

Neuroprotection by Mast Cell Stabilizers and Histamine H₁ Receptor Blockade in Rotenone-Induced Oxidative Stress and Nigrostriatal Damage

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Abdel-Salam OM et al. Reactive Oxygen Species 1(3):228–244, 2016; ©2016 Cell Med Press
<http://dx.doi.org/10.20455/ros.2016.833>

(Received: January 17, 2016; Revised: February 10, 2016; Accepted: February 10, 2016)

ABSTRACT | We studied the effect of mast cell stabilizers and histamine H₁ receptor blockade on the development of oxidative stress and nigrostriatal damage induced in the rat by systemic rotenone injection. Rotenone (1.5 mg/kg body weight) was administered subcutaneously every other day for two weeks with and without intraperitoneal administration of disodium cromoglycate (10 and 20 mg/kg body weight), ketotifen (1 and 2 mg/kg body weight), or loratadine (1 and 2 mg/kg body weight). Rotenone caused a significantly elevated oxidative stress condition in different brain regions. Malondialdehyde (MDA) and nitric oxide concentrations were significantly increased by 47.5%–57.7%, and 64.2%–80.7%, respectively, in the cerebral cortex, striatum, and the rest of the brain. In contrast, the level of reduced glutathione (GSH) and the activity of paraoxonase 1 (PON1) were decreased by 28%–36% and 59%–62.3%, respectively, in the above brain regions. Acetylcholinesterase (AChE) activity in the cortex was also significantly decreased by 57.2% after rotenone injection. Rotenone caused neuronal death in the cortex, striatum, and hippocampus. Prominent caspase-3 immunoreactivity was observed in the cortex and striatum after rotenone exposure. Rotenone-induced oxidative stress was ameliorated by co-treatment with cromoglycate, ketotifen, or loratadine. These agents decreased lipid peroxidation (MDA), inhibited nitric oxide formation, and restored GSH levels and PON1 activity in the different brain regions. The AChE activity in the cortex of the rotenone-exposed animals was significantly increased by co-treatment with ketotifen or loratadine, but further reduced after co-treatment with cromoglycate. Rotenone-induced histopathological changes and caspase-3 expression were attenuated after co-treatment with the above drugs. The improvement was most obvious in the groups co-treated with ketotifen. Collectively, these data suggest a role for mast cells and histamine H₁ receptors in mediating the pathological changes evoked by rotenone in the rat brain.

KEYWORDS | Cromoglycate; Hippocampus; Histamine H₁ antagonist; Ketotifen; Lipid peroxidation; Loratadine; Mast cell stabilizer; Mast cells; Neurotoxicity; Oxidative stress; Parkinson's disease; Parkinsonism; Rotenone; Striatum

ABBREVIATIONS | AChE, acetylcholinesterase; GSH, reduced glutathione; MDA, malondialdehyde; PD, Parkinson's disease; PON1, paraoxonase 1; ROS, reactive oxygen species; SNPc, substantia nigra pars compacta

CONTENTS

1. Introduction
2. Materials and Methods
2.1. Experimental Animals
2.2. Drugs and Chemicals
2.3. Study Design
2.4. Biochemical Analyses
2.4.1. Determination of Lipid Peroxidation
2.4.2. Determination of Reduced Glutathione Level
2.4.3. Determination of Nitric Oxide Level
2.4.4. Determination of Paraoxonase 1 Activity
2.4.5. Determination of Acetylcholinesterase Activity
2.5. Histopathological Examination
2.5.1. Hematoxylin and Eosin Staining
2.5.2. Toluidine Blue Staining
2.6. Statistical Analysis
3. Results
3.1. Biochemical Findings
3.1.1. MDA Level
3.1.2. Nitric Oxide Level
3.1.3. GSH Level
3.1.4. PON1 Activity
3.1.5. AChE Activity
3.2. Histopathological Findings
3.2.1. Hematoxylin and Eosin Staining
3.2.1.1. Cortex and Striatum
3.2.1.2. Hippocampus
3.2.2. Immunohistochemistry
3.2.3. Toluidine Blue Staining
4. Discussion

1. INTRODUCTION

Parkinson's disease (PD) is a common and progressive neurodegenerative disorder of old age that affects ~1% of individuals above the age of 65 years [1]. The disease is characterized by a triad of disordered voluntary motor activity in the form of (1) bradykinesia (slowness of movement) or even akinesia (absence of movement), (2) rigidity and postural instability, and (3) a resting tremor of the hands and less commonly the feet [2]. In PD, there is preferential loss of the dopamine-containing cells of the substantia nigra pars compacta (SNPc) which projects to

the striatum. This results in dopamine deficiency in the substantia nigra and striatum, and the development of the motor symptoms of the disease, which typically start following the loss of ~50–60% of SNPc and ~70% of the striatal dopaminergic neurons [3]. The genetic Mendelian forms of PD are rare, and ~95% of cases are sporadic. While the exact cause of sporadic PD is incompletely understood, the disorder is believed to largely result from exposure to an environmental toxin(s) in combination with a genetic susceptibility [4]. In this context, evidence from epidemiological studies implicates a number of insecticides and pesticides in increasing the risk for the

development of PD [5]. One such toxin, namely, rotenone—a pesticide and a mitochondrial complex I inhibitor—has been shown to replicate many features of PD including nigrostriatal cell loss and Lewy body-like inclusions when injected into rodents [6]. As such, rotenone is commonly used as a model chemical to create an animal model of Parkinsonism.

Increasingly accumulating evidence strongly implicates oxidative stress and neuro-inflammation in the pathological processes that lead to neuronal degeneration in PD [7]. Oxidative stress ensues when the cellular antioxidant mechanisms are faced with an overwhelming generation of oxygen-derived and other free radicals. This results in disordered cell signaling which might proceed to perturbed cellular machinery and even cause cell death owing to free radical attack on membrane lipids, proteins, and nucleic acids [8]. Several factors contribute to the high levels of oxidative stress in the brain of PD patients, such as decreased antioxidants (e.g., glutathione) [9], increased dopamine oxidation, increased redox activity of neuromelanin [10], and increased brain content of iron [11], a redox active metal that has the ability to generate highly reactive hydroxyl radicals [8].

Cell bodies of histaminergic neurons are present in the mammillary tubercles of the posterior hypothalamus from which they send projections to the rest of the brain and spinal cord [12]. In the central nervous system, histamine mediates its effects by interacting with histamine H₁, H₂ and H₃ receptors. The brain histaminergic system is involved in attention, sleep-wake cycle, memory, and learning, among other functions [13]. Brain histamine levels are increased in PD patients [14]. Histaminergic fibers also increase in the substantia nigra of the PD brain [15]. The striatum receives input from histaminergic neurons in the tuberomammillary nucleus [12], and there is evidence for a modulatory effect of histamine on basal ganglia output [16]. Histamine is also present within mast cells. Mast cells are bone marrow-derived immune cells found in all tissues and organs including the brain. The precursors of mast cells are derived from hematopoietic stem cells, released into blood, migrate to different tissues, where the cells differentiate and mature in the target tissue [17]. Mast cells are rich in cytoplasmic granules and release their preformed granular contents in response to a variety of stimuli (both immunogenic and non-immunogenic). The mast cell's principal granular content is histamine, but the granule also contains

serotonin, kinins, heparin, cytokines (e.g., tumor necrosis factor- α , interferon- γ , and interleukin-6), and proteases (e.g., cathepsins, chymases, and tryptases). The release of these mediators results in immediate inflammatory responses. Mast cells are also capable of synthesizing and releasing new inflammatory mediators, such as prostaglandins, leukotrienes, monocyte chemoattractant protein-1, nitric oxide, growth factors, and peptides (somatostatin and substance P). In this way, mast cells might be involved in the development of chronic inflammation [18]. Recently, a role of mast cells in the pathogenesis of neurodegenerative disorders has been revealed [19].

To elucidate the role of the histaminergic system and mast cells in the brain damage caused by rotenone injection, pharmacological blockade of mast cell activation with disodium cromoglycate or ketotifen has been employed in the present study. Moreover, the effect of histamine H₁ receptor blockade with the antihistaminic drug loratadine has been investigated in our study. Our results have demonstrated a potential role for mast cells and histamine H₁ receptors in mediating rotenone-induced oxidative stress and nigrostriatal damage.

2. MATERIALS AND METHODS

2.1. Experimental Animals

Sprague-Dawley rats (male, 130–140 g of body weight) obtained from the Animal House of the National Research Centre, Cairo were used in the study. The rats were housed under temperature- and light-controlled conditions and provided with standard laboratory food and water ad libitum. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre (Cairo, Egypt) and followed the recommendations of the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

2.2. Drugs and Chemicals

Loratadine, ketotifen, disodium cromoglycate, rotenone, and other chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Loratadine, ketotifen, and disodium cromoglycate were prepared in normal saline, and rotenone was

dissolved in 100% dimethyl sulfoxide. The doses of sodium cromoglycate (10 and 20 mg/kg body weight), ketotifen (1 and 2 mg/kg body weight), or loratadine (1 and 2 mg/kg body weight) used in the present were based on the literature [20–22].

2.3. Study Design

Rats were randomly allocated into eight groups, with six rats in each group. Group 1 received the vehicle (dimethyl sulfoxide); group 2 received a subcutaneous injection of rotenone (1.5 mg/kg body weight) three times per week; groups 3–8 received rotenone (1.5 mg/kg body weight subcutaneously three times per week) in combination with intraperitoneal injection of cromoglycate (10 and 20 mg/kg body weight), ketotifen (1 and 2 mg/kg body weight), and loratadine (1 and 2 mg/kg body weight), respectively. The drugs (cromoglycate, ketotifen, and loratadine) were given once every other day parallel to the rotenone injection. The injection volume was 0.2 ml per rat. The treatments were continued for 2 weeks, and rats were then euthanized under ether anesthesia for tissue collection. The brains were quickly dissected out on an ice-cold plate into different areas (the cerebral cortex, striatum, and rest of the brain), washed with ice-cold phosphate-buffered saline (pH 7.4), weighed, and stored at -80°C until the biochemical analyses. The tissues were homogenized in 0.1 M phosphate-buffered saline (pH 7.4) to give a final concentration of 10% (weight/volume) for the biochemical assays.

2.4. Biochemical Analyses

2.4.1. Determination of Lipid Peroxidation

Lipid peroxidation level in the brain homogenates was determined by measuring the level of malondialdehyde (MDA) using the method of Ruiz-Larrea et al. [23]. In this assay MDA (along with its equivalents) reacts with thiobarbituric acid to produce a pink colored complex which can be determined spectrophotometrically at 532 nm.

2.4.2. Determination of Reduced Glutathione Level

The level of the reduced glutathione (GSH) in the brain tissue homogenates was determined based on

the reduction of the Ellman's reagent by the sulfhydryl groups of GSH to 2-nitro-5-mercaptobenzoic acid, which has intense yellow colour and is determined spectrophotometrically at 412 nm [24].

2.4.3. Determination of Nitric Oxide Level

The level of nitrite, a stable end product of nitric oxide, in the supernatants of the brain tissue homogenates was determined using the Griess reagent, according to the method of Moshage et al. [25].

2.4.4. Determination of Paraoxonase 1 Activity

The arylesterase activity of paraoxonase 1 (PON1) was measured in the supernatants of the brain tissue homogenates using phenyl acetate as a substrate. In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate, resulting in the formation of phenol. The rate of the formation of phenol is measured spectrophotometrically by monitoring the increase in absorbance at 270 nm at 25°C [26].

2.4.5. Determination of Acetylcholinesterase Activity

The procedure described by Gorun et al. [27] was followed to measure the acetylcholinesterase (AChE) activity in the supernatants of the brain tissue homogenates. The principle of the method is based on the spectrophotometric measurement of the thiocholine formation resulting from the hydrolysis of acetylthiocholine by AChE.

2.5. Histopathological Examination

2.5.1. Hematoxylin and Eosin Staining

Brain sections were fixed in freshly prepared 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Paraffin sections of 5 μm thickness were prepared and stained with hematoxylin and eosin (H&E) for histopathological examination under a light microscope.

2.5.2. Toluidine Blue Staining

Rehydrated sections were treated with 0.1% toluidine blue, 7% ethanol, and 1% sodium chloride (pH of 2.0–2.5) for 5 min to identify mast cells and mast

cell granules. This results in a purple staining of the granules.

2.6. Statistical Analysis

Data are presented as mean \pm SEM. Statistical significance was determined using analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS software (SAS Institute Inc., Cary, NC). A probability (p) value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Biochemical Findings

3.1.1. MDA Level

Rotenone resulted in significant increases in MDA levels in the cerebral cortex (56.3%) (34.7 ± 1.2 versus 22.2 ± 0.16 nmol/g tissue), striatum (44.8%) (27.0 ± 0.91 versus 18.65 ± 0.6 nmol/g tissue), and in the rest of the brain (47.5%) (29.35 ± 1.7 versus 19.9 ± 1.0 nmol/g tissue) (**Figure 1A–1C**). In rotenone-exposed rats, co-administration of 1 mg/kg body weight of loratadine had no significant effect on the brain MDA level, compared to the values in the group exposed to rotenone only. Following co-administration of the higher dose of loratadine (2 mg/kg body weight), however, a significant decrease in MDA level by 19.6%, 23.0%, and 30.1% was observed in the cortex (27.9 ± 0.12 versus 34.7 ± 1.2 nmol/g tissue), striatum (20.8 ± 0.96 versus 27.0 ± 0.91 nmol/g tissue), and in the rest of the brain (20.52 ± 0.83 versus 29.35 ± 1.7 nmol/g tissue), respectively (**Figure 1A**). Co-administration of ketotifen at 1 and 2 mg/kg body weight to rotenone-exposed rats resulted in a decreased MDA level by 53% and 55%, respectively, in the cerebral cortex (16.3 ± 0.82 and 15.6 ± 0.77 versus 34.7 ± 1.2 nmol/g tissue), by 41.3% and 57.8%, respectively, in the striatum (15.83 ± 0.56 and 11.4 ± 0.66 versus 27.0 ± 0.91 nmol/g tissue), and by 41% and 58.4%, respectively, in the rest of the brain (17.34 ± 0.42 and 12.21 ± 0.71 versus 29.35 ± 1.7 nmol/g tissue) (**Figure 1B**). Co-administration of cromoglycate at 10 or 20 mg/kg body weight to rotenone-exposed rats significantly decreased the MDA level by 37.5% and 38.9%, respectively, in the cerebral cortex (21.7

± 1.1 and 21.2 ± 1.4 versus 34.7 ± 1.2 nmol/g tissue), by 18.1% and 34.7%, respectively, in the striatum (22.1 ± 0.7 and 17.63 ± 0.51 versus 27.0 ± 0.91 nmol/g tissue), and by 26.1% and 29.1%, respectively, in the rest of the brain (21.7 ± 0.80 and 20.8 ± 0.94 versus 29.35 ± 1.7 nmol/g tissue) (**Figure 1C**).

3.1.2. Nitric Oxide Level

Following injection of rotenone, a significant increase in the nitric acid level was found in the cortex, striatum, and the rest of the brain by 80.7% (37.84 ± 1.8 versus 20.94 ± 1.3 μ mol/g tissue), 64.4% (34.56 ± 1.6 versus 21.0 ± 0.18 μ mol/g tissue) and 74.9% (32.4 ± 1.9 versus 18.52 ± 1.0 μ mol/g tissue), respectively (**Figure 1D–1F**). Loratadine co-treatment at 1 and 2 mg/kg body weight reduced the nitric oxide level by 26 % and 47.4%, respectively, in the cerebral cortex, by 22.5% and 42.7%, respectively, in the striatum, and by 24.7% and 40.1%, respectively, in the rest of the brain, compared to the values in the group exposed to rotenone only (**Figure 1D**). Ketotifen at 1 and 2 mg/kg body weight decreased the nitric oxide level by 41.3% and 50.3%, respectively, in the cerebral cortex, by 38.8% and 46.9%, respectively, in the striatum, and by 29.6% and 38.0%, respectively, in the rest of the brain, compared to the values in the group exposed to rotenone only (**Figure 1E**). Similarly, co-administration of cromoglycate at 10 and 20 mg/kg body weight to rotenone-exposed rats significantly decreased the level of nitric oxide by 40.8% and 50.3%, respectively, in the cerebral cortex, by 29.7% and 51.1%, respectively, in the striatum, and by 24.4% and 38.0%, respectively, in the rest of the brain, compared to the values in the group exposed to rotenone only (**Figure 1F**).

3.1.3. GSH Level

In rats treated with rotenone, the levels of GSH decreased by 39%, 56.3%, and 71% in the cerebral cortex (3.41 ± 0.11 versus 4.74 ± 0.21 μ mol/g tissue), striatum (3.0 ± 0.11 versus 4.69 ± 0.13 μ mol/g tissue), and in the rest of the brain (2.96 ± 0.08 versus 4.6 ± 2.0 μ mol/g tissue), respectively (**Figure 2A–2C**). Co-administration of loratadine at 1 mg/kg body weight had no significant effect on the brain GSH levels in rotenone-exposed rats. The higher dose of loratadine (2 mg/kg body weight), however, almost restored the GSH level in the different brain

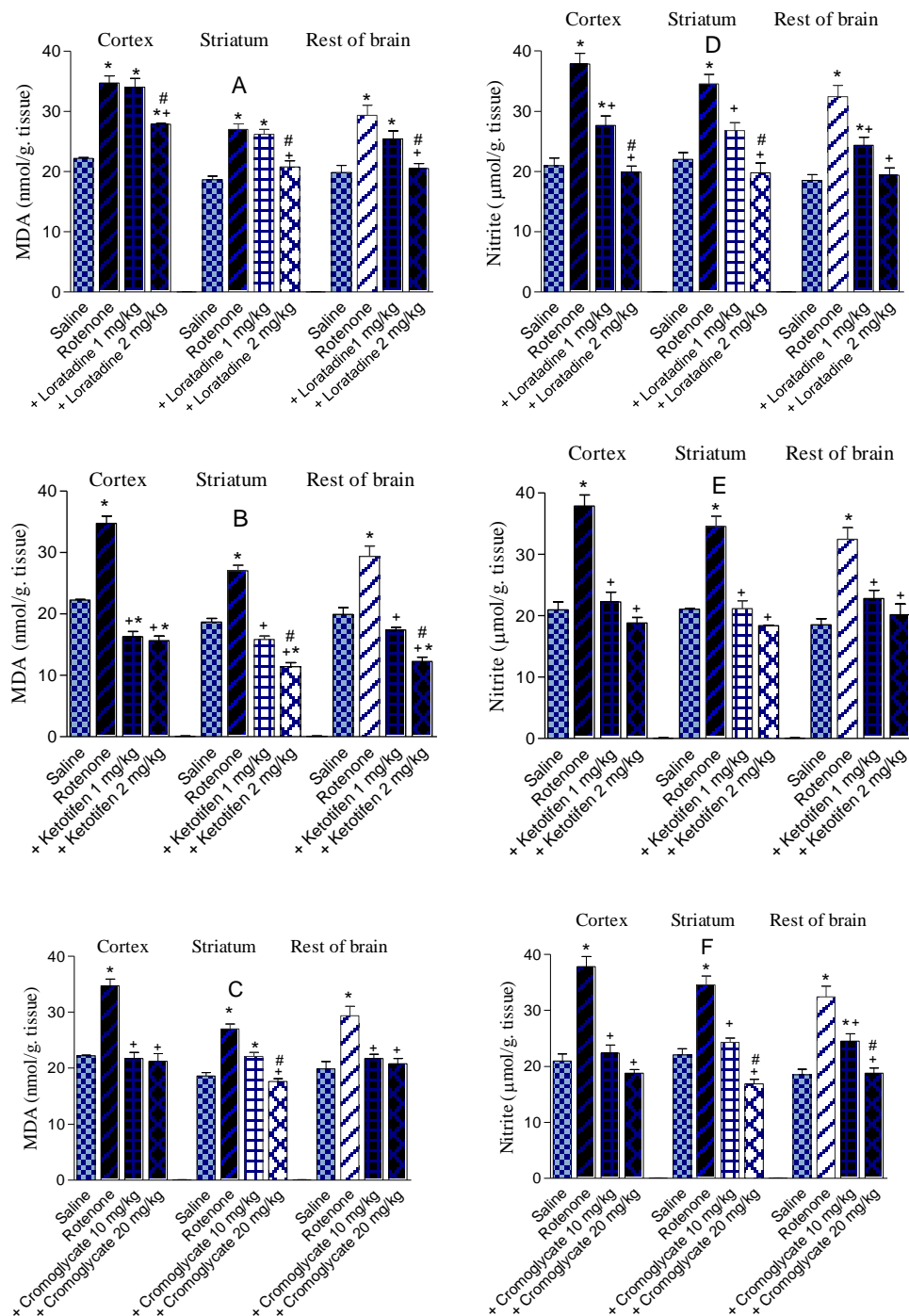


FIGURE 1. Effects of loratadine, ketotifen and cromoglycate on the levels of malondialdehyde (MDA) (A, B, C) and nitrite (D, E, and F) in the brain tissues of rotenone-exposed rats. Results are mean \pm SEM (n = 6). *, p < 0.05 versus saline; +, P < 0.05 versus rotenone only group; #, p < 0.05 versus the lower dose of the drug.

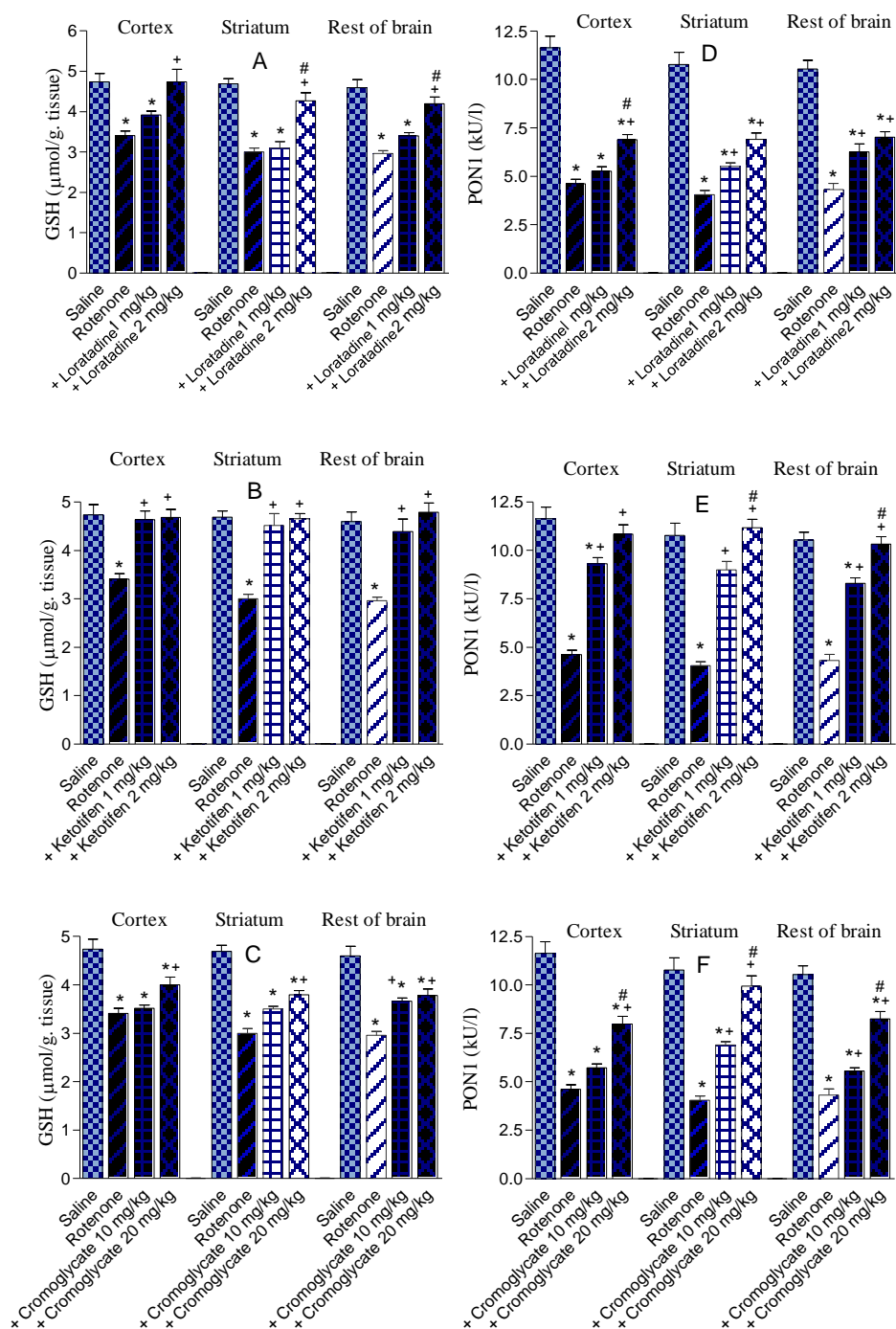


FIGURE 2. Effects of loratadine, ketotifen, and cromoglycate on the level of reduced glutathione (GSH) (A, B, and C) and the activity of paraoxonase 1 (PON1) (D, E, and F) in the brain tissues of rotenone-exposed rats. Results are mean \pm SEM ($n = 6$). *, $p < 0.05$ versus saline; +, $P < 0.05$ versus rotenone only group; #, $p < 0.05$ versus the lower dose of the drug.

regions to their corresponding control values (**Figure 2A**). Ketotifen co-treatment at 1 and 2 mg/kg body weight restored the GSH level in the cerebral cortex, striatum and the rest of the brain to their corresponding control values (**Figure 2B**). Co-administration of cromoglycate at 10 mg/kg body weight had no significant effect on the level of GSH in the cortex and striatum of rotenone-exposed rats. Co-treatment with cromoglycate at 20 mg/kg body weight, however, resulted in significant increases in the GSH levels in the cerebral cortex and striatum by 17.3% (4.0 ± 0.16 versus 3.41 ± 0.11 $\mu\text{mol/g}$ tissue) and 26.7% (3.8 ± 0.08 versus 3.0 ± 0.11 $\mu\text{mol/g}$ tissue), respectively. Co-treatment with cromoglycate at 10 and 20 mg/kg body weight significantly increased the GSH levels in the rest of the brain by 23.6% and 27.7% (3.66 ± 0.07 and 3.78 ± 0.14 versus 2.96 ± 0.08 $\mu\text{mol/g}$ tissue), respectively (**Figure 2C**).

3.1.4. PON1 Activity

Rotenone induced a significant decrease in PON1 activity by 60.3%, 62.3%, and 59% in the cerebral cortex (4.62 ± 0.23 versus 11.64 ± 0.59 kU/L), striatum (4.06 ± 0.21 versus 10.78 ± 0.63 kU/L) and in the rest of the brain (4.32 ± 0.31 versus 10.54 ± 0.40 kU/L), respectively (**Figure 2D–2F**). The PON1 activity in the rotenone-exposed rats showed a significant increase in the cortex by 49.1% following co-treatment with loratadine at 2 mg/kg body weight (6.89 ± 0.27 versus 4.62 ± 0.23 kU/L). The PON1 activity was increased by 36% and 70%, respectively, in the striatum and by 44.7 and 62.5%, respectively, in the rest of the brain after co-treatment with loratadine at 1 and 2 mg/kg body weight (**Figure 2D**). Co-treatment with ketotifen at both 1 and 2 mg/kg body weight caused dramatic increases in PON1 activity in all 3 regions of the brain tissues from rotenone-exposed rats with a complete restoration of PON1 activity at the higher dose (**Figure 2E**). Co-administration of cromoglycate at 10 and 20 mg/kg body weight to rotenone-exposed rats resulted in increased PON1 activities by 23.8% and 72.7%, respectively, in the cerebral cortex (5.72 ± 0.2 and 7.98 ± 0.41 versus 4.62 ± 0.23 kU/L), by 70% and 144.8%, respectively, in the striatum (6.9 ± 0.18 and 9.94 ± 0.53 versus 4.06 ± 0.21 kU/L), and by 28.9% and 90.7%, respectively, in the rest of the brain (5.57 ± 0.16 and 8.24 ± 0.39 versus 4.32 ± 0.31 kU/L) (**Figure 2F**).

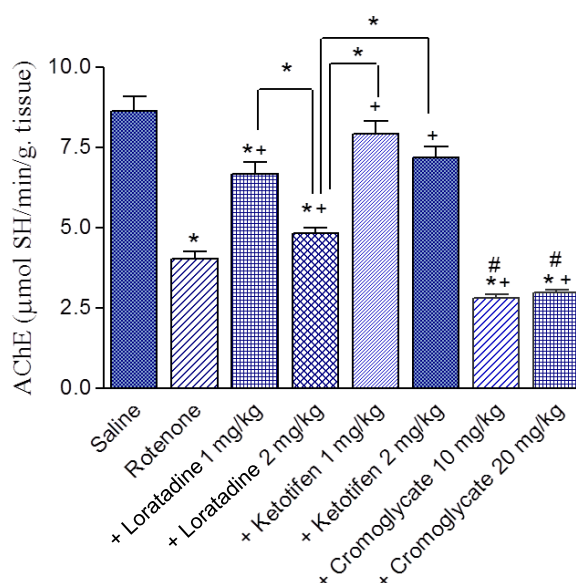


FIGURE 3. Effects of loratadine, ketotifen, and cromoglycate on acetylcholinesterase activity (AChE) in the brain tissues of rotenone-exposed rats. Results are mean \pm SEM (n = 6). *, p < 0.05 versus saline and between different groups as indicated in the figure; +, P < 0.05 versus rotenone only group; #, p < 0.05 versus other treatment groups.

3.1.5. AChE Activity

A significant inhibition in AChE activity by 53.2% was observed in the cerebral cortex of rotenone-exposed rats compared to the vehicle-control group (4.05 ± 0.21 versus 8.65 ± 0.46 $\mu\text{mol SH/min/g}$ tissue) (**Figure 3**). Co-administration of loratadine at 1 and 2 mg/kg body weight to rotenone-exposed rats resulted in increased AChE activity by 64.9% and 19.3% (6.68 ± 0.37 and 4.83 ± 0.18 versus 4.05 ± 0.21 $\mu\text{mol SH/min/g}$ tissue), respectively. A significant increase in AChE activity by 95.6% and 77.8% was also found after co-treatment with ketotifen at 1 and 2 mg/kg body weight, respectively (7.92 ± 0.40 and 7.2 ± 0.32 versus 4.05 ± 0.21 $\mu\text{mol SH/min/g}$ tissue). In contrast, co-administration of cromoglycate at 10 and 20 mg/kg body weight significantly inhibited AChE activity by 30.6% and 26.2%, respectively, compared to the group exposed to rotenone only (2.81 ± 0.13 and 2.99 ± 0.07 versus 4.05 ± 0.21 $\mu\text{mol SH/min/g}$ tissue) (**Figure 3**).

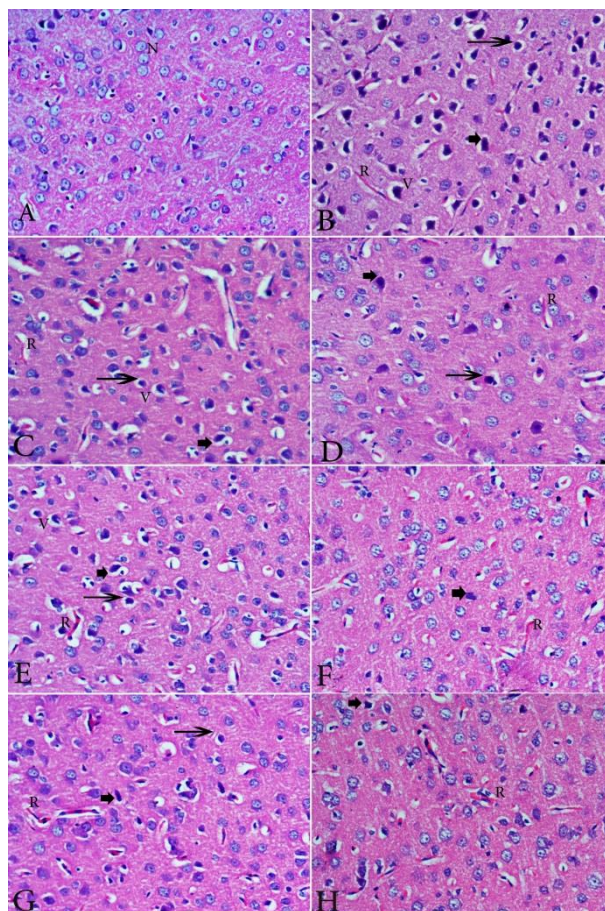


FIGURE 4. Representative photomicrographs of sections of the rat cortex. In (A), vehicle-control group showed normal neurons with round nuclei (N). In (B), rotenone-exposed group showed distortions in cellular architecture, pyknotic (arrowhead), and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V) and red neurons (R). In (C), rotenone plus loratadine (1 mg/kg body weight) group showed mild pyknotic (arrowhead) and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V) and a number of red neurons (R). In (D), rotenone plus loratadine (2 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic (arrowhead) and apoptotic nuclei (arrow) and a number of red neurons (R). In (E), rotenone plus cromoglycate (10 mg/kg body weight) group showed mild pyknotic (arrowhead) and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V) and a number of red neurons (R). In (F), rotenone plus cromoglycate (20 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic (arrowhead) and apoptotic nuclei and a number of red neurons (R). In (G), rotenone plus ketotifen (1 mg/kg body weight) group showed mild pyknotic (arrowhead) and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations and a number of red neurons (R). In (H), rotenone plus ketotifen (2 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic (arrowhead) and apoptotic nuclei and a number of red neurons (R). All images are 400× and H&E staining.

3.2. Histopathological Findings

3.2.1. Hematoxylin and Eosin Staining

3.2.1.1. CORTEX AND STRIATUM

In the vehicle-treated group, the cortical and striatal neurons were arranged in neat rows with abundant cytoplasm and round basophilic nuclei. Sections from rotenone-treated rats showed structural damage, pink shrunken neurons (considered as a sign of neuronal death), and apoptotic nuclei surrounded by perineuronal vacuolations. Moreover, a large number of red neurons were noted (**Figures 4 and 5**). In rotenone-treated rats, co-administration of loratadine at 1 mg/kg body weight resulted in less damage, with pink shrunken neurons, apoptotic nuclei surrounded by perineuronal vacuolations, and few red neurons. The higher dose of loratadine was associated with

slight degeneration with few pink shrunken neurons, apoptotic nuclei, and a small number of red neurons (**Figures 4 and 5**).

Sections from rats treated with rotenone along with ketotifen at 1 mg/kg body weight or cromoglycate at 10 mg/kg body weight showed mildly damaged neurons, with pink shrunken neurons, apoptotic nuclei surrounded by perineuronal vacuolations, and a small number of red neurons. Rats treated with the higher doses of ketotifen or cromoglycate showed improvement in cellular morphology compared with those in the group exposed to rotenone only. Notably, more remarkable improvement was observed after co-treatment with ketotifen, compared with cromoglycate co-treatment (**Figures 4 and 5**).

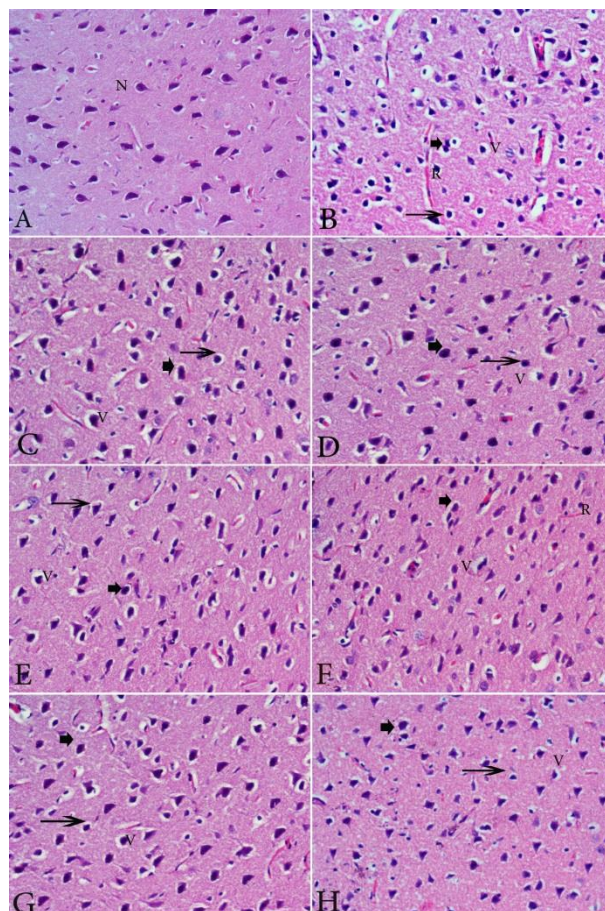


FIGURE 5. Representative photomicrographs of sections of the rat striatum. In (A), vehicle-control group showed normal neurons with round nuclei (N). In (B), rotenone-exposed group showed distortions in cellular architecture, pyknotic (arrowhead), and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V) with a number of red neurons (R). In (C), rotenone plus loratadine (1 mg/kg body weight) group showed mild pyknotic (arrowhead) and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V). In (D), rotenone plus loratadine (2 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic (arrowhead) and apoptotic nuclei (arrow) with perineuronal vacuolations (V). In (E), rotenone plus cromoglycate (10 mg/kg body weight) group showed mild pyknotic (arrowhead) and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V). In (F), rotenone plus cromoglycate (20 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic (arrowhead), perineuronal vacuolations (V), and a number of red neurons (R). In (G), rotenone plus ketotifen (1 mg/kg body weight) showed mild pyknotic (arrowhead) and apoptotic nuclei (arrow) surrounded by perineuronal vacuolations (V). In (H), rotenone plus ketotifen (2 mg/kg body weight) showed healthy appearance of neurons with some pyknotic (arrowhead) and apoptotic nuclei (arrow). All images are 400 \times and H&E staining.

3.2.1.2. HIPPOCAMPUS

In the vehicle-control group, the hippocampal pyramidal neurons were tightly arranged, with round nuclei and clear nucleoli (**Figure 6A**). Rats exposed to rotenone showed morphological changes of pyramidal neurons characterized by condensed shrunken morphology and pyknotic nuclei with eosinophilic staining (**Figure 6B**). The rotenone-induced morphological changes were markedly ameliorated following co-administration of the lower dose of each of the three drugs—loratadine, ketotifen, and cromoglycate (**Figure 6C**, **6E**, and **6G**). Following co-treatment with the higher dose of each of the above drugs, the hippocampal pyramidal neurons were largely rescued from rotenone-induced morphological damage, and the improvement was most obvious in ketotifen- and cromoglycate-treated groups (**Figure 6D**, **6F**, and **6H**).

3.2.2. Immunohistochemistry

Caspase-3 immunoreactivity in the cortex and striatum is shown in **Figures 7** and **8**. No caspase-3 expression was detectable in the neurons of the cortex and striatum of the vehicle-control rats. In contrast, the rotenone-treated group showed strong caspase-3 immunostaining. Rats co-treated with loratadine, ketotifen or cromoglycate showed dose-dependent decreases in caspase-3 expression in the cortex and striatum with the most obvious decrease being seen in rats co-treated with ketotifen.

3.2.3. Toluidine Blue Staining

In the vehicle-control group, mast cells were scarce in the brain tissue (**Figure 9A**). Mast cells with strong metachromatic stain were observed in the

brain tissue in rotenone-exposed group (**Figure 9 B**). After co-treatment with the lower doses of loratadine, cromoglycate, or kitotifen, there was mildly reduced metachromatic stain of mast cells which appeared partially degranulated (**Figure 9C, 9E, and 9G**). Following co-treatment with the higher doses of the above drugs, brain sections showed reduced mast cell accumulation, and these metachromatically stained cells appeared in granulated form (**Figure 9D, 9F, and 9H**).

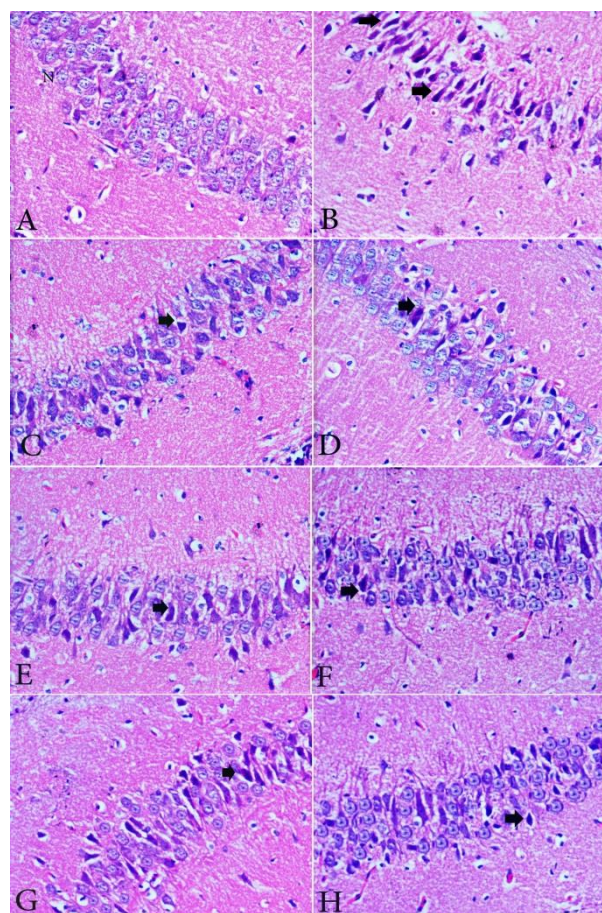
4. DISCUSSION

The findings of this study indicated that the subcutaneous administration of rotenone to rats resulted in increased oxidative stress in different brain regions. Histopathological and immunohistochemical studies of the striatum, cerebral cortex, and hippocampus revealed neuronal death and increased caspase-3 activity (an apoptotic marker) [28] in rotenone-exposed rats. The level of MDA, a marker of lipid peroxidation [8], was markedly increased in the striatum, cerebral cortex, and the rest of the brain after exposure to rotenone. Meanwhile, the levels of GSH, an important antioxidant and free radical scavenger [8], decreased in the brain following rotenone injection.

FIGURE 6. Representative photomicrographs of sections of the rat hippocampus. In (A), vehicle-control group showed normal pyramidal neurons with round nuclei (N). In (B), rotenone-exposed group showed distortions in cellular architecture and pyknotic nuclei (arrowhead). In (C), rotenone plus loratadine (1 mg/kg body weight) group showed mild pyknotic nuclei (arrowhead). In (D), rotenone plus loratadine (2 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic nuclei (arrowhead). In (E), rotenone plus cromoglycate (10 mg/kg body weight) group showed mild pyknotic nuclei (arrowhead). In (F), rotenone plus cromoglycate (20 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic nuclei (arrowhead). In (G) rotenone plus ketotifen (1 mg/kg body weight) group showed mild pyknotic nuclei (arrowhead). In (H), rotenone plus ketotifen (2 mg/kg body weight) group showed healthy appearance of neurons with some pyknotic nuclei (arrowhead). All images are 400× and H&E staining.

Consistent with previous observations [29], rotenone exposure resulted in increased nitric oxide levels in the brain. Excess nitric oxide produced by the inducible form of nitric oxide synthase during toxic and inflammatory conditions has been shown to cause neurotoxicity and induce apoptotic cell death [30]. Our findings thus confirmed previous studies suggesting oxidative stress and nitric oxide formation as contributing factors to rotenone-induced neurodegeneration [29, 31, 32]. In this context, it is noteworthy that the ability of rotenone to cause neuronal death in vitro could be prevented by the chain-breaking antioxidant α -tocopherol [31] or by *N*-acetyl-cysteine, a glutathione precursor [32].

Moreover, and in consistence with our previous findings [29], we showed here that rotenone was capable of inhibiting the activity of PON1 in the different brain regions. The PON1 enzyme is involved in the hydrolysis of the active metabolites of a number of organophosphate insecticides (such as diazoxon,



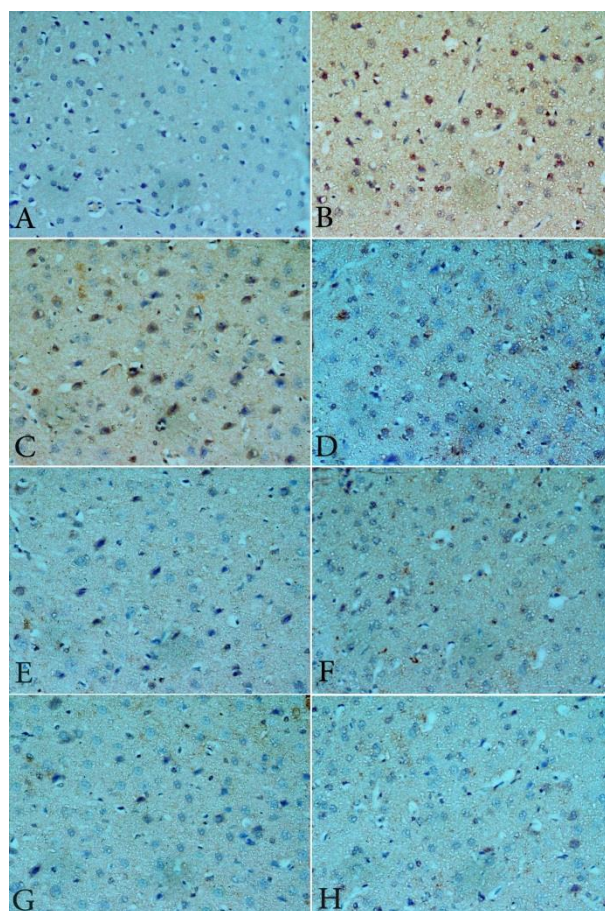


FIGURE 7. Representative photomicrographs of caspase-3 immunoreactivity in the rat cortex. In (A), vehicle-control group showed no detectable caspase-3 immunostaining. In (B), rotenone-exposed group showed positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (C), rotenone plus loratadine (1 mg/kg body weight) group showed mild positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (D), rotenone plus loratadine (2 mg/kg body weight) group showed little positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (E), rotenone plus cromoglycate (10 mg/kg body weight) group showed mild positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (F), rotenone plus cromoglycate (20 mg/kg body weight) group showed little positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (G), rotenone plus ketotifen (1 mg/kg body weight) group showed mild positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (H), rotenone plus ketotifen (2 mg/kg body weight) showed little positive immunoreaction for caspase-3 in the cytoplasm of neurons. All images are 400 \times and immunostaining for caspase-3.

paraoxon, dichlorvos, and chlorpyrifos oxon) and nerve agents [33]. Exposure to organophosphate insecticides has been implicated in the increased risk for developing PD [5]. Variation in PON1 enzyme activity could alter the susceptibility to organophosphates and increase the risk of developing PD in exposed subjects [34]. The low PON1 activity in the brains of rats treated with rotenone might be due to inactivation of the enzyme by the pesticide or by the increased generation of oxygen reactive metabolites. This latter effect has been demonstrated in previous studies [35].

In the present study, the increases in brain lipid peroxidation and nitric oxide levels, together with the decreases in GSH level and PON1 activity as well as the increases in neuronal death and caspase-3 activation due to rotenone exposure could all be attenuated by co-administration of the mast cell stabilizers cromoglycate and ketotifen or the antihistaminic

drug loratadine. These data implicated the brain histaminergic system and mast cells in mediating the neurotoxicity and neurodegeneration caused by rotenone in the rats. Loratadine is a non-sedating and a long-acting tricyclic antihistamine with selective histamine H_1 -receptor antagonistic activity. It belongs to the second generation H_1 receptor antagonists. These agents are highly selective for the histamine H_1 -receptor and have no anticholinergic effects. Because of their lipophilic properties, the H_1 antihistamines cross the blood-brain barrier, and bind to the cerebral H_1 receptors [36]. Loratadine and other H_1 antihistamines like cetirizine, fexofenadine, and meclizastine inhibit the activity of the H_1 receptor-mediated nuclear factor kappa-B (NF κ B) activation [37]. The latter is a transcription factor which orchestrates many mediators of inflammation [38]. H_1 -antihistamines displayed no scavenging action against superoxide anion, hydroxyl radical, or nitric oxide. Ketotifen and many other H_1 -antihistamines, however, inhibited the metabolic activity of phagocytes. This action might be beneficial in decreasing oxidative stress in pathological conditions where

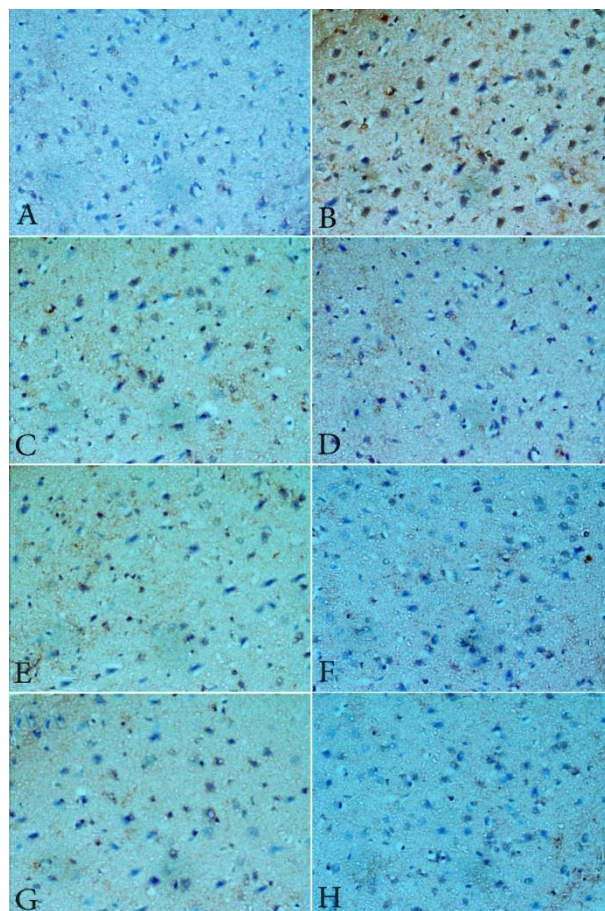


FIGURE 8. Representative photomicrographs of caspase-3 immunoreactivity in the rat striatum.

In (A), vehicle-control group showed no detectable caspase-3 immunostaining. In (B), rotenone-exposed group showed positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (C), rotenone plus loratadine (1 mg/kg body weight) group showed mild positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (D), rotenone plus loratadine (2 mg/kg body weight) group showed little positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (E), rotenone plus cromoglycate (10 mg/kg body weight) group showed mild positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (F), rotenone plus cromoglycate (20 mg/kg body weight) group showed little positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (G), rotenone plus ketotifen (1 mg/kg body weight) group showed mild positive immunoreaction for caspase-3 in the cytoplasm of neurons. In (H), rotenone plus ketotifen (2 mg/kg body weight) showed little positive immunoreaction for caspase-3 in the cytoplasm of neurons. All images are 400 \times and immunostaining for caspase-3.

there is excessive generation of reactive oxygen species (ROS) from the activated phagocytes [39].

Cromoglycate is used in asthma prophylaxis by virtue of its ability to stabilize the cell membrane of mast cells, thereby inhibiting their degranulation and the consequent release of the allergic and inflammatory mediators [40]. The drug also inhibited the activation of human neutrophils and eosinophils in vitro [41]. Mast cell stabilization with cromoglycate was found to decrease the extent of cerebral hemorrhage, brain swelling, and neutrophil infiltration caused by tissue plasminogen activator (tPA) after ischemia/reperfusion injury in rats. This suggests that mediators released from mast cells are involved in the initiation of neuronal damage following ischemia/reperfusion brain injury. Cromoglycate was given via the intracerebroventricular route assuming poor penetration of the drug across the blood–brain barrier [42]. In other studies, however, the drug ad-

ministered intravenously or subcutaneously was able to interfere with the development of cerebral damage in the ischemic brain [43] or after status epilepticus [44]. In their study, Jin et al. [43] found that mast cells increased in number and became activated in the immature rat brain early after brain ischemic/hypoxic injury. Cromoglycate given subcutaneously (50 mg/kg body weight) prevented the migration of mast cells, decreased brain damage, neuronal loss, astroglial activation, and brain atrophy. In rats administered pilocarpine to induce status epilepticus, treatment with cromoglycate (50 mg/kg body weight) via subcutaneous injection (s.c.) prevented the increase in the number of mast cells, decreased the release of histamine, and reduced neuronal damage in the hippocampus [44]. Our results further indicated the efficacy of cromoglycate in decreasing the toxic brain injury using the intraperitoneal route of administration. Cromoglycate has also been demonstrated to exert opiate receptor-dependent analgesic effects and potentiate morphine-induced analgesia (50 mg/kg body weight, s.c.) in mice [45], thereby suggesting an efficient penetration

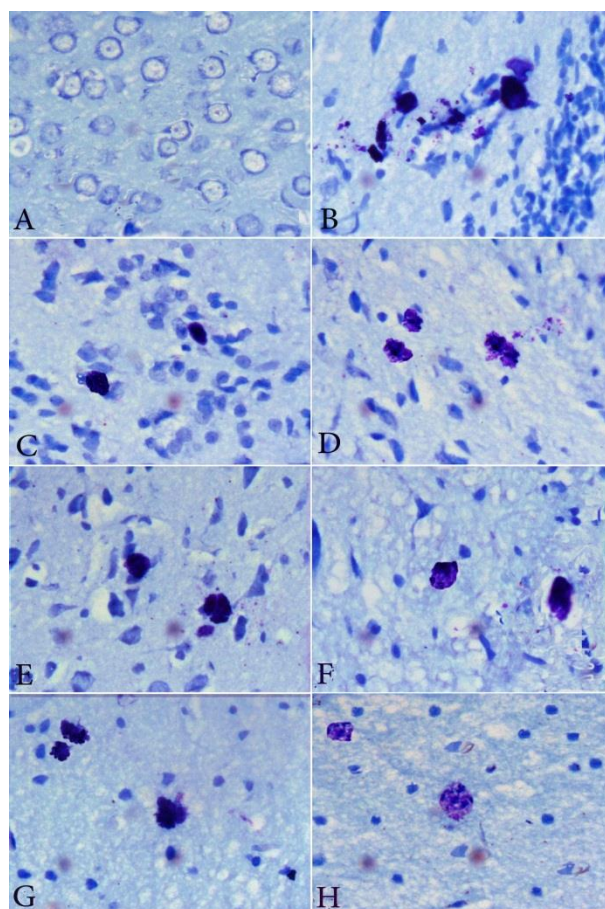


FIGURE 9. Representative photomicrographs of the rat brain sections stained with toluidine blue.

In (A), vehicle-control group showed no detection of positive mast cells. In (B), rotenone-exposed group showed strong metachromatically positive mast cells. In (C), rotenone plus loratadine (1 mg/kg body weight) group showed mildly positive degranulated mast cells. In (D), rotenone plus loratadine (2 mg/kg body weight) group showed mildly positive partially granulated mast cells. In (E), rotenone plus cromoglycate (10 mg/kg body weight) group showed positively stained degranulated mast cells. In (F), rotenone plus cromoglycate (20 mg/kg body weight) group showed positively stained partially degranulated mast cells. In (G), rotenone plus ketotifen (1 mg/kg body weight) group showed positively stained partially degranulated mast cells. In (H), rotenone plus ketotifen (2 mg/kg body weight) group showed positively stained granulated mast cells. All images are 1000 \times and toluidine blue staining.

of the drug across the blood–brain barrier. This indicates that peripheral administration of cromoglycate could be employed in brain pathologies that might be amenable to mast cell stabilization.

Ketotifen is used as an oral prophylactic agent for the treatment of asthma [46]. The drug acts as a mast cell stabilizer and possesses an antihistaminic action as well, thereby antagonizing the H_1 histamine receptors. It also showed marked inhibitory effect on the production of ROS from eotaxin-primed human eosinophils [47]. Similar to loratadine, ketotifen is a piperidine derivative and belongs to the first-generation of antihistamine receptor antagonists [36]. It is likely that this dual action of ketotifen underlies its ability to prevent neurotoxicity caused by rotenone. It is also possible that different kinetic properties accounting for better absorption and penetration of ketotifen into the brain have accounted for this difference. H_1 antihistamines like ketotifen which

readily cross the blood–brain barrier are however associated with impaired behavioral performance, frontal cortex activation [48], significant sleepiness, and declined psychomotor performance over the next day following their administration [49]. Ketotifen's undesirable side effect of sedation thus makes cromoglycate a better choice if mast cell stabilization is to be employed in neurodegenerative diseases.

In this study, exposure to rotenone was shown to result in significant inhibition of AChE activity in the cerebral cortex with respect to the control group. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine, terminating its action at the synapse [50]. The decreased AChE activity after rotenone exposure may result from the direct action of the pesticide on the enzyme or loss of cholinergic neurons. The significance of this action of rotenone and the resultant dysregulation in cholinergic neurotransmission are yet to be determined. The AChE activity in the cortex showed significant increase (though not restored to vehicle-control value) after ketotifen or loratadine treatment. This was especially evident with the lower dose of each drug. In contrast, the AChE activity showed further inhibition after co-treatment with cromoglycate. The brain histaminergic system is important in cognition and arousal [13]. The first generation histamine H_1 receptor antago-

nists are well known for their propensity to cause sedation, drowsiness, and slowed reaction time [36, 49]. Histamine and H₁ antagonists can influence brain cholinergic and dopaminergic neurotransmission [51]. Cromoglycate can also induce effects on brain neurotransmitters apart from mast cell stabilization. The drug has been shown to increase dopamine turnover in the striatum and frontal cortex and to decrease noradrenaline turnover in the hippocampus and striatum of mice [52]. The data in the present study might reveal an additional action for H₁ antagonists and cromoglycate on cholinergic neurotransmission via modulation of AChE activity.

In summary, the present study provides evidence for a neuroprotective effect of histamine H₁ antagonism by loratadine and mast cell stabilization with cromoglycate or ketotifen on the development of oxidative stress and neuronal death caused by rotenone. Since these agents possess anti-inflammatory action as well [37], it is possible that these drugs might be able to modulate the neurodegeneration in PD.

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