Clofibrate, a PPAR-α agonist, abrogates sodium fluoride-induced neuroinflammation, oxidative stress, and motor incoordination via modulation of GFAP/Iba-1/anti-calbindin signaling pathways

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ABSTRACT

Fluoride is an environmental contaminant that is ubiquitously present in air, water, and soil. It is commonly added in minute quantity to drinking water, toothpaste, and mouth rinses to prevent tooth decay. Epidemiological findings have demonstrated that exposure to fluoride

induced neurodevelopmental toxicity, developmental neurotoxicity, and motor disorders. The neuroprotective effect of clofibrate, a peroxisome proliferator-activated receptor alpha agonist, was investigated in the present study. Forty male Wistar rats were used for this study and randomly grouped into 10 rats per group as control, sodium fluoride (NaF) alone (300 ppm), NaF plus clofibrate (250 mg/kg), and NaF plus lisinopril (10 mg/kg), respectively, for 7 days. NaF was administered in drinking water while clofibrate and lisinopril were administered by oral gavage. Markers of neuronal inflammation and oxidative stress, acetylcholinesterase activity, and neurobehavioral (hanging wire and open field) tests were performed. Immunohistochemistry was performed on brain tissues, and they were probed with glial fibrillary acidic protein, ionized calcium-binding adaptor molecule 1, and cerebellar Ca^{2+}-binding protein calbindin-D28k. The results showed that NaF significantly increased of oxidative stress and neuroinflammation and inhibited AChE activity. Immunostaining showed reactive astrocytes, microgliosis, loss of dendritic spines, and arborization in Purkinje cells in rats administered only NaF. Neurobehavioral results showed that cotreatment of NaF with clofibrate improved muscular strength and locomotion, reduced anxiety, and significantly reduced astrocytic count. Overall, cotreatment of NaF with either clofibrate or lisinopril showed neuroprotective effects by mitigating neuronal inflammation and oxidative and motor incoordination. Hence, clofibrate could be seen as a novel drug candidate against neurodegeneration and motor disorders.

KEYWORDS: clofibrate, neuroinflammation, neuroprotection, oxidative stress, sodium fluoride

1 INTRODUCTION

Fluoride is an environmental contaminant that is ubiquitously present in air, water, and soil.1 Meanwhile, oxidative stress and free radical generation have been reported to play a fundamental role in the pathogenesis of sodium fluoride (NaF) toxicity.2-4 However, few studies have documented the toxic effects of NaF on the central nervous system (CNS) and motor incoordination.5-9

Recently, there has been a significant increase in the global incidence of hypertension and neurobehavioral deficit such as motor incoordination.10 Hypertension is a known important risk factor for the development of Alzheimer disease (AD) and cerebral small vessel disease.11 Hypertension, on the other hand, has been documented to enhance deposition of beta-amyloid (Aβ) protein, which can ultimately trigger AD and aging-related dementia.11 In other findings, attention-deficit hyperactivity disorder has been reported in spontaneously hypertensive rats.12-14

The involvement of oxidative stress and free radical generation has been linked to the pathophysiology of NaF-induced neurodegeneration.15 Previous research reports have documented neuronal apoptosis, developmental neurotoxicity in rat offspring, cytoskeleton damage and decreased signal, excessive endoplasmic reticulum stress and autophagic dysfunction, synaptic impairment, and developmental neurotoxicity following exposure to NaF.5, 7, 8, 16, 17 Furthermore, fragmentation and redistribution of mitochondria away from the axons of the cortical neurons18 decreased the expressions of glucose transporter 1 (GLUT1) and glial fibrillary acidic protein (GFAP),19 induction of S-phase cell-cycle arrest,

upregulation of NF-kappa B and DNA damage in primary rat hippocampal neurons,[20] behavioral deficit,[9] alteration of cognition, emotion, and synaptic plasticity in rat hippocampus.[21, 22]

Clofibrate is a peroxisome proliferator-activated receptor alpha (PPAR-α) agonist.[23] The novel neuroprotective effect of PPAR-α agonists such as pioglitazone and fenofibrate against neuroinflammation, neuronal damage, and axonal injury has been reported via antioxidant and anti-inflammatory properties.[24-26] However, there is a paucity of information on the novel neuroprotective properties of clofibrate; hence, this study seeks to unravel the neuroprotective effects of clofibrate and its molecular mechanism of action against NaF-induced neurotoxicity and neurobehavioral deficit.

2 MATERIALS AND METHODS

2.1 Chemicals

Clofibrate, lisinopril, trichloroacetic acid, sodium hydroxide, *O*-dianisidine, hydrogen peroxide (H_2O_2), xylenol orange (XO), potassium hydroxide, reduced glutathione (GSH), oxidized glutathione, NaF, thiobarbituric acid, and 1,2-dichloro-4-nitrobenzene were purchased from Sigma (St. Louis, Missouri). Normal goat serum, biotinylated antibody, and horse radish peroxidase system were purchased from KPL Inc (Gaithersburg, Maryland), anti-glial fibrillary acidic protein (GFAP; 1:1000) rabbit polyclonal antibodies from Dako (Denmark), anti-calbindin (1:12000) rabbit monoclonal antibody D-28k from Swant (Switzerland), and anti-ionized calcium-binding adapter molecule-1 (Iba-1; 1:800) rabbit monoclonal antibody from Wako, while 3,3′-diaminobenzidine (DAB, Wako, Richmond, USA) tablets were purchased from Amresco LLC (Ohio). All other chemicals used for this study were of analytical grade.

2.1.1 Experimental animals and design

In the study, forty male Wistar rats were randomly grouped into 10 rats per group as control, NaF alone (300 ppm), NaF plus clofibrate (250 mg/kg), and NaF plus lisinopril (10 mg/kg), respectively, for 7 days. NaF was administered using drinking water while clofibrate and lisinopril were administered by oral gavage. The animals were also fed with rat cubes ad libitum. The rats were kept in wire mesh cages under controlled light cycle (12 hours light/12 hours dark) and fed with commercial rat chow ad libitum and water was supplied liberally. The blood of the rats was taken on the eighth day and rats were euthanized on the ninth day. All the animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.

2.2 Hanging wire test

To test for the effect of treatments of rats on muscular strength, a hanging wire test was used. At the end of the treatment, rats were placed individually with their forepaws on a horizontally suspended wire (1 mm in diameter) and placed 47 cm above a soft foam landing area. Both ends of the hanging wire were tightly secured. Each of the experimental animals was placed with its forepaws on the wire. Rats were timed from the moment they were placed on the wire until they dropped from the wire. This reflected muscular strength in rats.[27] The

hanging wire was cleaned between each rat using 70% alcohol. Each rat was tested three times.

2.3 Open field test

The open field apparatus was a white box measuring 72 × 72 × 36 cm. One of the sides was covered with Plexiglass so that rats could be visible in the apparatus. Black lines divided the floor into sixteen 18 × 18 cm squares with a central square (18 × 18 cm) at the middle of the open field.28 Rats were placed at the center of the open field and allowed to explore the box for 5 minutes. Line crossing, center square entries, center square duration, rearing, stretched attend posture (SAP), grooming, freezing, and fecal pellet were recorded as previously described.29 The open field maze (OFM) was cleaned between each rat using 70% alcohol.

2.4 Blood sample collection and plasma preparation

Approximately 3 mL of blood was collected by retro-orbital venous puncture using heparinized capillary tubes into plain bottles and allowed to clot. The clotted blood was then centrifuged at 4000 rpm for 10 minutes. Clear serum was separated using a Pasteur pipette into another plain tube and then stored at 4°C until required.

2.5 Preparation of neuronal homogenates

At the end of treatment, rats were euthanized and the whole brain was excised, rinsed and homogenized using 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl. The homogenates were subjected to cold centrifugation at 4°C at a speed of 10 000 *g* for 15 minutes. The post mitochondrial fractions (PMFs) obtained from brain homogenates were used for biochemical assays.

2.6 Biochemical assays

2.6.1 Neuronal makers of oxidative stress

H_2O_2 generation was determined according to the method of Wolff.30 The reaction mixture was subsequently incubated at room temperature for 30 minutes. The mixtures were read at an absorbance of 560 nm and H_2O_2 generated was extrapolated from the H_2O_2 standard curve. The malondialdehyde (MDA) content as an index of lipid peroxidation was quantified according to the method of Varshney and Kale.31 The absorbance was measured against a blank of distilled water at 532 nm. Lipid peroxidation was calculated with a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. The absorbance of the sample was measured at 370 nm. Vitamin C contents were measured as earlier described.32 The protein and non-protein thiol (NPSH) contents were determined as described by Ellman.33 The activity of acetylcholinesterase (AChE) was measured as described by Ellman.33

2.6.2 Neuronal antioxidant defense system

The superoxide dismutase (SOD) assay was carried out using the method of Misra and Fridovich with slight modifications from our laboratory.34, 35 The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds. The one unit of SOD activity was

given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome. GSH was estimated by the method of Jollow et al.36 Glutathione peroxidase (GPx) activity was also measured according to Beutler et al.37 Glutathione *S*-transferase (GST) was estimated by the method of Habig et al38 using 1-chloro-2,4-dinitrobenzene as the substrate. The decrease in absorbance at 340 nm was measured, and enzymatic activity was calculated with a molar extinction coefficient of 6.1 mmol/L^{-1} cm. Protein carbonyl (PCO) contents in the brain tissues were measured using the method of Reznick and Packer.39 Protein concentration was determined using the Biuret method of Gornal et al40 using bovine serum albumin as a standard.

2.7 Immunohistochemical staining

The immunolocalization of cerebrum and cerebellum was described as earlier reported1 to determine the expressions of GFAP for astrocytes (cerebral cortex), ionized calcium-binding adaptor molecule 1 (Iba1) for microglia (cerebral cortex), and cerebellar Ca^{2+}-binding protein calbindin-D28k (CB) in the brain (cerebellum). Sections were observed using a light microscope (Leica LAS-EZ) with Leica software application suite version 3.4 equipped with a digital camera. Immunoreactivity was quantified using the Image J software by quantifying the total tissue area and the specific area of immunolocalization or immunopositivity. This was then followed by dividing the immunopositive reactions with total tissue area and then expressed as arbitrary units.

2.8 Statistical analysis

Data obtained were analyzed with one-way analysis of variance followed by Tukey's test to compare the mean of one group with the other. All values are expressed as mean ± SD. The test of significance between two groups was estimated by the Student's *t* test.

3 RESULTS

3.1 Behavioral studies

3.1.1 Hanging wire test

The hanging wire test was used to evaluate the motor function disorder induced by NaF. The hanging latency values in the control group were 99.92 ± 12.09 seconds, NaF alone of 26.39 ± 4.93 seconds, NaF plus clofibrate (250 mg/kg) of 82.39 ± 4.93 seconds, and NaF plus lisinopril (10 mg/kg) of 80.56 ± 12.13 seconds. The rats treated with NaF alone had significantly ($P \leq .05$) reduced hanging latency compared with other groups (Figure 1). The hanging latency of the NaF plus clofibrate and NaF plus lisinopril groups was similar to that of the control group. Rats coadministered with clofibrate or lisinopril and NaF held onto the wire for a longer duration while 90% of the rats administered with NaF alone fell before mid-way into the test.

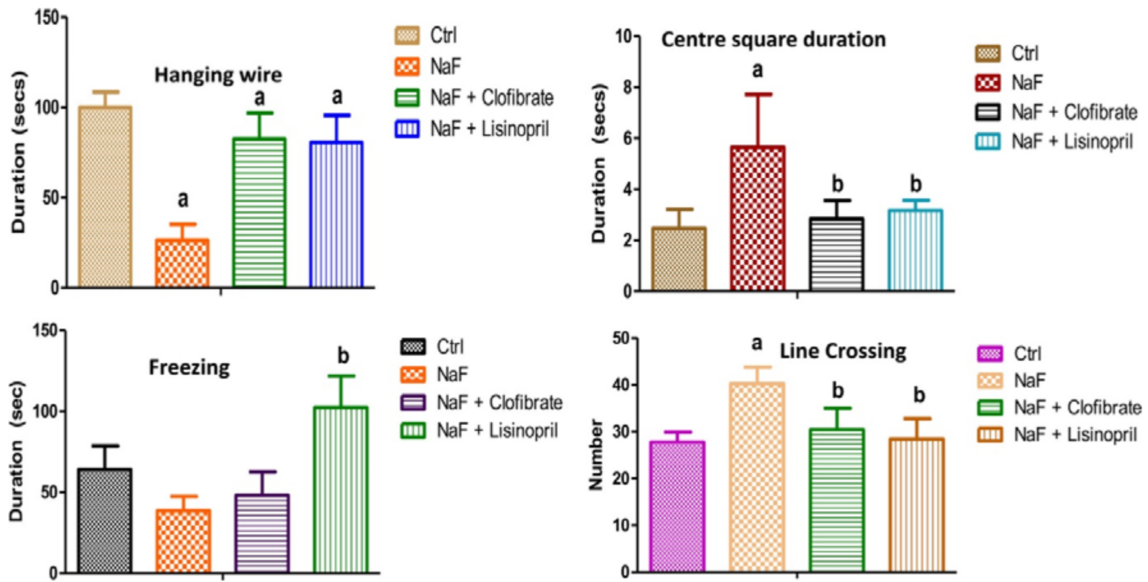


Figure 1. The effect of NaF on neurobehavioral tests. Alphabet "a" indicates significant difference when compared to the control at $P < .05$ while "b" indicates significant difference when compared to NaF only group at $P < .05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride

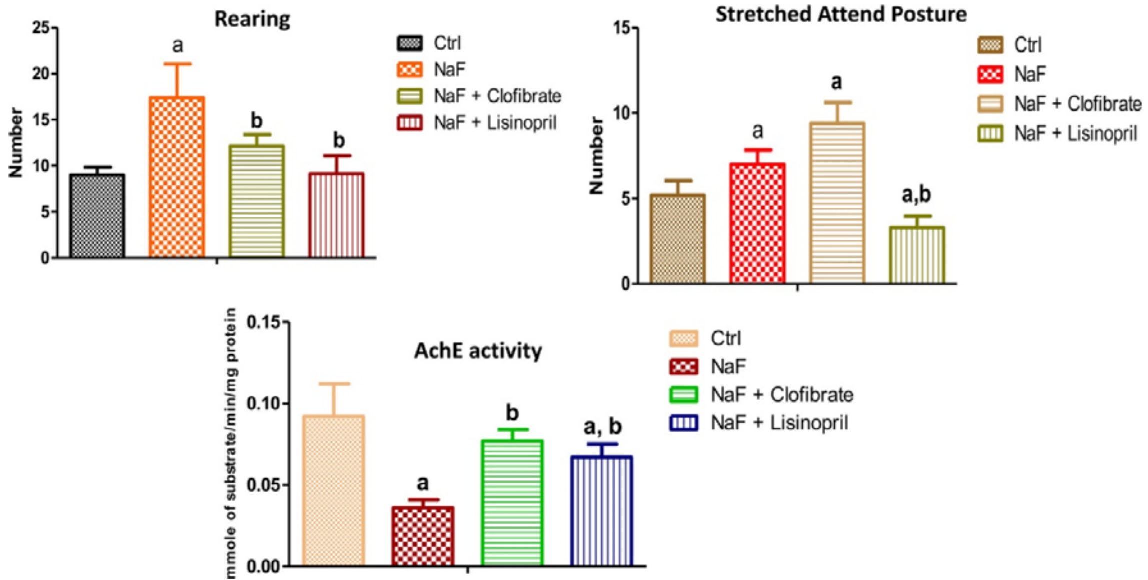


Figure 2. The effect of NaF on neurobehavioral tests and acetylcholinesterase (AChE) activity. Alphabet "a" indicates significant difference when compared to the control at $P < .05$ while "b" indicates significant difference when compared to NaF only group at $P < .05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride

3.1.2 Open field maze

Neither the neurotoxicant nor the test compounds modified ambulatory activity compared with the control group in the center square, line crossing, freezing, and hanging wire (Figure 1). Similar results were observed in center square duration and rearing (Figure 1). NaF alone or in combination with clofibrate significantly increased the SAP when compared with the control and cotreatment with lisinopril (Figure 2). Furthermore, in contrast to administration of NaF alone, every other group showed a significant decrease in rearing frequency in the OFM (Figure 2). The effects produced by lisinopril in the OFM were similar to those observed in the control group.

3.2 AChE activity and neuronal antioxidant defense system

The results as indicated in figures showed that NaF significantly inhibited AChE activity in comparison with the control and rats cotreated with clofibrate or lisinopril (Figure 2). The content of neuronal GSH fell significantly following the administration of NaF while a significant increase in GSH content was observed in rats coadministered with either clofibrate or lisinopril (Figure 3). The MDA content as an index of lipid peroxidation was found to increase significantly in the rats that received only NaF relative to the control (Figure 3). However, on the other hand, clofibrate or lisinopril coadministration led to a significant decrease in the values of MDA as shown in Figure 3. Similarly, the neuronal H_2O_2 generation and PCO content increased significantly in NaF alone treated rats as compared to control and other treatment groups (Figure 3). Interestingly, there was reduction in the activity of brain GPx. However, the reduction was not statistically significant across groups (Figure 4). The GST activity in the animals that received only NaF reduced significantly while improvement in the GST activity was recorded in the group that received NaF in combination with lisinopril (Figure 4). The activity of SOD increased significantly in groups that received NaF in combination with either clofibrate or lisinopril (Figure 4). The administration of NaF caused a significant increase in neuronal activity to control nitric oxide (NO) bioavailability while coadministration with either clofibrate or lisinopril caused a significant reduction in neuronal NO content (Figure 5). The protein thiol (PSH) content increased significantly in rats administered NaF when compared to the control, which was significantly reduced in groups that received either clofibrate or lisinopril along with NaF (Figure 5). Furthermore, the NPSH content was not significantly affected by the treatment as indicated in Figure 5. However, there was a significant increase in vitamin C levels in rats that received clofibrate along with NaF while NaF alone or lisinopril in combination with NaF did not alter the vitamin C content (Figure 5).

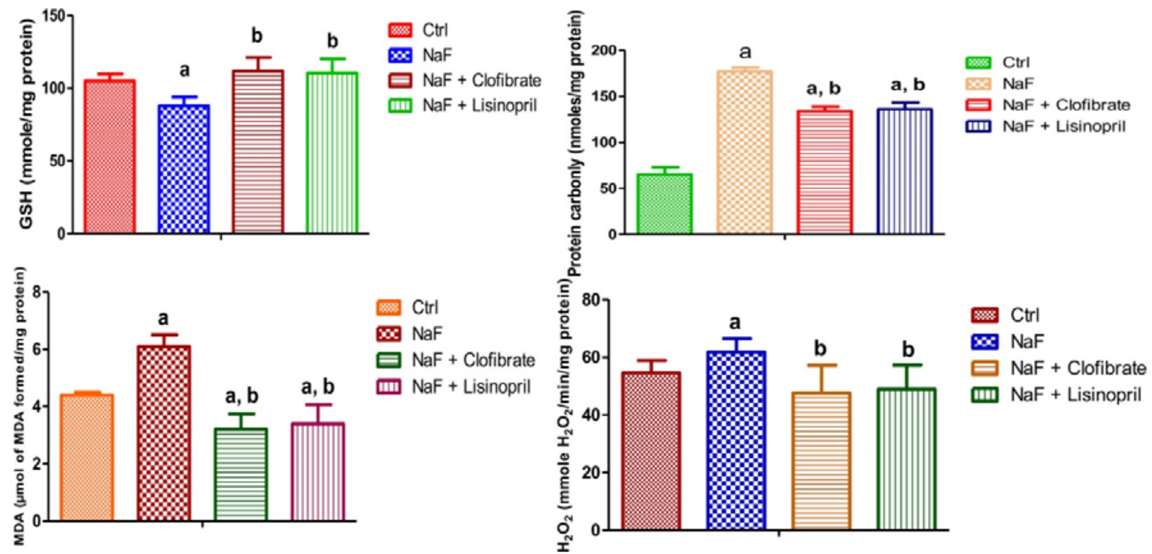


Figure 3. The effect of NaF on markers of oxidative. Alphabet "a" indicates significant difference when compared to the control at $P < .05$ while "b" indicates significant difference when compared to NaF only group at $P < 0.05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride

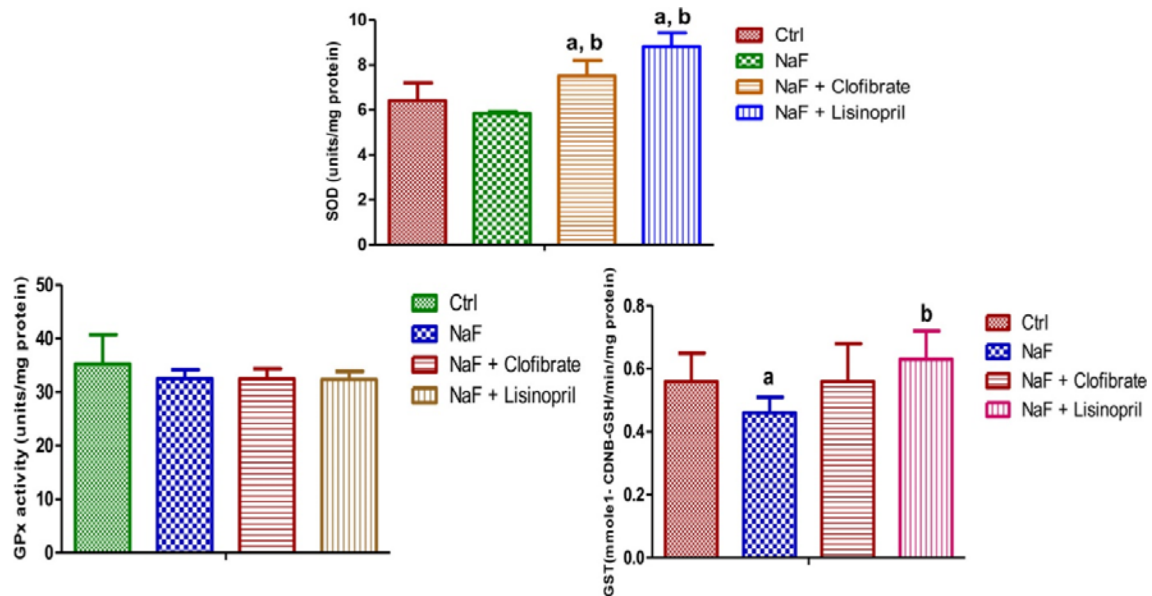


Figure 4. The effect of sodium fluoride (NaF) on the antioxidant defense system. Alphabet "a" indicates significant difference when compared to the control at $P < .05$ while "b" indicates significant difference when compared to NaF only group at $P < 0.05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride

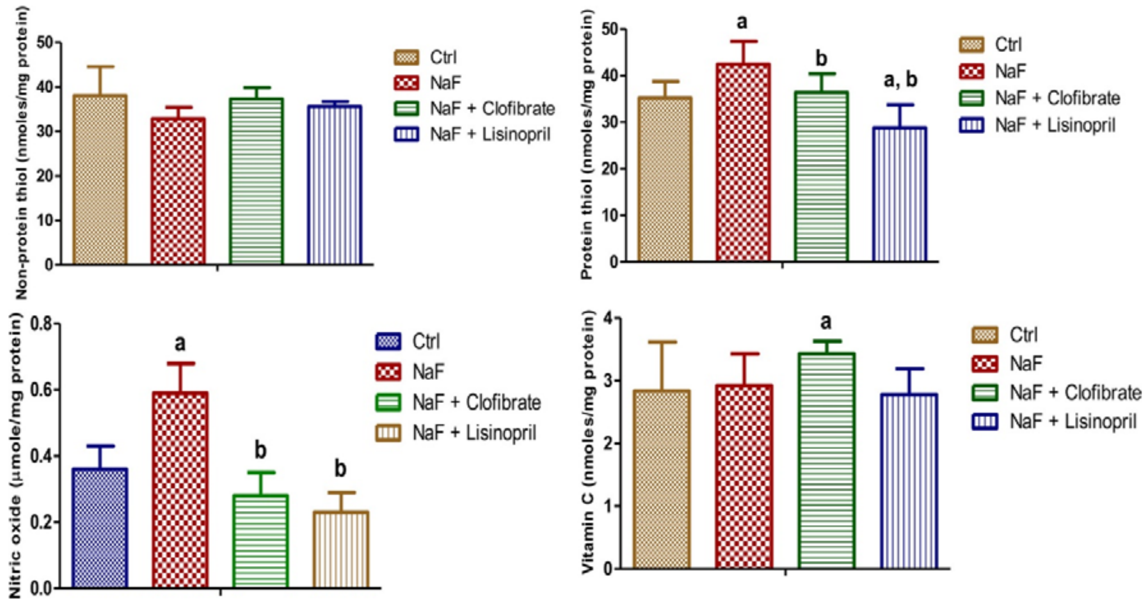
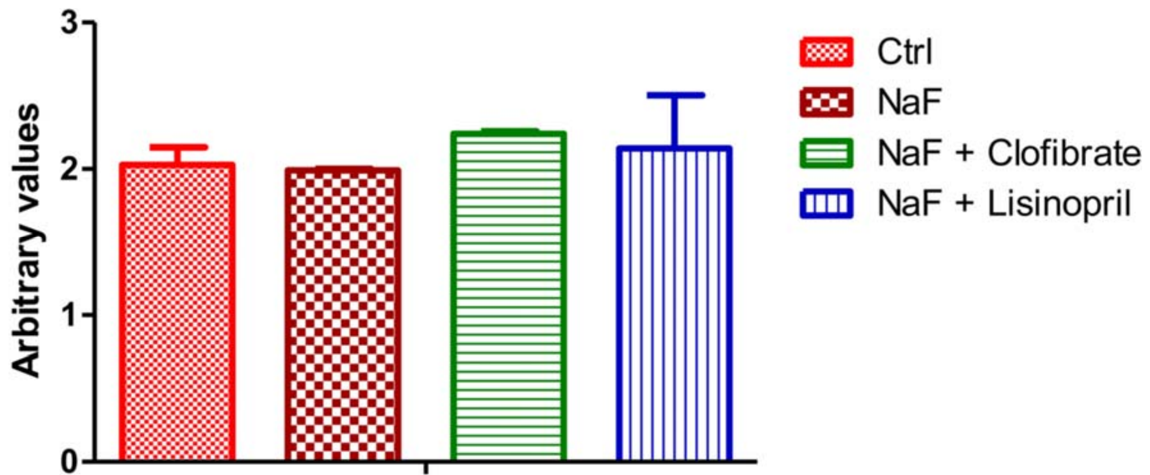


Figure 5. The effect of sodium fluoride (NaF) on non-enzymatic antioxidant and nitric oxide content. Alphabet "a" indicates significant difference when compared to the control at $P < .05$ while "b" indicates significant difference when compared to NaF only group at $P < 0.05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride

3.3 Immunohistochemistry

The immunohistochemistry of anti-calbindin showed a reduction in size of Purkinje cells in NaF alone treated rats relative to the control or groups that received either clofibrate or lisinopril in combination with NaF (Figure 6). However, the architecture of the Purkinje cells was slightly preserved in groups that received NaF in combination with either clofibrate or lisinopril (Figure 6). On the other hand, GFAP expressions showed significant astrocytosis in NaF alone treated rats relative to the control or groups that received either clofibrate or lisinopril combination with NaF (Figure 7). However, there was a significant reduction in the number of astrocytes in groups that received NaF in combination with either clofibrate or lisinopril (Figure 8). The immunolocalization of Iba1 showed significant microgliosis in NaF alone treated rats relative to the control (Figure 8). The cotreatment with either clofibrate or lisinopril caused a significant reduction in microgliosis (Figure 8).



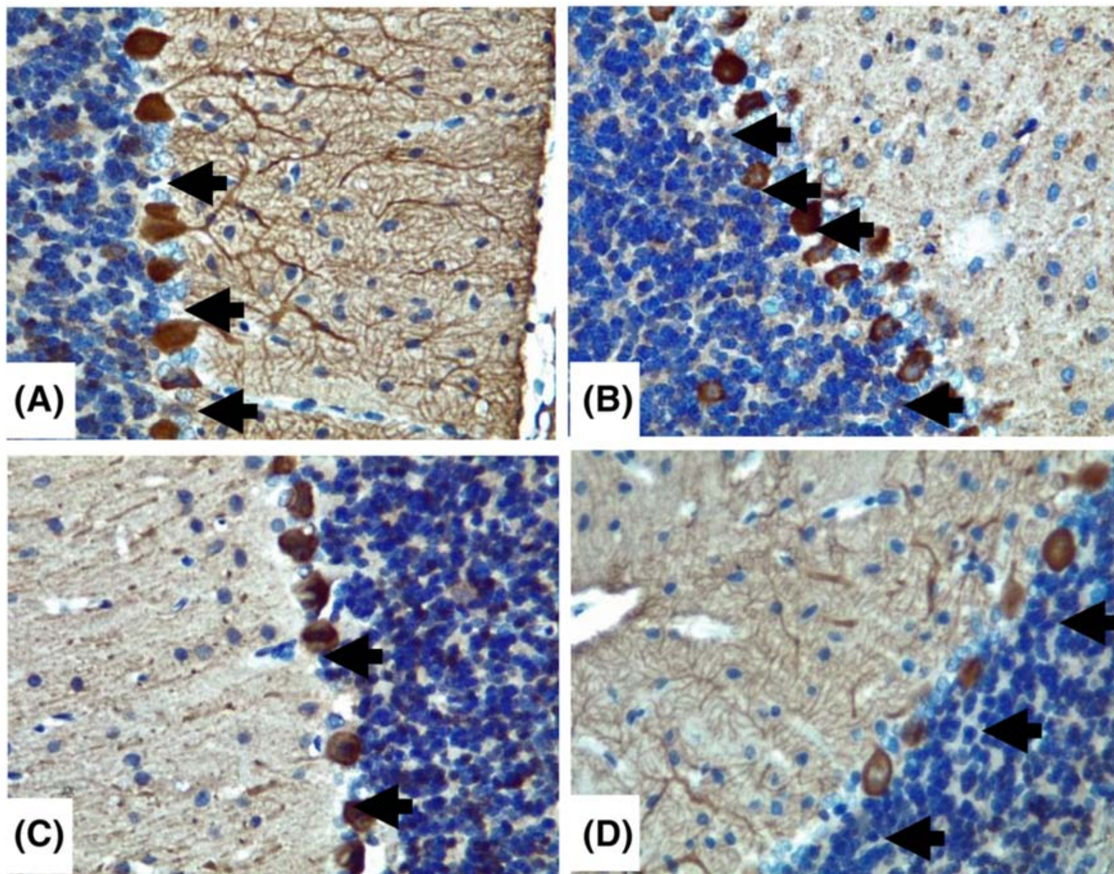
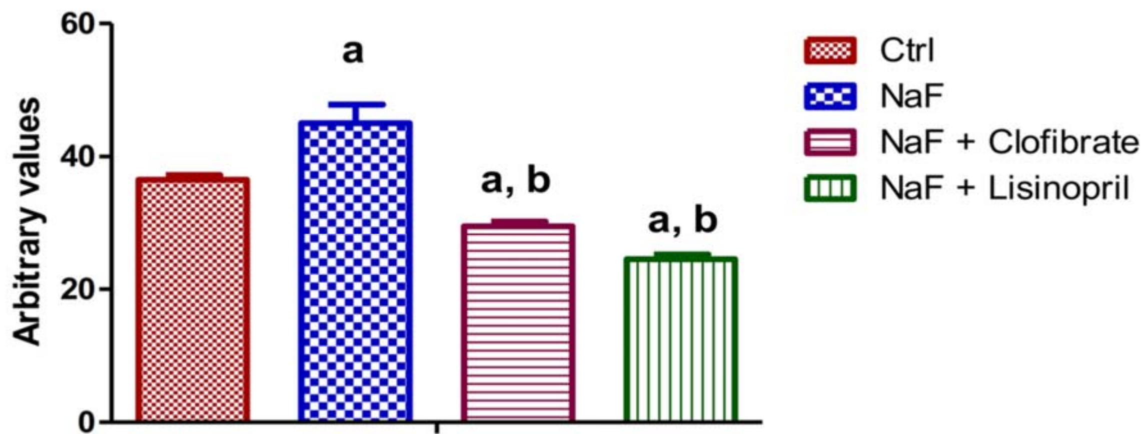


Figure 6. The immunolocalization of anti-calbindin on sodium fluoride (NaF)-induced neurotoxicity in the cerebellum. NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride. Alphabet "a" indicates significant difference when compared to the control while "b" indicates significant difference when compared to NaF only group at $P < .05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride



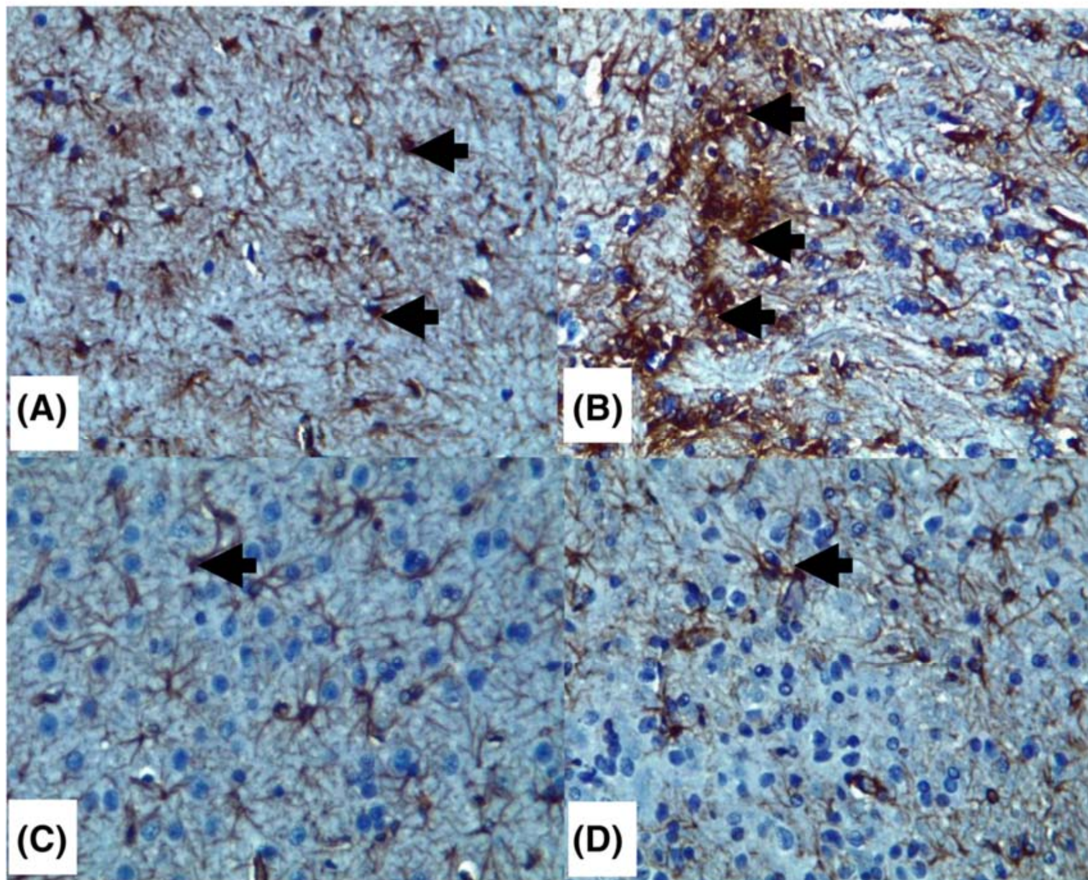
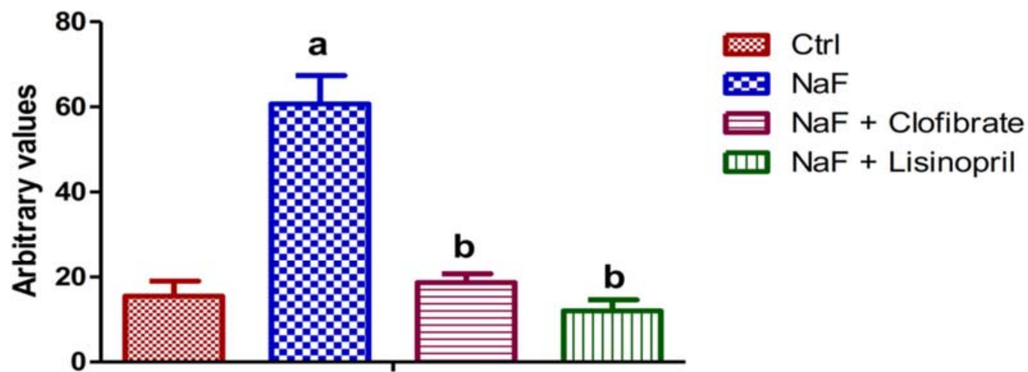


Figure 7. The immunolocalization of glial fibrillary acidic protein (GFAP) on sodium fluoride (NaF)-induced neurotoxicity in the cerebral cortex. Alphabet "a" indicates significant difference when compared to the control while "b" indicates significant difference when compared to NaF only group at $P < .05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride



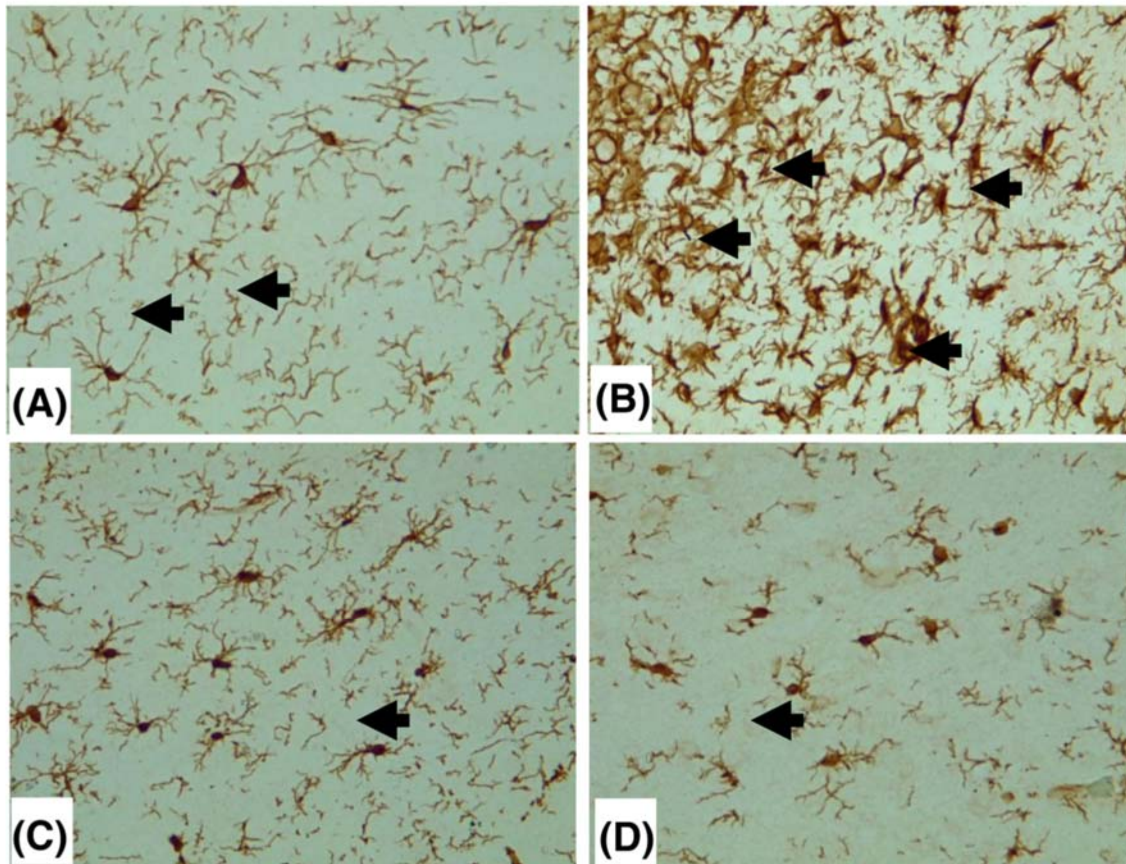


Figure 8. The immunohistochemistry of ionized calcium-binding adapter molecule 1 (IBA1) on sodium fluoride (NaF)-induced neurotoxicity in the cerebral cortex. Alphabet "a" indicates significant difference when compared to the control while "b" indicates significant difference when compared to NaF only group at $P < .05$ (n = 10). NaF (300 ppm), clofibrate (250 mg/kg), and lisinopril (10 mg/kg). Ctrl, control; NaF, sodium fluoride

4 DISCUSSION

The inhibition of AChE has been reported as one of the cardinal points of neurotoxicity in both aquatic and mammalian toxicity.41-44 AChE inhibition can lead to autonomic nervous crisis resulting in both sympathetic and parasympathetic symptoms.45 Neuropsychiatric disorders have been reported following exposure to organophosphates; these are major inhibitors of

AChE.[46], [47] AD is an advanced neurodegenerative disorder that is associated with AChE activation and amyloid plaque deposition.[48], [49]

From our results, NaF significantly inhibited AChE activity; this might be the hallmark of neurotoxicity observed in this study. Furthermore, coadministration with either clofibrate or lisinopril reactivates AChE inhibition to near normal activity. AChE reactivators have been reported as antidotes and treatment of choice for organophosphorus organophosphate pesticide intoxication.[46], [47], [50] Hence, clofibrate or lisinopril might be a novel cholinesterase reactivator in the near future.

Increasing evidence has shown the involvement of oxidative stress in the pathogenesis of Parkinson disease (PD) and potential drugs can be developed through modulation and inhibition of AChE activity or its antagonist.[51-54] The results obtained from the present study demonstrated the enhancement of oxidative stress by NaF intoxication as indicated by significant depletion of neuronal GSH, vitamin C, NPSH, SOD, and GST activities together with significant enhancement of neuronal MDA, H_2O_2 generated, and PSH and NO contents. The exaggerated production of lipid peroxidation products and depletion of enzymatic and non-enzymatic antioxidant defense system observed in this study contributed significantly oxidative stress, significantly to oxidative stress, neuroinflammation, and neurodegeneration. Our study is in tandem with previous studies that reported on how oxidative stress and neuroinflammation contributed to neurodegeneration and motor incoordination.[55] The novel antioxidant activity of clofibrate and lisinopril was demonstrated by a significant improvement in antioxidant defense system and mitigation of oxidative stress as indicated by significant reduction in markers of oxidative stress. The development of neurodegenerative disorders such as AD and PD has been positively correlated to oxidative stress and neuroinflammation.[56], [57] Similarly, oxidative fatty acid degradation in the brain has been reported as the signaling pathway in the development of Alzheimer-like pathology.[56] Therefore, enhanced production of neuronal NO in the present study played a major role in the observable neuroinflammation. Interestingly, coadministration of clofibrate or lisinopril clofibrate or lisinopril with NaF significantly attenuated excessive production of NO, which was indicative of anti-inflammatory properties of clofibrate and lisinopril. Lisinopril is a classic inhibitor of angiotensin-converting enzyme (ACE).

OFM is commonly used to measure mobility and anxiety-like behavior in animal models.[58] The different treatment protocols did not have any effect on the ambulatory ability of the rats; however, the animals moved freely in the box as displayed by similarity in the number of lines crossed across over the treatment groups. Rearing, an exploratory behavior, used as a measure of anxiety in the OFM increased in the NaF group, suggesting an increased anxiety level.[59] This anxiogenic potential of NaF is further confirmed by the significant increase in SAP. This indicates the reluctance of the rats to move reflecting an approach-avoidance tendency.[60] On the contrary, lisinopril administration significantly decreased this tendency showing its anxiogenic potential. The anxiety-stimulating effect of NaF reported in this study is in consonance with reports by Yuksel.[61] who in addition documented an increased number of defecations in the OFM following administration.

The immunohistochemistry of anti-calbindin revealed a significant loss of Purkinje fibers following NaF intoxication. Anti-calbindin is a 28KDa protein located in the cerebellar

cortex.[62] Previous studies have demonstrated link between Purkinje cell loss and neurobehavioral derangement such as motor incoordination and essential brain tremor of which PD is a sequela.[63-66] There is a growing evidence that Pukinje cell loss is associated with essential tremor.[67] The contribution of NaF to Purkinje cell loss in the present study was restored to near normal following coadministration of either clofibrate or lisinopril, which was indicative of their neuroprotective effects, thereby maintaining cerebellar architectural integrity. This study therefore supports a previous report on the possible neuroprotective of ACE inhibitor through the downregulation of kinin B1 receptors in the peripheral nervous system, which is responsible for neuropathic pain.[68] Oxidative stress and neuroinflammation might have contributed to the observable distortion and significant reduction in Purkinje cells in rats exposed to NaF alone.

The main intermediate filament protein in mature astrocytes is GFAP and is the critical component of the cytoskeleton during development.[69] Astrocytes are abundant in the brain and are the first reactive cells after any injury; thus, GFAP has been used as biomarker in several neurotoxicity studies.[70], [71] The significant increase in intermediate filament expressions, cellular hypertrophy, and proliferation observed in the cerebral cortex administered with NaF alone is indicative of astrocyte response to CNS insults. The astrocytic reaction is suggestive of defense against the observed increased oxidative stress induced by NaF. Astrocytes contain high concentrations of antioxidants upregulating glutathione following oxidative stress in a bid to restore CNS homeostasis.[72-74] On the other hand, astrocytosis with hypertrophied processes is compensatory for neuronal and synaptic degeneration as typified by the observed Purkinje cell degeneration in the rats.[75] Administration of either clofibrate or lisinopril was able to reduce astrocytic activation and expressions of GFAP, confirming their anti-inflammatory and neuroprotective properties as peroxisome proliferator-activated receptor-γ (PPARγ) agonist.[76]

In the present study, there was a significant increase in the expressions of Iba1. The Iba1 is a novel calcium-binding protein and is specifically expressed in microglia, and it has been reported to play a significant role in regulation of the function of microglia. Microglia constitute approximately 5% of all glial cells in the CNS. Activation of microglia commonly occurs in the early response of the CNS to a wide variety of pathological stimuli, including trauma, inflammation, degeneration, and ischemia.[77] They help in the elimination of microorganisms and deleterious debris by producing neurotrophic factors that are involved in neuroprotection.[78-80] On the other hand, microglia also exert a cytotoxic function by releasing reactive oxygen species, NO, or inflammatory cytokines, which ultimately triggered neuronal damage.[81] From the present study, administration of NaF led to a significant activation of microglia, and this might be associated with exaggeration exaggerated release of neuronal NO in NaF treated rats as compared to rats coadministered with either clofibrate or lisinopril. Therefore, clofibrate and lisinopril demonstrated their anti-inflammatory properties by mitigating microgliosis as observed in NaF alone. In conclusion, administration of NaF precipitated oxidative stress, Purkinje neuron degeneration, motor incoordination, and depletion of antioxidant defense system, while coadministration with either clofibrate or lisinopril attenuated oxidative stress, improved antioxidant defense status, and offered neuroprotection. Combining all, our findings demonstrate the neuroprotective effects of clofibrate and lisinopril as indicated in their ability to prevent astrocyosis, microgliosis, and Purkinje cell loss.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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