)−6.

2.2. Biochemical assays

MDA was assayed in the homogenates of the cerebrum and cerebellum as previously described [35]. GSH, SOD, and CAT were determined following the methods of Ellman [36], Marklund and Marklund [37], and Cohen et al. [38], respectively. NF-κB p65, TNF-α, and IL-6 were assayed using specific ELISA kits (MyBioSource, USA) according to the manufacturer's instructions.

2.3. Histopathology and immunohistochemistry

The cerebrum and cerebellum samples were fixed in 10% NBF for 24 h and then processed for paraffin embedding. Sections were cut, stained with hematoxylin and eosin (H&E), and were examined using a light microscope. The histopathological lesions were graded as follows; - score indicates normal tissue, + indicates mild lesions, ++ indicates moderate lesions, +++ indicates severe focal lesions, and ++++ indicates severe diffuse lesions. Other sections were dewaxed and incubated in a solution of 0.05 M citrate buffer (pH 6.8) for antigen retrieval. The sections were treated with 0.3% hydrogen peroxide and protein block, followed by overnight incubation with rabbit anti-aldehyde

dehydrogenase 1A1 (ALDH1A1) polyclonal antibody (MyBioSource, USA). After rinsing with PBS, the slides were incubated with goat anti-rabbit secondary antibody (Cat. no. K4003, EnVision+™ System HRP-Labelled Polymer; Dako) for 30 min at room temperature. The slides were visualized with a DAB kit and eventually stained with Mayer's hematoxylin as a counterstain. The staining intensity was measured by ImageJ (version 1.32j, NIH, USA).

2.4. qRT-PCR

The mRNA abundance of Bax and Bcl-2 in the cerebrum and cerebellum of rats was determined using qRT-PCR. RNA was isolated using RNA purification kit (ThermoFisher Scientific, USA). The isolated RNA was quantified and samples with A260/A280 $\geq$ 1.8 were selected for reverse transcription into cDNA. Synthesis of cDNA was accomplished using a high-capacity cDNA reverse transcription kit (ThermoFisher Scientific, USA), and the obtained cDNA was amplified using Maxima SYBR Green/ROX qPCR master mix (ThermoFisher Scientific, USA) and the primers in Table 1. The obtained data were analyzed using the $2^{-\Delta\Delta Ct}$ method [39] and normalized to β-actin.

2.5. Western blotting

The samples were homogenized in RIPA buffer supplemented with proteinase inhibitors. The homogenate was centrifuged, and the supernatant was collected, and its protein content was determined using the Bradford protein assay kit (BioBasic, Canada). Fifty μg protein was subjected to 10% SDS/PAGE followed by electro-transfer to nitrocellulose membranes. After blocking in 5% milk in TBST, the membranes were incubated with mouse anti-retinoic acid receptor alpha (RARα) (Santa Cruz, USA; Cat. No. sc-515796), mouse anti-caspase-3 (Santa Cruz, USA; Cat. no. sc-56053), and mouse anti-β-actin (Santa Cruz, USA; Cat. no. sc-8432) overnight at 4°C. Following washing, the membranes were probed with rabbit anti-mouse secondary antibody (Biospes, China; Cat. no. A1038), washed, and then developed using BCIP/NBT substrate detection kit (GeneMed Biotechnologies, USA). The bands were visualized and quantified using ImageJ (version 1.32j, NIH, USA).

2.6. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical analysis and multiple comparisons were performed by one-way ANOVA and Tukey's post-hoc test using GraphPad Prism 8. A P value < 0.05 was considered significant.

3. Results

3.1. ALCAR and/or CoQ10 prevent tissue injury in PPA-administered rats

Microscopic examination of H&E-stained sections in the cerebral cortex of control rats showed normal histological structure with normal pyramidal cells (Fig. 1). In contrast, the cerebral cortex of PPA-administered rats revealed neuronal ischemic injury, degenerative changes, and perivascular and pericellular edema (Fig. 1 and Table 2). Treatment with ALCAR and/or CoQ10 prevented PPA-induced cerebral tissue injury in rats (Fig. 1).

Histopathological examination of the cerebellum of control rats revealed normal histological features, with normal granular, Purkinje and molecular layers (Fig. 1). Sections in the cerebellum of PPA-administered rats showed distortion of the granular cell layer, reduction in the size of cells in the molecular layer, reduced number and shrinkage of Purkinje cells, perivascular edema, malacia, congestion, neurophagia, and marked gliosis. Treatment of the rats with ALCAR and/or CoQ10 prevented PPA-induced tissue injury and the cerebellum showed almost normal histological features, and well-defined molecular, granular and Purkinje layers (Fig. 1 and Table 2).

Table 1
Primers used for qRT-PCR.

Gene	Forward Primer Sequence (5'−3')	Reverse Primer Sequence (5'−3')
BAX	AGGACGCATCCACCAAGAAG	CAGTTGAAGTTGCCGTCTGC
BCL2	ACTCTTCAGGGATGGGGTGA	TGACATCTCCCTGTTGACGC
Actb	AGGAGTACGATGAGTCCGGC	CGCAGCTCAGTAACAGTCCG

3.2. ALCAR and/or CoQ10 attenuate oxidative stress in PPA-administered rats

To evaluate the protective effect of ALCAR and/or CoQ10 against PPA-induced oxidative stress, MDA, GSH, SOD, and CAT were determined in the cerebrum and cerebellum of rats. As shown in Figs. 2A and 3A, PPA increased MDA significantly in the cerebrum and cerebellum, respectively, as compared to the control group ($P < 0.001$). Concurrent treatment with ALCAR or CoQ10 markedly decreased MDA in the cerebrum and cerebellum of PPA-administered rats ($P < 0.001$). The combined therapy decreased cerebral MDA significantly when compared with ALCAR ($P < 0.001$) and cerebellar MDA when compared with either ALCAR ($P < 0.001$) or CoQ10 ($P < 0.05$).

PPA administration decreased cerebral GSH (Fig. 2B), SOD (Fig. 2C), and CAT (Fig. 2D) significantly as compared to the control group ($P < 0.001$). Likewise, cerebellar GSH (Fig. 3B), SOD (Fig. 3C), and CAT (Fig. 3D) were significantly ($P < 0.001$) decreased in PPA-administered rats. Treatment with either ALCAR or CoQ10 increased cerebral and cerebellar GSH ($P < 0.001$; $P < 0.001$), SOD ($P < 0.001$; $P < 0.01$), and CAT ($P < 0.001$; $P < 0.01$) in PPA-administered rats. The combined treatment increased cerebral and cerebellar SOD ($P < 0.001$; $P < 0.001$) and CAT ($P < 0.05$; $P < 0.001$) when compared with ALCAR, and cerebellar GSH ($P < 0.01$), SOD ($P < 0.05$) and CAT ($P < 0.001$) as compared to CoQ10.

3.3. ALCAR and/or CoQ10 mitigate inflammation in PPA-administered rats

PPA-administered rats exhibited an increase in NF-κB p65 in the cerebrum (Fig. 4A) and cerebellum (Fig. 4B) as compared to the control group ($P < 0.001$). Cerebral and cerebellar TNF-α (Fig. 4C-D) and IL-6 (Fig. 4E-F) were elevated in PPA-treated rats ($P < 0.001$). Treatment with ALCAR and/or CoQ10 ameliorated NF-κB p65, TNF-α, and IL-6 in both cerebrum and cerebellum of PPA-administered rats. The combined therapy was more effective in ameliorating these inflammatory markers in PPA-administered rats.

3.4. ALCAR and/or CoQ10 prevent apoptosis in PPA-administered rats

mRNA abundance of Bax and Bcl-2 and protein expression of caspase-3 were assayed to determine apoptotic changes induced by PPA and the protective effect of ALCAR and/or CoQ10. Bax mRNA showed a significant increase in the cerebrum (Fig. 5A) and cerebellum (Fig. 5B) of PPA-administered rats ($P < 0.001$). Treatment with ALCAR and/or CoQ10 significantly downregulated cerebral and cerebellar Bax mRNA with the combined therapy was the most effective. Bcl-2 exhibited a different pattern where its mRNA was decreased in both cerebrum (Fig. 5C) and cerebellum (Fig. 5D) of PPA-administered rats ($P < 0.001$), an effect that was reversed by ALCAR and/or CoQ10. The effect of the combined treatment on Bcl2 was significant when compared with CoQ10. Caspase-3 was upregulated in the cerebrum (Fig. 5E) and cerebellum (Fig. 5F) of PPA-treated rats ($P < 0.001$). Concurrent treatment with ALCAR and/or CoQ10 decreased caspase-3 in the cerebrum and cerebellum, with the combined treatment being the most effective.

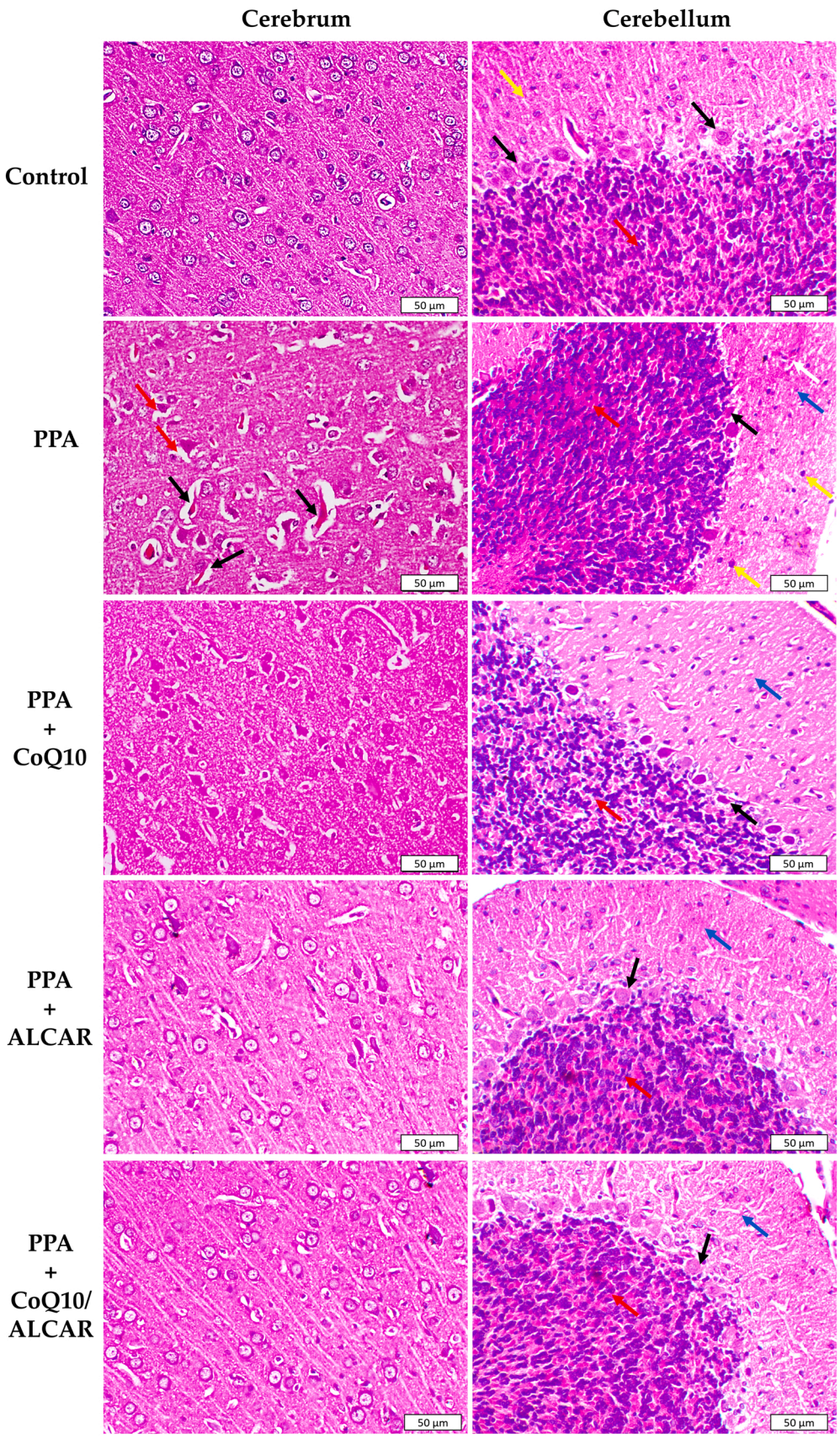

Fig. 1. ALCAR and/or CoQ10 prevented tissue injury in PPA-administered rats. Photomicrographs of H&E-stained sections in the cerebral cortex of control rats showing normal histological structure with normal cells, and PPA-administered rats showing neuronal ischemic injury, degenerative changes (black arrow), and perivascular and pericellular edema (red arrow). Treatment of the PPA-administered rats with ALCAR and/or CoQ10 prevented tissue injury. The cerebellum of control rats showing normal histological features, with normal granular (red arrow), Purkinje cells (black arrow) and molecular layers (yellow arrow), PPA-administered rats showing distortion of the granular cell layer (red arrow), reduction in the size of cells in the molecular layer (blue arrow), hyperchromatic glial nuclei (yellow arrow), few and shrunken Purkinje neurons with hyperchromatic nuclei (black arrow), and edema (white arrow), and PPA-administered rats treated with CoQ10 showing well-defined molecular (blue arrow), granular (red arrow) and Purkinje layers (black arrow), and some shrunken Purkinje cells with hyperchromatic nuclei (black arrow). Sections in the cerebellum of PPA-administered rats treated with ALCAR or ALCAR/CoQ10 revealing almost normal histological features, well-defined molecular (blue arrow), granular (red arrow) and Purkinje layers (black arrow). (Scale bar = 50 μm).

Table 2
Scoring of the histopathological lesions.

	Control	PPA	PPA + CoQ10	PPA + ALCAR	PPA + CoQ10/ ALCAR
Congestion	–	++++	+	–	–
Perivascular edema	–	++++	+	–	–
Gliosis	–	++++	+	–	–
Malacia	–	++	–	–	–
Neurophagia	–	++++	+	+	–

3.5. *ALCAR and/or CoQ10 upregulate ALDH1A1-RA-RARα signaling in PPA-administered rats*

Immunostaining revealed positive expression of ALDH1A1 in the cerebral cortex and the granular and Purkinje cell layers of the cerebellum of normal rats, as represented in Fig. 6A. PPA administration resulted in downregulation of ALDH1A1 ($P < 0.001$) whereas treatment with ALCAR and/or CoQ10 significantly increased its expression in cerebral cortex and the layers of cerebellar cortex ($P < 0.001$; Fig. 6A-B).

The expression of RARα revealed a significant downregulation in the cerebrum (Fig. 6C) and cerebellum (Fig. 6C) of PPA-treated rats ($P < 0.001$). The supplementation of ALCAR and/or CoQ10 increased RARα significantly ($P < 0.001$) in the cerebrum and cerebellum of PPA-administered rats (Fig. 6C).

4. Discussion

To date, there is no specific treatment for autism despite the vast number of clinical trials. Current treatment for ASD targets the disease symptoms, such as anxiety, depression, and seizure. Additionally, there is yet no effective therapy for ASD due to the lack of clear understanding of the pathogenesis of this disorder. In the present study, we explored the beneficial effects of liposomal CoQ10 and/or ALCAR in an autistic rat model induced by PPA, focusing on oxidative injury, inflammation, and ALDH1A1-RA-RARα signaling.

The short-chain fatty acid PPA is endogenous to the human body and several reports have pinpointed its association with the development of ASD [7,8]. This notion was supported by several *in vivo* studies and clinical trials. For instance, ingestion of refined wheat and dairy products that contain PPA increased behavioral symptoms in autistic children [40]. The consumption of these products can increase the bacterial fermentation of undigested food and the production of PPA [41]. In rats, PPA systemic administration provoked aversive internal cues [42], and its intracerebroventricular injection resulted in neuropathological, behavioral, and biochemical alterations similar to those recorded in autistic cases [7,8]. Here, PPA administration caused histopathological alterations in the cerebral cortex and cerebellum of rats. While the control rats showed normal histological features, the cerebral cortex revealed degenerative and edematous changes, and the cerebellum exhibited a reduction in cell size, gliosis, and distortion of the granular layers in PPA-administered rats. Interestingly, ALCAR, CoQ10, and their combination prevented cerebral and cerebellar tissue injury in PPA-administered. The neuroprotective efficacy of ALCAR has been

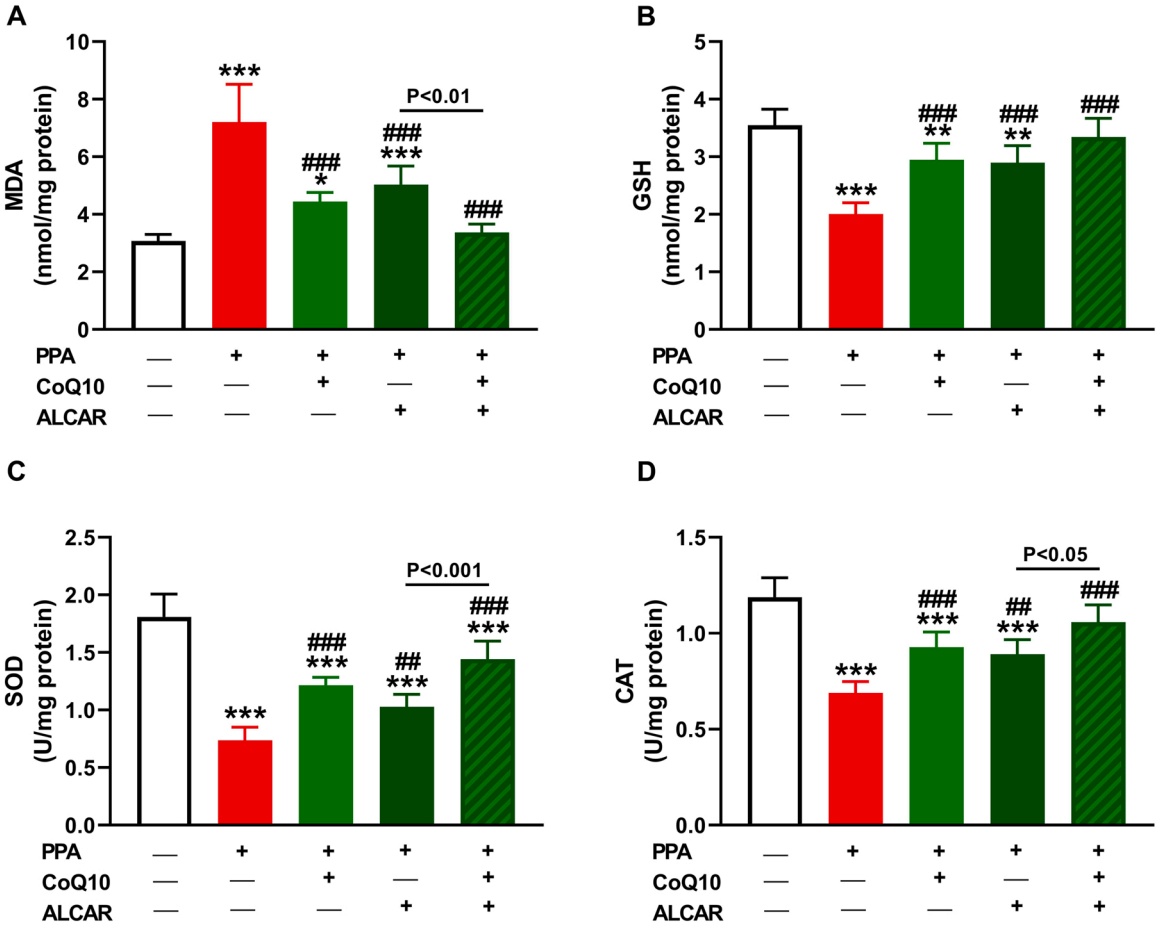

Fig. 2. ALCAR and/or CoQ10 attenuated cerebral oxidative stress in PPA-administered rats. Treatment of the PPA-administered rats with ALCAR and/or CoQ10 decreased (A) MDA, and increased (B) GSH, (C) SOD, and (D) CAT. Data are mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus Control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus PPA.

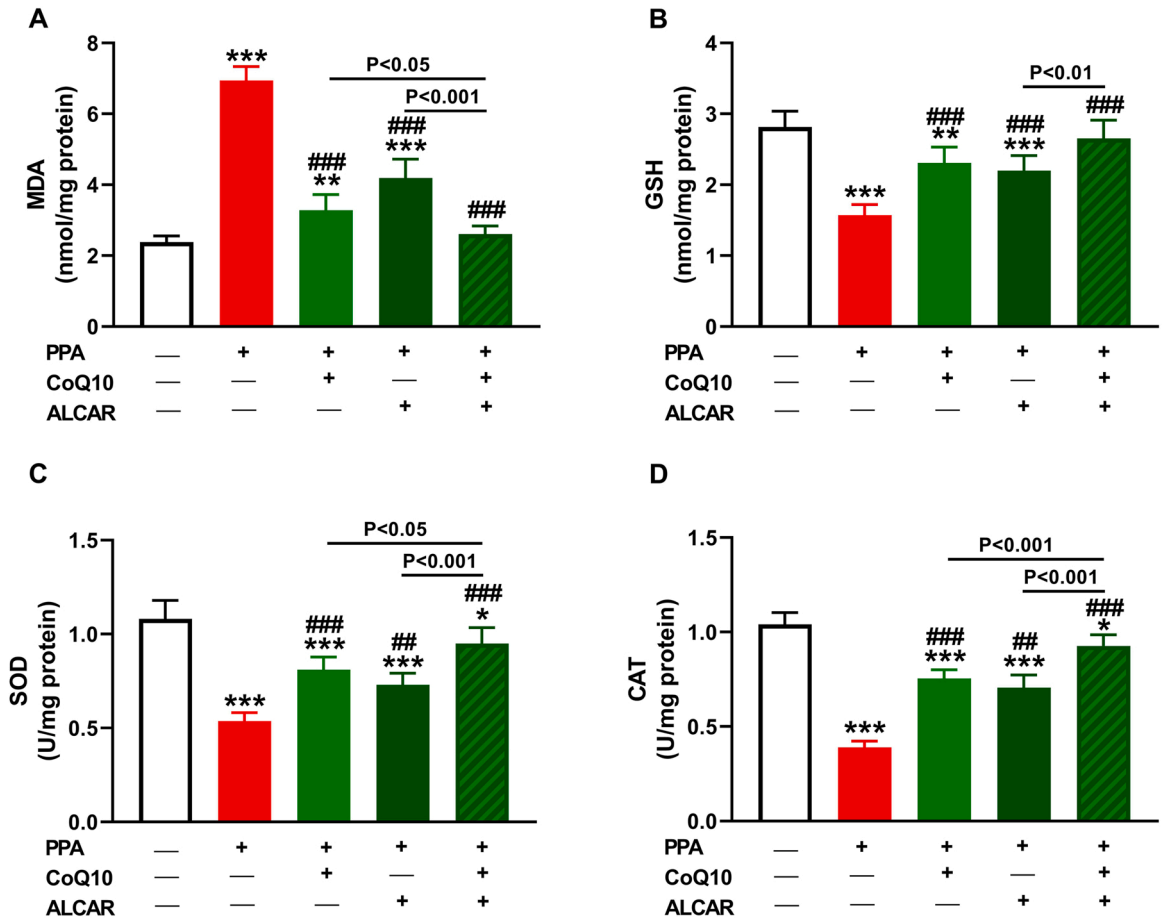

Fig. 3. ALCAR and/or CoQ10 attenuated cerebellar oxidative stress in PPA-administered rats. Treatment of the PPA-administered rats with ALCAR and/or CoQ10 decreased (A) MDA, and increased (B) GSH, (C) SOD, and (D) CAT. Data are mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus Control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus PPA.

reported in aged rats [23], immature rat brain after traumatic injury [43], ischemia-induced brain injury in neonatal rats [44], and dementia and other cognitive disorders [45]. ALCAR is absorbed in the jejunum by simple diffusion and enters the cells through active transport. ALCAR, administered orally or intravenously, reaches equilibrium in the plasma via the carnitine acetyl-transferase activity [46], and the corresponding increase in cerebrospinal fluid (CSF) indicates that it can cross the BBB [45]. The neuroprotective efficacy of CoQ10 has been demonstrated in cognitive dysfunction [28], Parkinson's disease [29], and several neurodegenerative disorders [47].

Given their antioxidant efficacy, we assumed that the neuroprotective effects of ALCAR and CoQ10 in PPA-administered rats were mediated mainly via the suppression of oxidative stress. Systemic uptake and absorption of PPA occur passively due to its weak acidic nature, easing its cross through the BBB and being retained by neurons [11]. Accumulation of PPA in neurons leads to intracellular acidification which affects neuronal function and communication by inducing systemic inflammation, triggering mitochondrial dysfunction [5], and altering neurotransmitter synthesis and release, leading to distorted social behavior and cognition [48]. Several clinical and preclinical studies revealed the involvement of oxidative stress in psychiatric disorders [49]. For instance, GSH was decreased whereas its oxidized form was increased in autistic children [17]. Although the role of ROS in ASD is still far to be explored, oxidative stress is highly implicated in considerable cellular damage and provokes severe mitochondrial dysfunction in autistic patients [50]. Lipid peroxidation (LPO) markers showed a two-fold increase in erythrocytes in ASD children when compared with age-matched healthy controls [51]. In autistic brains, oxidative damage was found to be accelerated in the cortical areas which serve communication and language [52]. Consistently, the current study showed increased MDA and declined GSH, SOD, and CAT in the cerebrum and cerebellum of PPA-administered rats. Treatment of the rats with either CoQ10 or ALCAR prevented cerebral and cerebellar oxidative injury as shown by the decrease in MDA and enhancement of the antioxidant defenses. These findings added support to previous studies demonstrating the improvement of symptoms in autistic patients following treatment with antioxidants [53,54]. ALCAR has been shown to suppress cerebral oxidative stress, excitotoxicity, cell death, and ischemia-induced neuronal injury [55]. Besides nerve regeneration and protection, ALCAR conferred antioxidant, antiapoptotic as well as analgesia in patients with peripheral neuropathy [56]. CoQ10 is a component of the electron transport chain (ETC) that acts as a potent antioxidant [47]. CoQ10 prevented neurodegeneration by suppressing mitochondrial dysfunction and oxidative stress [47], as shown in disease models of cognitive dysfunction [28] and Parkinson's disease [29].

Besides oxidative stress, neuroinflammation has been implicated in the development of autism, and inflammatory responses are often found in autistic children [57]. Oxidative stress is the major cause of neuroinflammation and impaired astrocyte-neuron crosstalk in ASD [58]. Elevated ROS elicits the activation of NF-κB, a redox-sensitive transcription factor that stimulates the transcription of pro-inflammatory cytokines, including TNF-α and IL-6. Accordingly, PPA-administered rats revealed a significant increase in cerebral and cerebellar NF-κB p65, TNF-α, and IL-6, demonstrating an inflammatory response. TNF-α is positively correlated to the severity of ASD [59], and IL-6 impairs synapse formation, and neuronal circuit balance [60]. Similar findings have

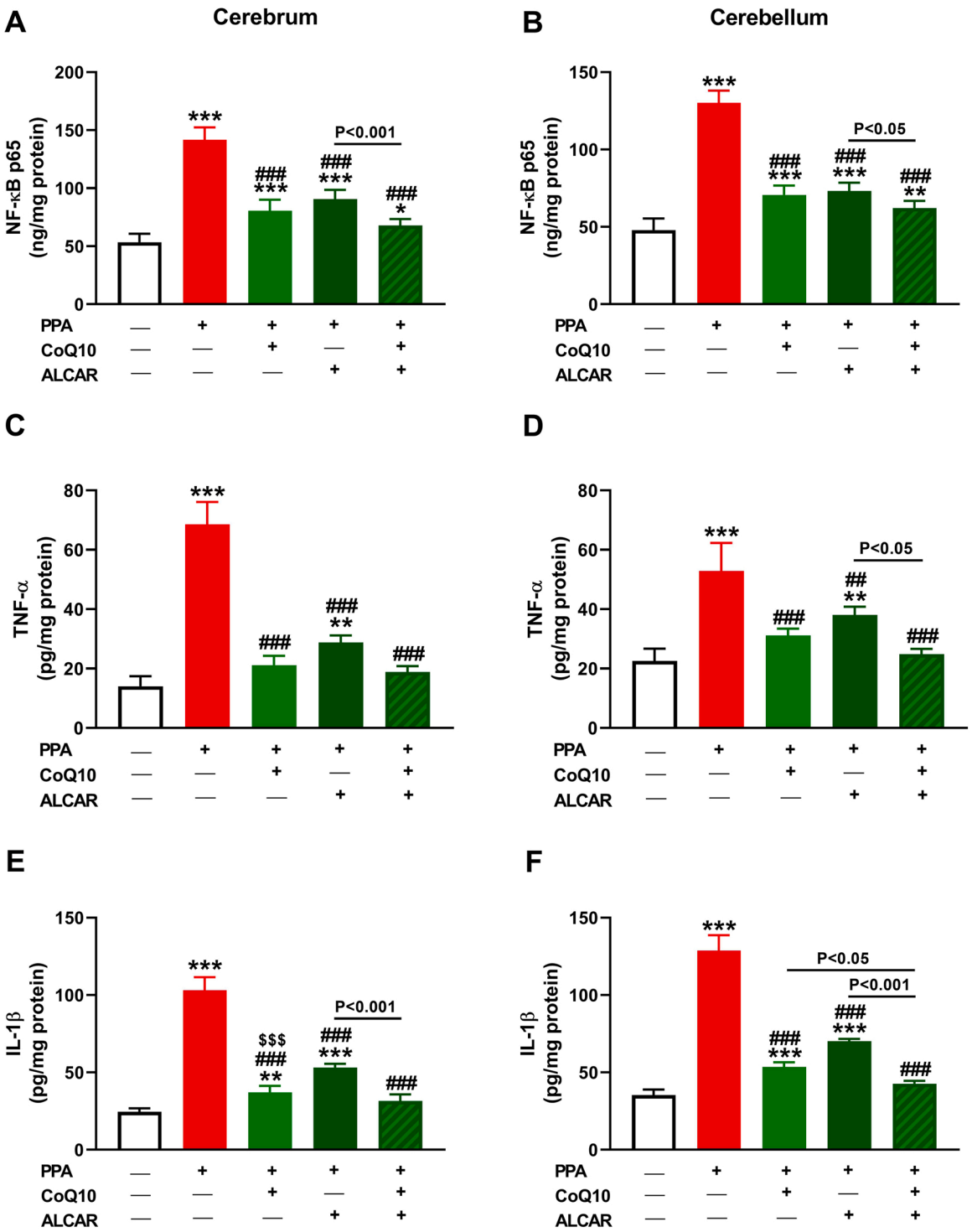

Fig. 4. ALCAR and/or CoQ10 mitigated inflammation in the cerebrum and cerebellum of PPA-administered rats. ALCAR and/or CoQ10 decreased (A,B) NF-κB p65, (C,D) TNF-α, and (E,F) IL-6 in the cerebrum and cerebellum of PPA-administered rats, respectively. Data are mean ± SEM ($n = 6$). *P < 0.05, **P < 0.01 and ***P < 0.001 versus Control. ##P < 0.01 and ###P < 0.001 versus PPA. $$$P < 0.001 versus ALCAR.

been reported in the brain of autistic mice where TNF-α and IL-6 were increased in the hippocampus and amygdala [15]. ALCAR, CoQ10, and their combination protected the rats against PPA-induced activation of NF-κB and the release of TNF-α and IL-6, pinpointing their potent anti-inflammatory activity which could be a direct consequence of their antioxidant efficacies. Moreover, both ALCAR and CoQ10 prevented apoptosis in the cerebrum and cerebellum of PPA-administered rats. Oxidative stress and inflammation work in concert to promote apoptotic cell death. Here, PPA-induced rats exhibited upregulation of the pro-apoptotic factors Bax and caspase-3, whereas Bcl-2 was down-regulated in the cerebrum and cerebellum. Increased ROS levels can activate Bax which provokes the release of mitochondrial cytochrome c due to loss of mitochondrial membrane potential [61]. Within the cytoplasm, cytochrome c initiates a sequence of events that finally activate caspase-3 resulting in cell death [62]. On the other hand, Bcl-2 prevents the release of mitochondrial cytochrome c and apoptosis [63].

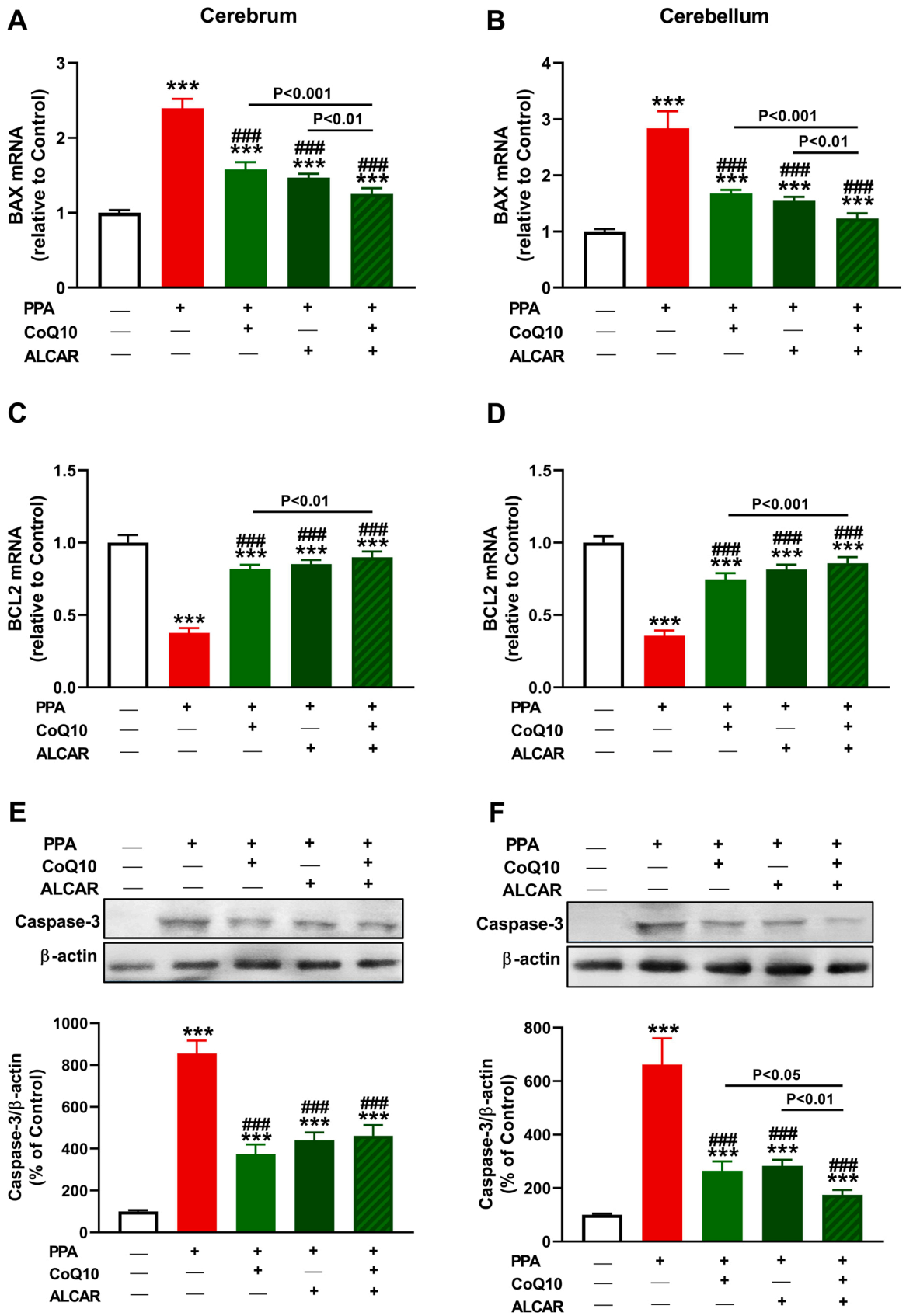

Fig. 5. ALCAR and/or CoQ10 prevented apoptosis in the cerebrum and cerebellum of PPA-administered rats. ALCAR and/or CoQ10 decreased BAX mRNA (A,B), upregulated BCL-2 mRNA (C,D), and decreased caspase-3 protein (E,F) in the cerebrum and cerebellum of PPA-administered rats, respectively. Data are mean ± SEM ($n = 6$). $^{***}P < 0.001$ versus Control, and $^{###}P < 0.001$ versus PPA.

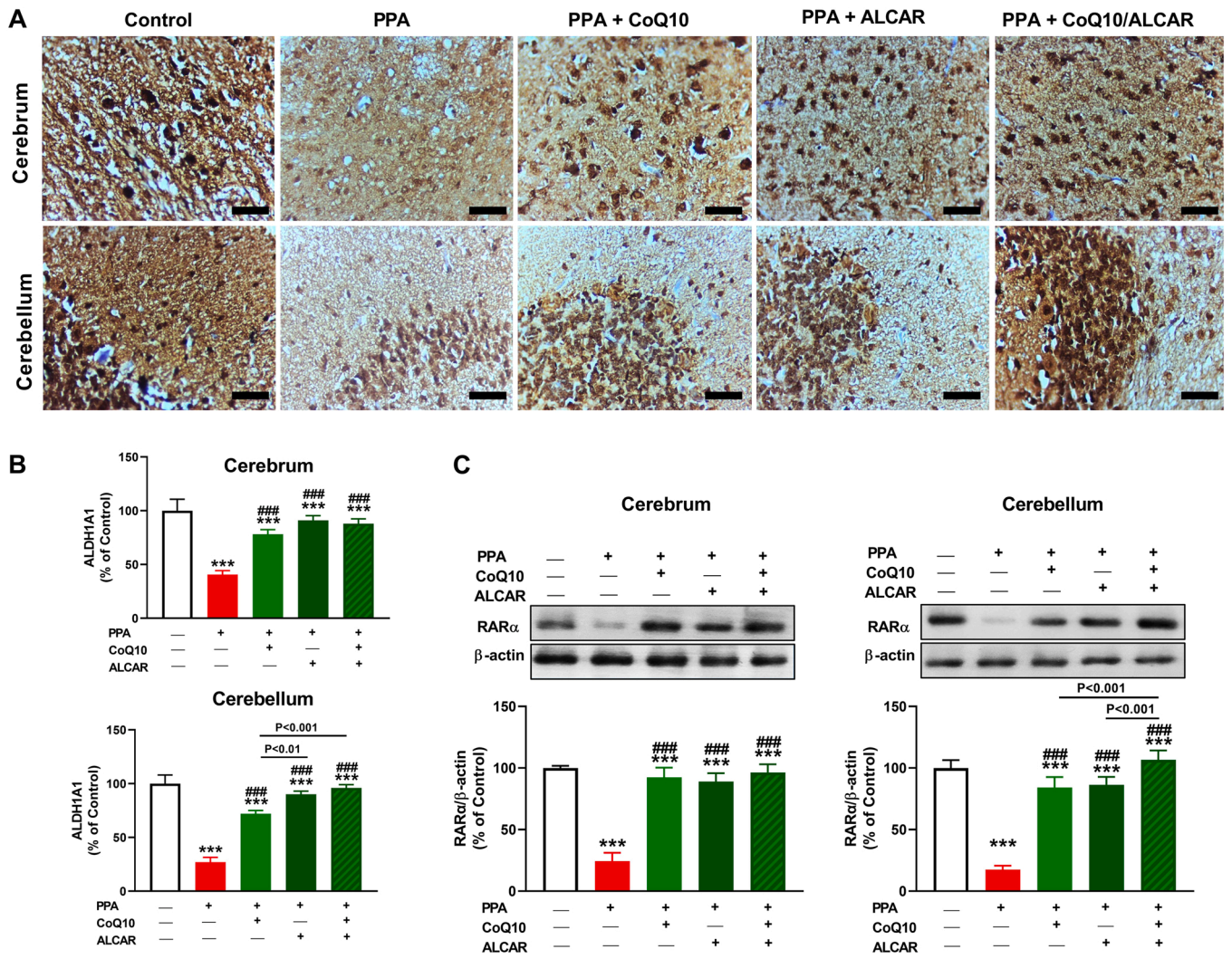

Fig. 6. ALCAR and/or CoQ10 upregulated ALDH1A1-RA-RARα signaling in PPA-administered rats. (A) Photomicrographs of sections in the cerebral and cerebellar cortex stained with anti-ALDH1A1 showing decreased expression in PPA-administered rats and upregulation in rats treated with ALCAR and/or CoQ10. (x400). (B) Image analysis of ALDH1A1 staining showed significant decrease in the cerebrum and cerebellum of PPA-induced rats and upregulation in ALCAR and/or CoQ10-treated groups. (C) Western blotting revealed downregulation of RARα in the cerebrum and cerebellum PPA-administered rats and upregulation in rats treated with ALCAR and/or CoQ10. Data are mean ± SEM ($n = 6$). $^{***}P < 0.001$ versus Control, and $^{\#\#\#}P < 0.001$ versus PPA.

Upregulation of Bcl-2 and downregulation of Bax and caspase-3 following treatment with ALCAR and/or CoQ10 demonstrated their anti-apoptotic activity, an effect that could be directly attributed to the suppression of oxidative stress and inflammation.

To further explore the mechanism underlying the beneficial effects of ALCAR and CoQ10 in PPA-induced autistic rats, we evaluated changes in the expression of ALDH1A1 in the cerebrum and cerebellum. The results revealed downregulation of both cerebral and cerebellar ALDH1A1 in PPA-administered rats. ALDH1A1 is the rate-limiting enzyme for the oxidation of retinaldehyde to retinoic acid (AR) that binds extranuclear RARα and regulates homeostatic synaptic plasticity (HSP) [64,65]. In ASD, synaptic abnormalities can lead to dysregulation of HSP [66]. Therefore, we assumed that altered ALDH1A1-RA-RARα signaling is implicated in PPA-induced ASD in rats and that modulation of this pathway might be involved in the neuroprotective efficacy of ALCAR and CoQ10. The results revealed downregulation of ALDH1A1 and RARα in the cerebrum and cerebellum of PPA-administered rats. Accordingly, a recent study by Liu et al. reported the downregulation of ALDH1A1-RA-RARα signaling in the prefrontal cortex of valproic acid-induced autism in rats [67]. In the same context, genetic variations in the ALDH1A family are clinically associated with ASD [68], and the administration of ALDH1A antagonists in mice provoked autism-like symptoms [69]. Interestingly, ALCAR and/or CoQ10 upregulated ALDH1A1-RA-RARα signaling in the cerebrum and cerebellum of PPA-administered rats. These findings introduced new information that PPA causes downregulation of ALDH1A1-RA-RARα signaling and upregulation of this pathway mediated the beneficial effects of ALCAR and CoQ10 against ASD. However, the lack of behavioral assessment could be considered a limitation of this study.

5. Conclusions

This study introduced new information on the beneficial effects of ALCAR and/or CoQ10 against ASD, pinpointing the possible involvement of ALDH1A1-RA-RARα signaling. Administration of PPA resulted in histopathological alterations, oxidative stress, and inflammatory response in the cerebrum and cerebellum of adult rats. In addition, PPA provoked apoptosis manifested by the downregulation of Bcl-2 and upregulation of Bax and caspase-3 in both the cerebrum and cerebellum of rats. ALCAR, CoQ10, and their combination prevented histological alterations, oxidative stress, inflammatory response, and apoptotic cell death in PPA-administered rats. These effects were associated with

upregulation of ALDH1A1-RA-RARα signaling. Therefore, ALCAR and CoQ10 could be promising candidates to prevent autism, pending further studies to explore the exact mechanism(s) underlying their beneficial effects.

Ethics approval

The experimental protocol was approved by the Animal Care Committee at King Saud University (Approval number: KSU-SE-19-15).

Funding

This research project was supported by the Research Center of the Female Scientific and Medical Colleges, Deanship of Scientific Research, King Saud University, Saudi Arabia.

Conflict of interest statement

All authors declare no conflict of interests in relation to the manuscript.

Data Availability

The manuscript contains all data supporting the reported results.

Acknowledgements

The authors extend their appreciation to the Research Center of the Female Scientific and Medical Colleges, Deanship of Scientific Research, King Saud University, Saudi Arabia for funding this study.

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