



Non-Steroidal Anti-Inflammatory Drugs and Vitamin C in the Rotenone Induced Nigrostriatal Damage in Mice

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Abstract: The nigrostriatal pathway is a dopaminergic pathway that connects the substantia nigra with the dorsal striatum. Loss of dopamine neurons in the substantia nigra is one of the main pathological features of Parkinson's disease, leading to a marked reduction in dopamine function in this pathway. This study aimed at evaluating the protective role of two anti-inflammatory drugs, indomethacin and nimesulide separately or in combination with vitamin C against biochemical disturbances, brain damage and motor impairment in rotenone-induced mice model of Parkinson's disease. Animals were divided into 7 groups. 1st received the vehicle (DEMSO); 2nd received rotenone (1.5 mg/kg); 3rd received rotenone then were left for two weeks recovery; 4th rotenone + indomethacin (10 mg/kg); 5th received rotenone + indomethacin in combination with vitamin C (25 mg/kg). 6th received rotenone + nimesulide (10 mg/kg); group 7 received rotenone + nimesulide in combination with vitamin C. All treatments were given subcutaneously three times per week for one month. Rotenone treatment caused significant Increases in brain malondialdehyde (MDA), nitric oxide (NO), but induced significant decreases in brain reduced glutathione (GSH) level, acetylcholinesterase (AChE) activity, dopamine (DA), norepinephrine (NE) and serotonin (5-HT) levels. These changes lasted for two weeks after the termination of rotenone treatment. Histologically, Rotenone caused degeneration of neurons in striatum, cellular infiltration, atrophy, pyknosis, necrosis, as well as focal gliosis in cerebral cortex and pyknosis of pyramidal cells in the hippocampus. Furthermore, rotenone treatment caused a significant impairment in the motor function of the mice (stair test). Co-administration of indomethacin or nimesulide separately or in combination with vitamin C to rotenone treated mice resulted in alleviation of biochemical and motor activity but not the histological disturbances caused by rotenone treatment alone.

Keywords: Parkinson's Disease, Substantia Nigra, Striatum, Nigrostriatal, Rotenone, Indomethacin, Nimesulide

1. Introduction

Parkinson's disease (PD) is a late-onset, progressive motor syndrome, marked by selective degeneration of the dopaminergic neurons of the substantia nigra (SN) in the midbrain [1, 2]. After losing about 50% of the dopaminergic neurons and approximately 75 to 80% of striatal dopamine (DA), progressive bradykinesia, resting tremor and rigidity start to appear [3, 4]. Degeneration of dopamine-containing cells in the SN and depigmentation of this brain area are

considered as the main pathological features of PD [5]. Although the loss of dopaminergic neurons of the SNpc is regarded as the best pathological feature of PD, many other types of neurons are degenerated such as neurons in the serotonergic raphe nuclei, the noradrenergic locus coeruleus, and regions of the cerebral cortex and the peripheral nervous system [6], and a marked loss of both noradrenergic and serotonergic neurons occurs [7], so neurotransmitters like norepinephrine and serotonin are affected by the DA deficiency in PD [8]. The exact cause leading to the selective

loss of dopamine secreting cells of the SN is not yet known but an interaction between environmental and genetic factors is suggested to be involved in the etiology of PD [9], and researchers have reported the correlation between the exposure to insecticides and herbicides such as rotenone and paraquat and the increasing risk of PD [10, 11]. Among the molecular mechanisms involved in the pathogenesis of PD, oxidative stress, mitochondrial dysfunction, and neuroinflammation are the most strongly implicated [12, 13, 14]. Mitochondria are regarded as the main source of endogenous reactive oxygen species [15, 16]. These highly reactive oxygen intermediates lead to apoptosis and cell death by interacting with proteins, DNA and RNA [17, 18]. The free radical NO^o is a biological molecule that plays a key role in many physiological conditions [19]. Increased production of NO^o, however, can be associated with oxidative and/or nitrative stress and subsequent neuronal damage. This has been attributed to its reaction with superoxide radical and the production of the highly reactive peroxynitrite radical [20]. The excess free radicals result in lipid peroxidation (LP) and oxidative stress in the SN and finally lead to neurodegeneration [21]. Increased lipid peroxidation, and DNA oxidation have been reported in the brain tissue from PD patients post-mortem [22]. A major antioxidant in the brain, that is glutathione (GSH) is very beneficial for the cells as it plays an important role in maintaining their redox status [23]. Glutathione was reported to be decreased in brains of PD patients [24]. The reduction of the GSH content in the brain is a marker of oxidative damage [25].

There is growing evidence that points to the significant role of neuroinflammation in the pathogenesis of PD [26]. The inflammatory process in the SN is mainly characterized by the presence of activated microglial cells and the secretion of proinflammatory and neurotoxic factors [27]. Cytokines are involved in the pathogenesis of PD [28] as they induce the production of cyclooxygenase enzymes (COXs) which generate important mediators during the inflammatory reaction [29]. The cyclooxygenase enzyme catalyzes the first two steps of the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid [29]. It converts arachidonic acid to prostaglandin H₂ (PGH₂), the precursor of prostaglandin E₂ (PGE₂) and other prostanoids [30]. It was later revealed that cyclooxygenase enzyme had two distinct isoforms, identified as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) with distinct cellular functions [31].

Non-steroidal anti-inflammatory drugs (NSAIDs) are compounds which nevertheless share common mechanism of action [32]. They have the ability to inhibit the PGs synthesis [33]. NSAIDs are one of the most commonly prescribed drugs in the world that are known to treat pain and inflammation [34]. Several studies suggest that anti-inflammatory drugs generally have a protective effect on PD in humans, possibly by reducing neuroinflammation [35, 36]. Other researchers, however, failed to demonstrate a benefit from NSAIDs in relation to the development of PD [37, 38,

39]. Indomethacin is a non-selective NSAID which is widely used in the treatment of various rheumatic conditions [40]. It was demonstrated that indomethacin protected dopaminergic neurons in the SN, protected against MPTP induced neurotoxicity and decreased microglial activation in the MPTP mouse model of PD but the drug appeared to be toxic at high doses [41]. COX-2 inhibitors (COXIBs) are regarded as the most widely used medications nowadays because these lack the adverse effects of the classic non-selective drugs on the stomach and kidney [42]. Nimesulide is an analgesic, antipyretic and anti-inflammatory drug and it is relatively selective COX-2 inhibitor [43]. These effects of nimesulide are based on its inhibition of PGs synthesis [44]. According to many experimental studies, nimesulide has neuroprotective effect and produces a long lasting neuroprotection [45, 46].

The present study investigates two NSAIDs, namely indomethacin and nimesulide on their ability to modulate brain oxidative stress, brain damage and motor impairment induced in the mouse by systemic rotenone injection. Rotenone is a pesticide that has been widely used in rodents to model human PD. It is a complex I inhibitor [47], and was shown to cause nigrostriatal cell death and pathological and behavioral changes that resemble those found in the brain of patients with PD [48].

2. Materials and Methods

2.1. Animals

Swiss male albino mice, weighing 25-26 g were obtained from the breeding colony maintained at the animal house of National Research Center, Cairo, Egypt. Animal procedures were performed in accordance with the ethics committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Drugs and Chemicals

Rotenone was obtained from Sigma-Aldrich (St Louis, MO, USA). Indomethacin (Kahira Pharm. and Chem. Ind. Co., Cairo, Egypt), nimesulide (Hikma Pharma, S. A. E., 6th of October city, Egypt) and vitamin C ampoules (Pharm. and Chem. Ind. Co., Cairo, Egypt) were used. Rotenone was freshly prepared in 100% dimethyl sulfoxide. Indomethacin, nimesulide and vitamin C were dissolved in distilled water to obtain the necessary doses. All the used chemicals and reagents in the present study were of analytical grade and obtained from Sigma-Aldrich.

2.3. Experimental Design

The mice were randomly divided into seven groups (6 animals each), Group 1 received the vehicle (DEMSO) three times a week; group 2 received a subcutaneous injection of rotenone (1.5 mg/kg) three times per week for one month; group 3 received rotenone for one month then left for two weeks for recovery; group 4 received subcutaneous injection

of rotenone 1.5 mg/kg + indomethacin 10 mg/kg three times per week; group 5 received the same of group 4 but in combination with vitamin C 25 mg/kg subcutaneously; group 6 received subcutaneous injection of rotenone 1.5 mg/kg + nimesulide 10 mg/kg three times per week; group 7 received the same of group 6 but in combination with vitamin C 25 mg/kg subcutaneously. All animals received the treatments for one month and were then euthanized by decapitation under ether anesthesia; brains were then quickly dissected out on an ice-cold plate into right halves and left halves, weighed, washed with ice-cold phosphate-buffered saline (PBS pH 7.4), and stored at -80°C until biochemical analyses. The right halves were used for the determination of neurotransmitters, namely dopamine, norepinephrine and serotonin. The left halves were used for the determination of glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO). The tissues were homogenized with 0.1 M phosphate buffer saline at pH 7.4, to give a final concentration of 10% w/v for the biochemical assays. Homogenization was performed using a homogenizer (ULTRA-TURAX, IKA T10 basic, Germany) at speed 5000 rpm for 30 seconds. For the determination of monoamine neurotransmitters, frozen samples were homogenized in cold 0.1 N perchloric acid.

2.4. Biochemical Analysis

2.4.1. Determination of Lipid Peroxidation

Lipid peroxidation was assayed by measuring the level of malondialdehyde in brain tissues. Malondialdehyde was determined by measuring thiobarbituric acid-reactive species according to the method devised by Ruiz-Larrea et al (1994) [49], in which thiobarbituric acid-reactive substances react with thiobarbituric acid to produce a red-colored complex having peak absorbance at 532 nm. In brief, 2.25 mL of working reagent (one volume of 0.8 g thiobarbituric acid dissolved in 100 mL of 10% perchloric acid and three volumes of 20% trichloroacetic acid) were added to 0.25 mL of sample, incubated for 20 minutes in a boiling water bath, and then left to cool at room temperature before centrifugation at 3,000 rpm for 5 minutes at 0°C . The pink color was measured using a ultraviolet (UV)-VI8 recording spectrophotometer (Shimadzu Corporation, Rydalmere, Australia) at a 532 nm wavelength against the blank solution, which was prepared by addition of 0.25 mL of distilled water to 2.25 mL of working reagent.

2.4.2. Determination of Reduced Glutathione

Reduced glutathione was determined in the supernatants by Ellman's method (1959) [50]. This procedure is based on the reduction of Ellman's reagent by $-\text{SH}$ groups of glutathione to form 2-nitro-s-mercaptobenzoic acid; the nitromercaptobenzoic acid anion has an intense yellow color which can be determined spectrophotometrically using a UV-VI8 recording spectrophotometer. The reduced glutathione concentration was calculated by comparison with a standard curve.

2.4.3. Determination of Nitric Oxide

Nitric oxide measured as the nitrite was determined using Griess reagent, according to the method devised by Moshage et al (1995) [51], which is based on measurement of endogenous nitrite concentration as an indicator of nitric oxide production. It depends on the addition of Griess reagent which converts nitrite into a deep purple azo compound, the absorbance of which is read at 540 nm.

2.4.4. Determination of Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity was determined according to the modification in Ellman et al method (1961) [52], as described by Gorun et al (1978) [53]. The principle of the method involves measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color was read immediately at 412 nm. The following reagents were pipetted in a cuvette: 0.14 mL of phosphate buffer 20 mM (pH 7.6), 0.05 mL of 5mM acetylthiocholine iodide, and 0.01 mL of tissue homogenate. After 10 minutes of incubation at 38°C , the reaction was stopped with 1.8 mL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)-phosphate ethanol reagent. The DTNB-phosphate ethanol reagent was prepared by dissolving 12.4 mg of DTNB in 120 mL of 96% ethanol, 80 mL of distilled water, and 50 mL of 0.1 mM phosphate buffer (pH 7.6). Glutathione 2.5 mM was used as the standard.

2.4.5. Determination of Monoamine Levels

Estimation of 5-HT, NE and DA was carried out according to the fluorometric method described by Ciarlone (1978) [54]. The method involves extraction of the monoamines into butanol, return of the amine to an aqueous phase and conversion to a fluorescent derivative by oxidation [55].

2.5. Motor Activity

Stair test was applied to assess skilled reaching, mice were placed at the bottom of a stair (30 cm in length) placed at an angle of 55° above the bench, and the latency to climb the stair is recorded for each mouse [56].

2.6. Histopathological Studies

The brain tissues (right half) were immediately fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene and embedded in paraffin. Sections (4 μm) were prepared and then stained with hematoxylin and eosin (H&E) dye for photomicroscopic observations. Sections were examined using a light microscope.

2.7. Statistical Analysis

The data are expressed as the mean \pm standard error of the mean. The data were analyzed by one-way analysis of variance followed by Tukey's test, using SPSS software (SPSS Inc., Chicago, IL, USA). A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Malondialdehyde

The administration of rotenone (1.5 mg/kg) significantly ($P < 0.05$) increased MDA level by 82.2% compared to vehicle-treated control group. After 2 weeks recovery period from rotenone treatment, MDA level was not significantly decreased ($P > 0.05$) compared to rotenone-treated mice. Indomethacin administration at a dose of 10 mg/kg had no effect on MDA level relative to rotenone-treated mice ($P > 0.05$), while was still significant ($P < 0.05$) from vehicle-treated control group. On the other hand, treatment with indomethacin (10 mg/kg) combined with vitamin C (25 mg/kg) significantly ($P < 0.05$) decreased MDA level by 20.5% relatively to rotenone-treated group. Nimesulide administration at a dose of 10 mg/kg caused marked ($P < 0.05$) decrease of MDA level by 26.2% in relation to rotenone-treated group. After treatment with nimesulide (10 mg/kg) combined with vitamin C (25 mg/kg) MDA level did not significantly ($P > 0.05$) change compared to rotenone-treated group, while was still significant ($P < 0.05$) from vehicle group (Figure 1).

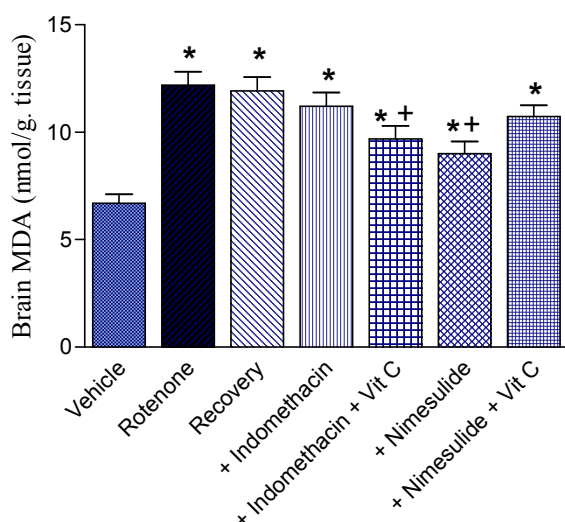


Figure 1. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on malondialdehyde (MDA) in mice brain after rotenone treatment. * $P < 0.05$ compared with the vehicle group. + $P < 0.05$ compared with rotenone control group.

3.2. Reduced Glutathione

The administration of rotenone (1.5 mg/kg) induced a significant ($P < 0.05$) reduction to GSH level by 35.2% compared to vehicle-treated control group. After 2 weeks recovery period from rotenone treatment GSH level was not restored ($P > 0.05$) compared to rotenone-treated mice. GSH level was markedly ($P < 0.05$) elevated by 57.5% in comparison to rotenone-treated mice after Indomethacin administration at a dose of (10 mg/kg). Treatment with indomethacin (10 mg/kg) combined with vitamin C (25 mg/kg) markedly ($P < 0.05$) increased GSH level by 30.1%

relatively to rotenone-treated group. Nimesulide administration at a dose of (10 mg/kg) caused significant ($P < 0.05$) elevation to GSH level by 55.43% in relation to rotenone-treated group. On the other hand, after treatment with nimesulide (10 mg/kg) combined with vitamin C (25 mg/kg) GSH level markedly ($P < 0.05$) elevated by 65.5% (Figure 2).

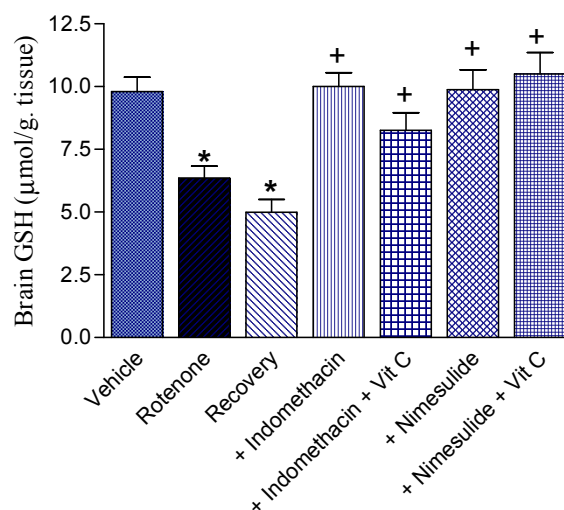


Figure 2. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on reduced glutathione (GSH) in mice brain after rotenone treatment. * $P < 0.05$ compared with the vehicle group. + $P < 0.05$ compared with rotenone control group.

3.3. Nitric Oxide

The administration of rotenone (1.5 mg/kg) significantly ($P < 0.05$) increased nitric oxide level by 60.91% compared to vehicle-treated control group. After 2 weeks recovery period from rotenone treatment nitric oxide level was significantly ($P < 0.05$) decreased by 20.4% compared to rotenone-treated mice. Nitric oxide level was markedly ($P < 0.05$) decreased by 24.1% in relation to rotenone-treated group after indomethacin administration at a dose of (10 mg/kg). However, nitric oxide level was not significantly ($P > 0.05$) affected compared to rotenone induced changes after treatment with indomethacin (10 mg/kg) combined with vitamin C (25 mg/kg), but was still significant ($P < 0.05$) from vehicle-treated control value. After nimesulide administration at a dose of (10 mg/kg) nitric oxide level was not markedly ($P > 0.05$) affected compared to rotenone induced changes, while was still significant ($P < 0.05$) from vehicle-treated control group. After treatment with nimesulide (10 mg/kg) combined with vitamin C (25 mg/kg) nitric oxide level significantly ($P < 0.05$) decreased by 36.8% relatively to rotenone-treated group (Figure 3).

3.4. Acetylcholinesterase Activity

In rotenone-treated mice (1.5 mg/kg) a significant ($P < 0.05$) reduction in AChE level was observed by 26.2% relatively to vehicle-treated control group. AChE level was not restored ($P > 0.05$) after 2 weeks recovery period from

rotenone treatment compared to rotenone-treated mice. AChE level was highly ($P<0.05$) increased compared to rotenone-treated mice by 130.1% after the administration of indomethacin alone (10 mg/kg) and by 80.8% after the administration of indomethacin (10 mg/kg) combined with vitamin C (25 mg/kg). AChE level after administration of indomethacin (10 mg/kg) to rotenone-treated mice was significant ($P<0.05$) from AChE level after administration of indomethacin (10 mg/kg) + vitamin C (25 mg/kg). AChE level was markedly ($P<0.05$) increased by 63.1% after treatment with nimesulide alone at (10 mg/kg) and by 42.1% after treatment with nimesulide (10 mg/kg) combined with vitamin C (25 mg/kg) in relation to rotenone-treated group (Figure 4).

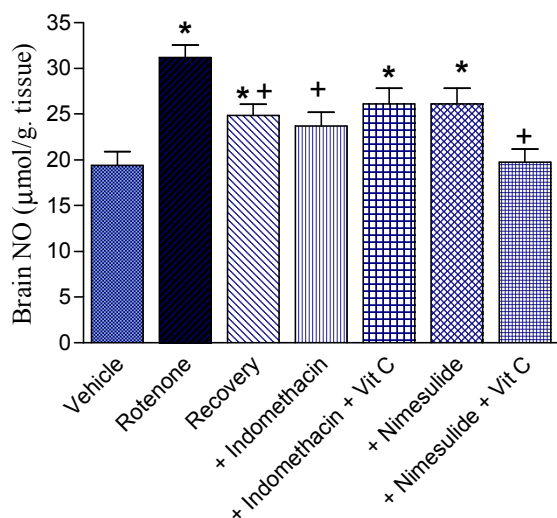


Figure 3. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on nitrite in mice brain after rotenone treatment. * $P<0.05$ compared with the vehicle group. + $P<0.05$ compared with rotenone control group.

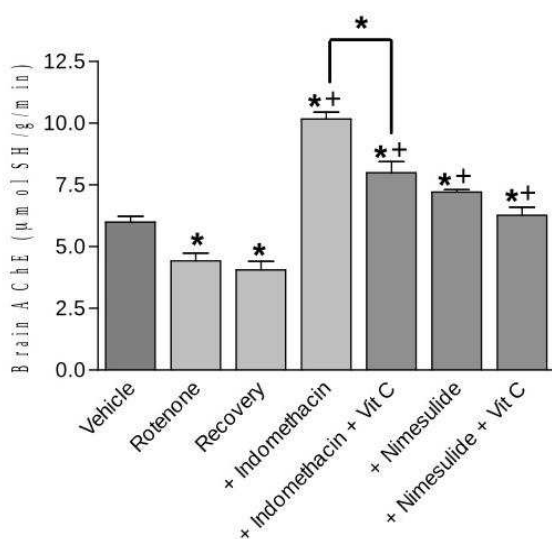


Figure 4. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on acetylcholinesterase activity in mice brain after rotenone treatment. * $P<0.05$ compared with the vehicle group. + $P<0.05$ compared with rotenone control group.

3.5. Dopamine

Rotenone administration induced significant ($P<0.05$) reduction of DA content in the mice brain by 14.8% in comparison to vehicle-treated control group. After 2 weeks recovery period from rotenone treatment DA content was restored ($P<0.05$) by 47.8% in comparison to rotenone-treated mice. Treatment with indomethacin at (10 mg/kg) markedly ($P<0.05$) elevated DA content by 88.5% compared to rotenone-treated group. On the other hand, administration of indomethacin (10 mg/kg) combined with vitamin C (25 mg/kg) caused significant ($P<0.05$) elevation to DA content by 68.3% relatively to rotenone-treated mice. Nimesulide administration at (10 mg/kg) significantly ($P<0.05$) elevated DA content by 60% compared to rotenone-treated mice, and administration of nimesulide (10 mg/kg) combined with vitamin C (25 mg/kg) markedly ($P<0.05$) increased DA content by 77.5% in relation to rotenone-treated group (Figure 5).

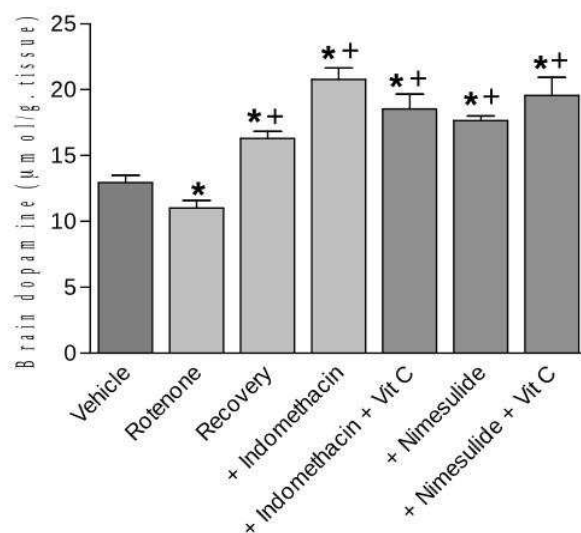


Figure 5. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on dopamine in mice brain after rotenone treatment. * $P<0.05$ compared with the vehicle group. + $P<0.05$ compared with rotenone control group.

3.6. Norepinephrine

After rotenone administration NE content was significantly ($P<0.05$) reduced by 15.3% in comparison to vehicle-treated control group. After 2 weeks recovery period from rotenone treatment NE content was significantly restored ($P<0.05$) by 45.1% in comparison to rotenone-treated mice. Treatment with indomethacin markedly ($P<0.05$) elevated NE level by 26.2% compared to rotenone-treated group. However, NE level did not alter ($P>0.05$) compared to rotenone-treated group after administration of indomethacin combined with vitamin C, while was still significant ($P<0.05$) from vehicle group. NE content was not markedly affected ($P>0.05$) by nimesulide administration compared to rotenone-treated group, while was still significant ($P<0.05$) from vehicle-treated control group. NE content also did not alter ($P>0.05$)

after administration of nimesulide combined with vitamin C compared to rotenone induced changes (Figure 6).

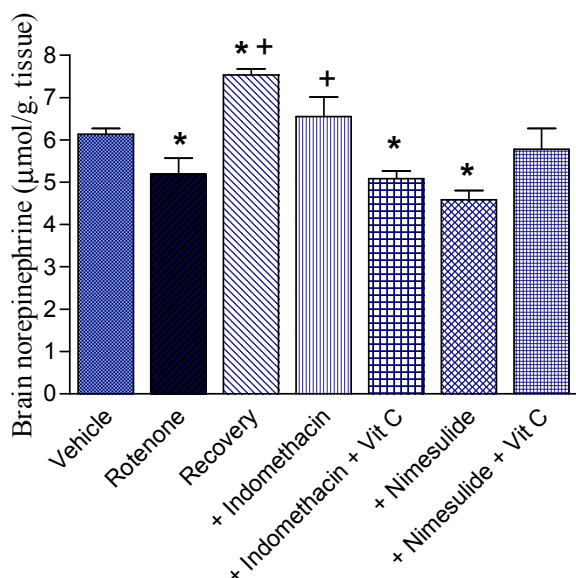


Figure 6. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on norepinephrine in mice brain after rotenone treatment. * $P < 0.05$ compared with the vehicle group. + $P < 0.05$ compared with rotenone control group.

3.7. Serotonin

Rotenone administration induced significant ($P < 0.05$) reduction of 5-HT content by 49.1% in comparison to vehicle- treated control group. 5-HT content was normalized ($P < 0.05$) by 91.4%, compared to rotenone-treated mice. Treatment with indomethacin markedly ($P < 0.05$) elevated 5-HT by 49.1% compared to rotenone-treated group. On the other hand, administration of indomethacin with vitamin C caused significant ($P < 0.05$) elevation to 5-HT level by 42.2% relatively to rotenone- treated mice. 5-HT level was significantly ($P < 0.05$) elevated by 29.3% compared to rotenone-treated mice after nimesulide administration. Administration of nimesulide combined with vitamin C markedly ($P < 0.05$) increased 5-HT content by 45.7% in relation to rotenone-treated group (Figure 7).

3.8. Motor Activity

In order to assess skilled reaching, mice were placed at bottom of a stair (30 cm in length) placed at an angle of 55° above the bench and the latency of climbing the stair was recorded for three trials for each mouse. The time spent by mice to ascend a stair inclined to a 55° angle was markedly ($P < 0.05$) increased by 123.6% after rotenone administration (1.5 mg/kg) compared to vehicle-treated control value. The administration of indomethacin alone (10 mg/kg) or indomethacin (10 mg/kg) combined with vitamin C (25 mg/kg) to rotenone-treated mice resulted in significant ($P < 0.05$) decrease in the time to ascend by 45% and 39.3% respectively, compared to rotenone only-treated group. Meanwhile, nimesulide alone (10 mg/kg) or nimesulide (10

mg/kg) combined with vitamin C (25 mg/kg) treated mice exhibited significant ($P < 0.05$) decreases in the time to ascend by 16.4% and 17.1% respectively, in comparison to rotenone only-treated group (Figure 8).

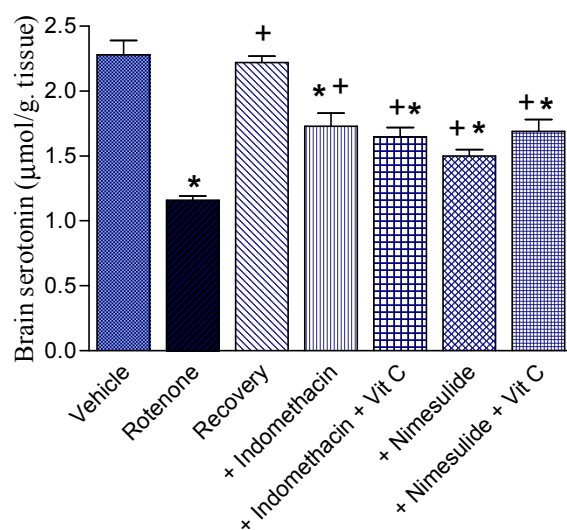


Figure 7. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on serotonin in mice brain after rotenone treatment. * $P < 0.05$ compared with the vehicle group. + $P < 0.05$ compared with rotenone control group.

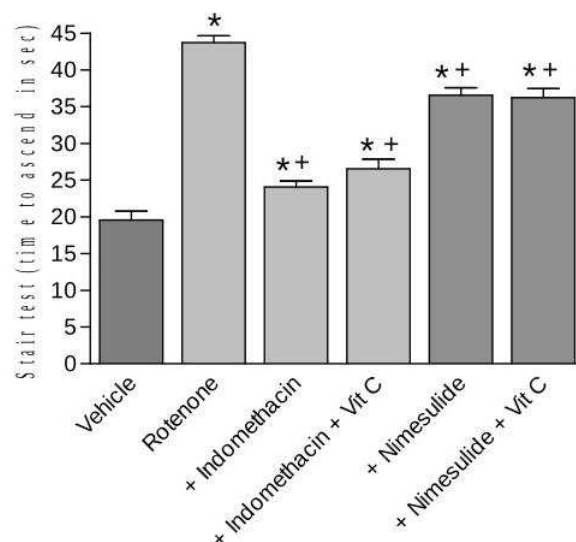


Figure 8. Effect of treatment with indomethacin or nimesulide alone or combined with vitamin C on the time to ascend (sec) in the stair test after rotenone treatment. Columns represent the mean of three consecutive measurements. * $P < 0.05$ compared with the vehicle group. + $P < 0.05$ compared with rotenone control group.

3.9. Histopathological Results

3.9.1. Cortex

The histological study of the control brains showed normal cellularity and round nuclei of the cerebral cortex (Figure 9A). Histopathological changes of the cerebral cortex of rotenone treated mice included cellular infiltration, atrophy, pyknosis, necrosis, congestion of cerebral blood vessels, as

well as focal gliosis. Perinuclear cytoplasmic vacuoles were observed (Figure 9 B). The most prominent observations of the examined sections of mice treated with rotenone and indomethacin were atrophy, pyknosis, shrunken cells and hemorrhage as well as fragmentation and condensation of the nuclei compared to control. Perinuclear cytoplasmic vacuoles and focal gliosis were also noted (Figure 9 C). However, in the group treated with rotenone, indomethacin and vitamin C, there were milder alterations such as perinuclear cytoplasmic vacuoles in neurons and pyknotic nuclei (Figure 9 D). Mice treated with nimesulide exhibited degenerated neurons in cortex which appeared shrunken with pyknotic nuclei and vacuolation. Hemorrhage were also observed (Figure 9 E). Sections from mice treated with rotenone + nimesulide + vitamin C displayed preservation of most of the neurons with an almost normal appearance. Few cells only were seen affected (Figure 9 F).

3.9.2. Striatum

The histological study of the control brains showed normal neurons with normal nuclei and prominent nucleoli (Figure 10 A). In group treated with rotenone, degenerated neurons appeared shrunken with dark cytoplasm and pyknotic nuclei. Pericellular vacuolation of the neurons and presence of eosinophilic lesions and hemorrhage were also observed (Figure 10 B). Sections from mice treated with rotenone and indomethacin revealed extensive neuronal damage. Neurons appeared smaller and shrunken compared to the control section. Vacuolations were also observed (Figure 10 C). In contrast, the brain of mice treated with rotenone, indomethacin and vitamin C showed attenuated histopathological changes except pyknosis of some neurons (Figure 10 D). On the other hand, sections from mice administered rotenone and nimesulide still showed marked degeneration indicated by decreased cell size, vacuolations, and hyperchromatic cells with congested capillaries (Figure 10 E). These changes were, however, attenuated after treatment with vitamin C, except few cells that were seen affected (Figure 10 F).

3.9.3. Hippocampus

The hippocampus from the control mice showed pyramidal cells of normal appearance (Figure 11 A). The hippocampus of rotenone-treated mice showed pyknosis of pyramidal cells (Figure 11 B). This tissue damage increased following the administration of both rotenone, indomethacin (Fig.11 C) but attenuated in mice treated with indomethacin and vitamin C (Figure 11 D). Pyknosis of pyramidal cells was also seen in mice treated with rotenone and nimesulide (Figure 11 E). In contrast, in mice treated with vitamin C, along with nimesulide and rotenone, there was decreased neuronal degeneration with only few neuronal cells showing pyknosis (Figure 11 F).

4. Discussion

In the present investigation we aimed at comparing between the effects of the non-selective COX inhibitor

indomethacin and the selective COX-2 inhibitor nimesulide on a rotenone-induced PD animal model, where each drug was given alone or combined with vitamin C. The findings of the present study provide evidence that treatment with indomethacin or nimesulide each administered alone or combined with vitamin C can decrease many biochemical changes evoked by rotenone, a pesticide and complex I inhibitor, in mice. Rotenone is widely used to induce experimental models of PD through different routes and doses of administration [47]. Continuous administration of rotenone in rats causes nigrostriatal dopaminergic loss, formation of cytoplasmic inclusions containing α -synuclein (α -syn) resembling Lewy bodies (LBs) that found in humans with PD and behavioral symptoms of PD including decreased locomotion, flexed posture, and rigidity [57].

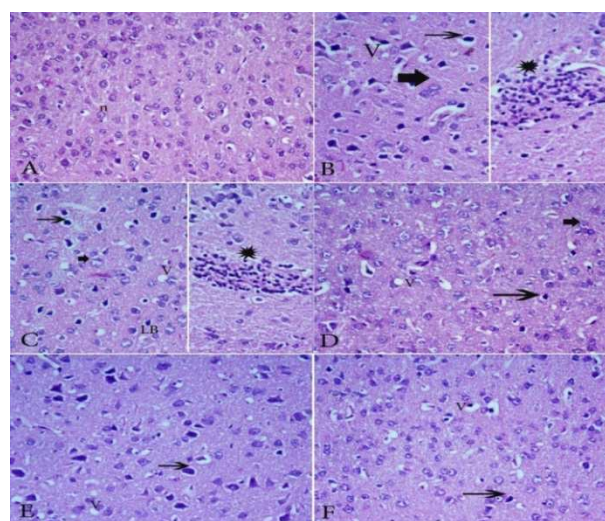


Figure 9. Hematoxylin and eosin-stained sections of mice cerebral cortex after treatment with: (A) Vehicle. (B) Rotenone. (C) Rotenone+ indomethacin. (D) Rotenone+ indomethacin+ vitamin C. (E) Rotenone+ nimesulide. (F) rotenone+ nimesulide + vitamin C.

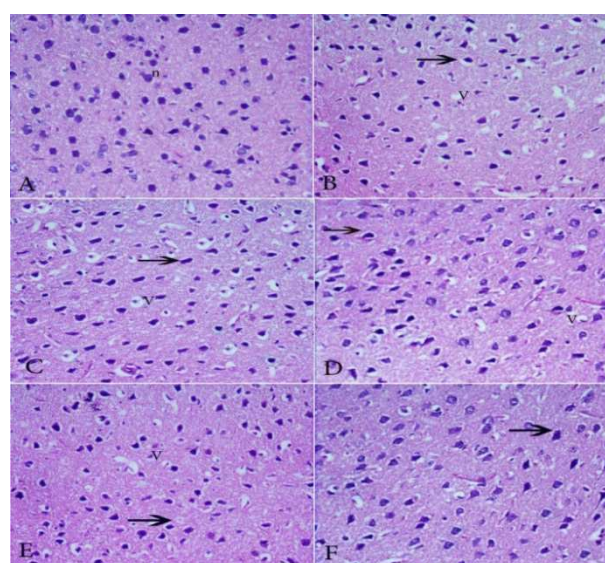


Figure 10. Hematoxylin and eosin-stained sections of mice striatum after treatment with: (A) Vehicle. (B) Rotenone. (C) Rotenone+ indomethacin. (D) Rotenone+ indomethacin+ vitamin C. (E) Rotenone+ nimesulide. (F) rotenone+ nimesulide + vitamin C.

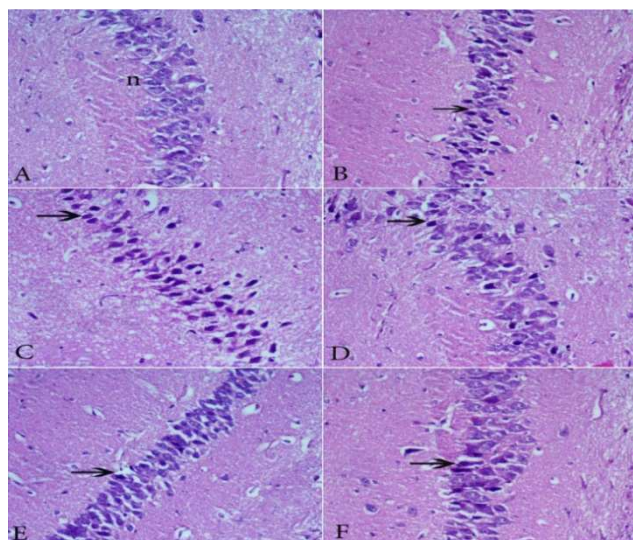


Figure 11. Hematoxylin and eosin-stained sections of mice Hippocampus after treatment with: (A) Vehicle. (B) Rotenone. (C) Rotenone+ indomethacin. (D) Rotenone+ indomethacin+ vitamin C. (E) Rotenone+ nimesulide. (F) rotenone+ nimesulide + vitamin C.

In the present study it was found that subcutaneous administration of rotenone in mice induced several features of PD including reduction of DA concentration, nigrostriatal degeneration, increase in the oxidative stress indices and impairment of motor performance expressed in stair test. Strong evidence supports the role of increased oxidative stress and mitochondrial dysfunction in the pathogenesis of PD [14, 58]. Inhibition of mitochondrial complex I by rotenone treatment may also induce the production of reactive oxygen species [59]. Several studies found that MDA levels are increased in brain of rotenone treated animals compared to vehicle treated animals [60]. Administration of rotenone caused depletion of GSH in rat brains [61]. Microglia are activated by rotenone administration and once activated by rotenone, microglial cells increase the generation of reactive oxygen species [62]. In the present work, subcutaneous injection of rotenone caused increased oxidative stress in the whole brain as shown by significant reduction of GSH in brain and significant increase of malondialdehyde (MDA) which is a marker of lipid peroxidation. MDA production reflects oxidative damage of lipids [63]. MDA indicates increased free radical production attack on membrane lipids [64]. The level of nitric oxide also was elevated in the brain after rotenone treatment. Production of nitric oxide is a marker of oxidative stress [18]. Elevated nitric oxide levels were detected in brains of rotenone-treated mice [60]. Nitric oxide plays a key role in rotenone-induced nigrostriatal injury in rats, as chronic rotenone administration caused significant injury to the nigrostriatal system which mediated by increased generation of nitric oxide [48]. High levels of nitric oxide inhibit respiratory chain complexes by peroxynitrite (ONOO^-) that formed by superoxide anion (O_2^-) and nitric oxide (NO) [65]. Reduced glutathione (GSH) is one of the most important antioxidants in the brain that plays an important role in the prevention of oxidative stress and

protecting cells from oxidative damage [66]. Reduction of the GSH level in the brain is correlated to the increase in oxidative stress induced by rotenone. The excess production of free radicals leads to consumption of GSH, the scavenger molecule. The effects of rotenone on oxidative stress in the brain were reduced by coadministration of indomethacin or nimesulide alone or combined with vitamin C. Thus, indomethacin alone or nimesulide combined with vitamin C resulted in significant reduction of nitric oxide level, while indomethacin combined with vitamin C or nimesulide alone caused significant decrease in MDA level, and the GSH content was markedly elevated after administration of each drug alone or combined with vitamin C, suggesting decreased oxidative and nitrosative stress on administration of the drugs.

NSAIDs in general are considered to be beneficial by inhibiting COX enzyme, scavenging reactive oxygen and nitrite radicals and inhibiting the activation of $\text{TNF}\alpha$ [67]. Different types of studies were conducted for evaluating anti-parkinson activities of Indomethacin. Indomethacin can protect the complex-I enzymes and increase the efficacy of the neuronal mitochondria [68]. It has an ability to scavenge NO radicals [69]. It was shown that nimesulide has therapeutic potential in treatment of PD and it was shown to evaluate neuroprotective effect. Moreover it was reported that chronic administration of nimesulide (5 or 10 mg/kg) attenuated the alterations induced by MPTP in the animal brains of a PD model and inhibited the oxidative stress induced by MPTP [70]. It was suggested that during oxidative stress induced by the toxin, glutathione peroxidase which is responsible for scavenging hydrogen peroxide radicals under normal conditions is not enough to attenuate the oxidative alterations, so the level of oxidized glutathione (GSSG) elevates by continuous administration of the cellular toxin and so the redox state of the DA neurons is altered. Treatment of nimesulide restored the antioxidant enzymes and normalized the redox state [70].

In the present study, subcutaneous administration of rotenone in mice resulted in a significant reduction of brain dopamine content. Our results are also supported by other data obtained using rotenone as an experimental PD model [47, 71, 72]. Because of the ability of rotenone to freely enter all cells, the high susceptibility of the dopaminergic neurons to rotenone suggested that dopaminergic neurons are sensitive to complex I inhibition [47]. DA degeneration mainly entails formation of reactive species, so additional oxidative stress caused by microglial activation, increases the vulnerability of the nigrostriatal dopaminergic neurons to the oxidative damage [73]. DA metabolism produces hydrogen peroxide and superoxide radicals, and auto-oxidation of DA produces quinone products [74]. Oxidative stress causes oxidation of DA to quinone products that cause damage to the mitochondria of the brain [75]. The formation of the potent oxidant peroxynitrite which is produced by the interaction between ROS especially superoxide with NO [76] may directly oxidize DA [75] and lead to damage of dopaminergic neurons [78]. On the other hand,

administration of indomethacin alone or combined with vitamin C resulted in a marked increase in the DA level in the brain. NSAIDs in general have been shown to reduce dopaminergic neuron degeneration in animal models of PD [35]. Results from animal models of PD displayed that indomethacin has the ability to prevent MPTP-induced loss of striatal DA in mice like those of Kurkowska-Jastrzebska et al. (2002) [41] who used MPTP-induced mice as a PD model and they found that treatment with indomethacin at a dose of 1mg/kg protects against MPTP-induced neural damage of dopaminergic neurons and they demonstrated that this effect is associated with diminished microglial activation in the damaged areas. Pathology of PD involves neuroinflammatory processes including increased expression of COX and elevated prostaglandin E2 (PGE2) levels [79]. Generally, the ability of NSAIDs to suppress inflammation is based on their ability to inhibit the COX enzyme [33]. Indomethacin inhibits COX by inhibiting PG synthesis, so it was suggested that the neuroprotective activity of indomethacin depends on PG inhibition [68].

We also found that nimesulide administered alone elevated the DA level in the mice brain. Increased DA level indicated that antioxidant effect of nimesulide might be responsible for inhibiting the oxidation of DA which induced by rotenone treatment. Nimesulide might have prevented the mitochondrial dysfunction and oxidative stress induced by rotenone and thus prevented the inhibition of the cell damaging pathways by inhibiting the inflammatory mediators according to its selective inhibition of COX-2.

In the present investigation, subcutaneous rotenone administration induced a marked reduction of the NE level in the brain. This result is consistent with those of Abdel-Rahman et al. (2008) [80]. We also found that rotenone administration significantly decreased the 5-HT level in the brain, a result that goes in line with those of He et al. (2003) [48] and Verma and Nehru (2009) [60] who also used rotenone treatment. It was revealed that rotenone causes a multisystem degeneration incorporating 20–30% losses in the striatum of serotonergic fibers and about 30% loss of noradrenergic neurons in the locus coeruleus and substantia nigra pars compacta (SNpc) cells [71]. Degenerations of NE neurons of the locus coeruleus and 5-HT neurons of the dorsal raphe are considered as pathological changes in PD [81]. The cognitive and behavioral impairments observed in PD suggest a role of the 5-HT and NE systems [82]. Our results revealed that the levels of NE and 5-HT were also elevated by administration of indomethacin alone while its combination with vitamin C did not improve its action. Treatment with nimesulide caused elevation of 5-HT content in the brain and combination with vitamin C did not improve the action of nimesulide. Nimesulide neither administered alone nor combined with vitamin C has a significant effect on NE level in the mice brain. The rise of NE is directly linked to the elevation of DA levels, as the increased availability of DA as a precursor of NE, enhanced the synthesis of the latter, as displayed in our investigation after treatment with indomethacin in the rotenone-treated mice.

In the current investigation, AChE activity was markedly reduced in the mice brain after administration of rotenone, a result that goes in line with those of Zhang et al. (2013) [83], who used MPTP-induced mice as a model of PD. In vivo studies of AChE have been shown cholinergic projection losses in the magnitude of 5% to 25% in PD subjects [84]. Cholinergic degeneration may play a key role in the cognitive decline in PD [85]. AChE has been regarded as a reliable marker for brain cholinergic pathways in the human brain [86]. The enzyme AChE is responsible for degrading acetylcholine (ACh) [87] which is the major neurotransmitter related to memory [88]. Administration of the drugs, indomethacin and nimesulide alone or combined with vitamin C resulted in a marked elevation in the activity of AChE in the mice brain.

In our histological results it was obvious that indomethacin alone did not decrease the injury of the neurons and nimesulide alone did not markedly attenuate the neuronal injury, but the interesting finding is that vitamin C combination significantly protected the neurons and attenuated the neuronal injury in the striatum, hippocampus and cortex. Vitamin C (ascorbic acid) at low doses is an exogenous powerful antioxidant molecule which acts with endogenous antioxidant systems within tissue cells to scavenge the formed ROS [89]. The presence of vitamin C as an antioxidant in the different brain regions makes the brain tissue susceptible to oxidative attack [90]. It was reported that administration of vitamin C to systemically injected MPTP mice increased the striatal DA level [91].

In the current study, the stair test was used for assessment of the skilled reaching and evaluating the motor performance. We found that rotenone treatment caused a marked increase of the time spent by mice to ascend the stair which inclined to a 55° angle, and we concluded that it reflected the slowness of movement (bradykinesia) as a main feature of PD that induced by rotenone administration. According to Betarbet et al. (2000) [47], rotenone-treated animals exhibited motor and postural deficits characteristic of PD and all animals with a dopaminergic lesion became hypokinetic and had unsteady movement. They concluded that rotenone may act via the inhibition of mitochondrial complex I leading to selective damage of the DA neurons associated with the PD motor and postural deficits. Motor functions impairment is correlated to deficiency of DA [92] as DA deficiency in the striatum is associated clinically with motor symptoms of PD including bradykinesia, tremor, rigidity and postural instability [4]. In the basal ganglia one of the mechanisms leading to motor changes is the balance between cholinergic and dopaminergic systems, as ACh and DA play a key role in the control of motor functions [93]. In the present study, rotenone treatment caused marked decrease in DA and reduction in AChE contents in the brain (increase ACh), which resulted in imbalance between DA and ACh contents in the brain leading to impaired motor functions. The imbalance between DA and ACh is a consequence of excessive ACh concentrations as well as DA deficiency [94]. In the present investigation, the motor function of mice was

markedly improved by administration of indomethacin or nimesulide, alone or combined with vitamin C. The non-selective COX inhibitor and the selective COX-2 inhibitor significantly restored the motor activity. Thus according to our results we concluded that the antioxidant effect of both indomethacin and nimesulide might be responsible for inhibition of DA oxidation and prevented the motor impairments caused by rotenone treatment by restoring the normal balance between DA and NE in the brain.

In conclusion, our study revealed that non-selective inhibition of COX-1 and COX-2 by indomethacin and also selective inhibition of COX-2 by nimesulide is effective in decreasing oxidative stress (decrease MDA and NO, and increase GSH). On the other hand, selective COX-2 inhibition was effective in decreasing brain damage in rotenone-treated mice. Moreover, Vitamin C at low doses showed an additive effect when combined with either indomethacin or nimesulide both biochemically and histologically and so it can be used safely with NSAIDs in PD patients.

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