

RESEARCH PAPER

Role of LOX/COX pathways in 3-nitropropionic acid-induced Huntington's Disease-like symptoms in rats: protective effect of licofelone

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BACKGROUND AND PURPOSE

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by a degeneration of striatal neurons. The possible role of COX and lipoxygenase (LOX) pathways has been well-documented in the pathology of several neurodegenerative disorders including HD. Licofelone is a competitive inhibitor of COX-1- and COX-2 and 5-LOX isoenzymes. Therefore, the present study was designed to investigate possible neuroinflammatory and apoptotic mechanisms in the neuroprotective effect of licofelone against 3-nitropropionic acid (3-NP)-induced HD-like symptoms in rats.

EXPERIMENTAL APPROACH

Rats were administered 3-NP (10 mg·kg⁻¹·day⁻¹, i.p.) for 14 days. Licofelone (2.5, 5 and 10 mg·kg⁻¹, p.o.) was given once a day, 1 h before 3-NP treatment for 14 days. Body weight and behavioural parameters (locomotor and rotarod activity) were assessed on the 1st, 5th, 10th and 15th day post-3-NP administration. Malondialdehyde, nitrite concentration, endogenous antioxidant enzymes (superoxide dismutase and catalase levels), mitochondrial enzyme complexes, pro-inflammatory compounds (TNF- α , IL-6, NF- κ B), PGs (PGE₂ and PGF_{2 α}) and caspase-3 activity were measured on day 15 in the striatum.

KEY RESULTS

Systemic 3-NP treatment significantly reduced body weight, locomotor activity, oxidative defence, mitochondrial enzyme complex activities and increased TNF- α , IL-6, caspase-3 activity, NF- κ B and PGE₂ and PGF_{2 α} levels in the striatum. Licofelone (2.5, 5 and 10 mg·kg⁻¹) significantly attenuated the impairment in behavioural, biochemical and mitochondrial, pro-inflammatory and pro-apoptotic markers as compared with vehicle-treated group.

CONCLUSIONS AND IMPLICATIONS

The results demonstrate the involvement of pro-inflammatory compounds and the apoptotic cascade in the neuroprotective effect of licofelone against 3-NP-induced neurotoxicity.

Abbreviations

3-NP, 3-nitropropionic acid; BDNF; brain-derived neurotrophic factor; CMC, sodium carboxymethyl cellulose; CREB, cAMP response element binding protein; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; HD, Huntington's disease; LDH, lactate dehydrogenase; LOX, lipoxygenase; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; NBT, nitrazobluetetrazolium; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SOD, superoxide dismutase

Introduction

Huntington's disease (HD) is a neurodegenerative disorder of complex neuropathology. It is characterized by ataxia, choreiform movements and dementia, and affects medium spiny neurons in the striatum (Gil and Rego, 2008). Recently, oxidative stress has been shown to be involved in the neurobiology of HD, although the cause of oxidative stress is uncertain (Brown *et al.*, 1999). Recent reports show that oxidative stress activates the apoptotic and neuroinflammatory processes that trigger cell death in neurodegenerative diseases (Liot *et al.*, 2009). In addition, results have been obtained that suggest excitotoxic events and mitochondrial dysfunction are involved in the pathophysiology of HD (Keller *et al.*, 1998; Kumar and Kumar, 2009a). Apoptosis is a complex process involving activation of proteases of the caspase family, alterations in plasma membrane phospholipids, nuclear DNA condensation and fragmentation (Keller *et al.*, 1998; Yang *et al.*, 2004). Apoptosis has also been implicated in neurodegenerative diseases including HD and associated with mitochondrial dysfunction (Portera-Cailliau *et al.*, 1995; Keller *et al.*, 1998). Neuroinflammation is a key event involved in neuronal death and axonal degeneration (Mrak and Griffin, 2005). Prostaglandins are important mediators of the inflammatory process and a potent indicator of *in vivo* COX-mediated inflammatory processes (Mrak and Griffin, 2005). Clinical evidence also suggests that excess TNF- α is centrally involved in the pathogenesis of neurological diseases (Tobinick and Gross, 2008). A greater understanding of the apoptotic and neuroinflammatory cascades that mediate these effects, as well as their molecular interaction, can be used to elucidate the pathology of HD and aid the development of new drugs suitable to treat this and other neurodegenerative diseases. 3-Nitropropionic acid (3-NP) is a fungal toxin that produce HD-like symptoms both in animals and humans (Ludolph *et al.*, 1991; Kumar and Kumar, 2009b). 3-NP irreversibly blocks the enzyme succinate dehydrogenase (SDH), decreases ATP levels and accelerates neuronal apoptosis (Coles *et al.*, 1979; Tunez *et al.*, 2006). 3-NP induces oxidative stress, neuroinflammation and excitotoxicity, all of which are associated with the pathogenesis of HD.

Licofelone ([2,2-dimethyl-6-(4-chlorophenyl-7-phenyl-2,3-dihydro-1H-pyrazolone-5-yl) acetic acid), a competitive inhibitor of 5-lipoxygenase (5-LOX), COX-1 and COX-2, is currently in the clinical developmental phase for the treatment of osteoarthritis. Licofelone decreases significantly the production of pro-inflammatory mediators (leukotrienes and prostaglandins), which are involved in the pathophysiology of several neurological disorders (Singh *et al.*, 2006). Both COX and 5-LOX inhibitors, alone or in combination, have been reported to have neuroprotective effects against several motor disorders (Bishnoi *et al.*, 2005; 2006; 2007; Kalonia *et al.* 2009a,b,c). However, their exact cellular mechanism is still not understood.

Therefore, the present study was designed to investigate the possible neuroinflammatory and apoptotic mechanisms in the neuroprotective effect of licofelone against 3-NP-induced HD-like symptoms in rats.

Methods

Animals

Male Wistar rats (300–350 g) bred in the Central Animal House of Panjab University, Chandigarh, were used. Animals were acclimatized to laboratory conditions prior to experimentation. The animals were kept in plastic cages (two to three in each cage) under hygienic conditions with a 12 h light and dark cycle with food and water *ad libitum*. All experiments were carried out between 09 h 00 min and 15h00 min. The protocol was approved by the Institution's Animal Ethics Committee and was carried out in accordance with the Indian National Science Academy Guidelines for use and care of animals.

Materials

3-NP (Sigma Chemicals, St. Louis, MO), licofelone animals (rats) from Central Animal House, Panjab University; sodium carboxymethyl cellulose (CMC) (Hi-Media, New Delhi, India), saline, BSA (Hi-Media, New Delhi); EGTA, ELISA kit; actophotometer (IMCORP, Ambala, India); rotarod apparatus (Techno, Ambala, India); spectrophotometer (Norwalk, CT).

Drugs and treatment schedule

The following drugs were used in the present study. 3-NP was dissolved in saline (adjusted to pH 7.4) and administered *i.p.* in a volume of 0.5 mL 100 g⁻¹ animal body weight. Licofelone was suspended in a 0.5% (w v⁻¹) CMC solution and administered *p.o.*, through a cannula, in a constant volume of 0.5 mL 100 g⁻¹ body weight. Animals were randomly divided into six groups, consisting of 10–15 rats in each.

Group 1, vehicle-treated group ($n = 15$); group 2, vehicle + diet restriction-treated group ($n = 10$); group 3 received 3-NP (10 mg·kg⁻¹, *i.p.*) for 14 days ($n = 15$); group 4 received licofelone (10 mg·kg⁻¹ *p.o.*) alone ($n = 12$); groups 5 to 7 received licofelone (2.5, 5 and 10 mg·kg⁻¹) + 3-NP (10 mg·kg⁻¹, *i.p.*) for 14 days ($n = 15$ in each group).

Diet restriction protocol

All animals received food and water *ad libitum*. One week before the beginning of the experiment, food intake was recorded in order to know the average daily consumption of each rat. In the restricted diet-treated groups, animals received 15 g of commercial diet, which corresponded to 50% of the average amount of diet consumed by the animals in the previous week.

Measurement of body weight

Animal body weight was recorded on the first and last days of experiment; % change in body weight was calculated:

$$[\text{Body weight (1st day} - 15\text{th day)} \times 100] / [1^{\text{st}} \text{ day body weight}]$$

Behavioural assessments

Assessment of gross behavioural activity (locomotor activity). The locomotor activity was monitored using an actophotometer. The motor activity was detected by infrared beams above the floor of the testing area. Animals were placed individually in the activity chamber for a 3 min accli-

matization period before the actual activity tasks were started. Each animal was observed over a period of 5 min, and activity was expressed as counts 5 min^{-1} (Kumar *et al.*, 2007).

Rotarod activity. The motor co-ordination and grip performance of the animals were evaluated using the rotarod apparatus. The rats were exposed to a prior training session to acclimatize them to rotarod performance. Rats were placed on a rotating rod with a diameter of 7 cm (speed, 25 r.p.m.). The cut-off time was 180 s, and each rat performed three separate trials after a 5 min gap. The average time of the fall was recorded (Kumar and Kumar, 2009a).

Dissection and homogenization. On the 15th day, animals were randomly divided into two groups, one for biochemical estimations and the other for mitochondrial enzyme complex estimations immediately after the behavioural assessments. The brains were dissected out. The striatum was separated and placed on ice. A 10% ($w v^{-1}$) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at $10\,000\times g$ for 15 min. Aliquots of the supernatant was separated and used for biochemical estimations.

Measurement of oxidative stress parameters

Measurement of lipid peroxidation. The quantitative measurement of lipid peroxidation in the brain striatum was performed according to the method of Wills (1966). The amount of malondialdehyde (MDA), a measure of lipid peroxidation, was measured by reaction with thiobarbituric acid at 532 nm using a Perkin Elmer lambda 20 spectrophotometer. The values were calculated using the molar extinction coefficient of the chromophore ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed as a percentage of the vehicle-treated group (the protein concentration was $0.876 \text{ mg}\cdot\text{mL}^{-1}$).

Estimation of nitrite. The accumulation of nitrite in the striatum supernatant, an indicator of the production of NO, was determined by a colorimetric assay with Greiss reagent [0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulphanimide and 2.5% phosphoric acid] as described by Green *et al.* (1982). Equal volumes of supernatant and Greiss reagent were mixed, and this mixture was incubated for 10 min at room temperature in the dark. Absorbance at 540 nm was measured with a Perkin Elmer lambda 20 spectrophotometer. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and expressed as a percentage of the vehicle-treated group (the protein concentration was $0.876 \text{ mg}\cdot\text{mL}^{-1}$).

Catalase estimation. Catalase activity was assayed by the method of Luck (1971), in which the breakdown of hydrogen peroxide (H_2O_2) is measured at 240 nm. Briefly, the assay mixture consisted of 12.5 mM H_2O_2 in phosphate buffer (50 mM of pH 7.0) and 0.05 mL of supernatant from the striatum tissue homogenate (10%), and the change in absorbance was recorded at 240 nm. The results are expressed as mM of H_2O_2 decomposed $\text{mg}^{-1} \text{ protein min}^{-1}$ (the protein concentration was $0.876 \text{ mg}\cdot\text{mL}^{-1}$).

Superoxide dismutase (SOD) activity. SOD activity was assayed according to the method of Kono (1978), in which the inhibition of the reduction of nitrazobluetetrazolium (NBT) by SOD is measured at 560 nm using a spectrophotometer. Briefly, the reaction was initiated by the addition of 20 mM of hydroxylamine hydrochloride to the mixture containing 96 mM of NBT and 0.1 mL of the sample (striatum homogenate). The results are expressed as $\text{U mg}^{-1} \text{ protein}$ (the protein concentration was $0.876 \text{ mg}\cdot\text{mL}^{-1}$).

Protein estimation. The protein concentration was measured by the Biuret method using BSA as a standard (Gornall *et al.*, 1949).

Mitochondrial complex estimation

Isolation of rat brain mitochondria. Rat brain mitochondria were isolated by the method of Berman and Hastings (1999). The brain striatum was homogenized in isolation buffer with EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). The homogenate was centrifuged at $13\,000\times g$ for 5 min at 4°C . The pellet was resuspended in isolation buffer with EGTA and spun again at $13\,000\times g$ for 5 min. The resulting supernatant was transferred to new tubes and topped off with isolation buffer containing EGTA and spun again at $13\,000\times g$ for 10 min. The pellet containing pure mitochondria was resuspended in isolation buffer without EGTA (the protein concentration was $0.676 \text{ mg}\cdot\text{mL}^{-1}$).

Complex I (NADH dehydrogenase activity). Complex I was measured spectrophotometrically by the method of King and Howard (1967). The method involves the catalytic oxidation of NADH to NAD^+ with subsequent reduction of cytochrome C. The reaction mixture contained 0.2 M glycyl glycine buffer, pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer and 10.5 mM cytochrome C. The reaction was initiated by the addition of a requisite amount of solubilized mitochondrial sample. The absorbance change at 550 nm was followed for 2 min (the protein concentration was $0.676 \text{ mg}\cdot\text{mL}^{-1}$).

Complex II (SDH activity). SDH was measured spectrophotometrically according to the method of King (1967). The method involves the oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid and 0.03 M potassium ferricyanide. The reaction was initiated by addition of the striatum mitochondrial sample, and the absorbance change at 420 nm was followed for 2 min (the protein concentration was $0.676 \text{ mg}\cdot\text{mL}^{-1}$).

Complex IV (cytochrome oxidase assay). Cytochrome oxidase activity was assayed in brain mitochondria according to the method of Sottocasa *et al.* (1967). The assay mixture contained 0.3 mM reduced cytochrome C in 75 mM phosphate buffer. The reaction was initiated by addition of the solubilized striatum mitochondrial sample, and absorbance change at 550 nm was followed for 2 min (the protein concentration is $0.676 \text{ mg}\cdot\text{mL}^{-1}$).

MTT assay. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-H-tetrazolium bromide (MTT) assay is based on the reduction of MTT by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess reducing activity of the mitochondrial samples in isolated striatum mitochondria by the method of Liu *et al.* (1997). Briefly, 100 μ L striatum mitochondrial samples were incubated with 10 μ L MTT for 3 h at 37°C. The blue formazan crystals were solubilized with dimethyl sulphoxide and measured by an ELISA reader with a 580 nm filter (Model 680 Microplate Reader, Bio-Rad, Japan) (the protein concentration was 0.676 mg·mL⁻¹).

Preparation of lysates from tissue

(i) **Cytoplasmic fraction collection.** Striatum was weighed and cut into small pieces using a clean razor blade followed by wash with 5 mL of cold 1X PBS–PMSF. The striatal pieces were placed in a clean homogenizer, 5 μ L of 1 M DTT and 500 μ L of 10% detergent solution was added to 4.495 mL of ice-cold 1X hypotonic buffer g⁻¹ tissue and then homogenized. The homogenate was incubated with ice for 15 to 30 min (whole-cell lysate), centrifuged for 10 min at 5590 \times g at 4°C, and the supernatant (cytoplasmic fraction; concentration, 1.1 mg·mL⁻¹) was transferred to a 15 mL tube and stored at 4°C. The pellet was the nuclear fraction and concentration was 1 mg·mL⁻¹.

(ii) **Nuclear fraction collection.** The nuclear pellet (concentration 1 mg·mL⁻¹) was resuspended in 500 μ L nuclear lysis buffer by pipetting up and down. The suspension was vigorously vortexed and incubated at 4°C for 30 min on a rocking platform. The suspension was centrifuged at 10956 \times g for 10 min at 4°C in a microcentrifuge. The supernatant (nuclear fraction; concentration is 0.91 mg·mL⁻¹) was transferred into a pre-chilled microcentrifuge tube and stored at –80°C until further use. The protein concentration of the nuclear extract was 1 mg·mL⁻¹.

Estimation of TNF- α and IL-6 in striatum. The quantifications of TNF- α and IL-6 were done by rat TNF- α and IL-6 immunoassay kit (R&D Systems, Minneapolis, MN). The Quantikine rat TNF- α and IL-6 immunoassay is a 4.5 h solid phase ELISA designed to measure rat TNF- α and IL-6 levels. It is a solid-phase sandwich ELISA using a microtitre plate reader. Concentrations of TNF- α were calculated from the standard curves. The protein concentration of the nuclear extract was 1 mg·mL⁻¹.

Caspase-3 colorimetric assay in striatum. Caspase-3, also known as CPP-32, Yama or Apopain, is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis. The enzymatic reaction for caspase activity was carried out as using an Imgenex (San Diego, CA) caspase-3 colorimetric kit. The protein concentration of the nuclear extract was 1 mg·mL⁻¹.

Quantification of NF- κ B p65 subunit in striatum. The NF- κ B/p65 Activ ELISA (Imgenex) kit was used to measure NF- κ B-free p65 in the nuclear lysate of the rat striatum. The nuclear levels of p65 may correlate positively with the activation of NF- κ B pathway. The anti-p65 antibody-coated plate captures

free p65, and the amount of bound p65 was detected by adding a second anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody using colorimetric detection in an ELISA plate reader at 405 nm. The protein concentration of the nuclear extract was 1 mg·mL⁻¹.

Estimation of PGE₂ levels in striatum. The quantitative determination of PGE₂ levels was done by using a PGE₂ assay kit (R&D Systems). Briefly, the assay is based on the competitive binding technique in which PGE₂ present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labelled PGE₂ for sites on a mouse monoclonal antibody. During the incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The colour development is stopped, and the absorbance is read at 450 nm. The intensity of the colour is inversely proportional to the concentration of PGE₂ in the sample. The protein concentration of the nuclear extract was 1 mg·mL⁻¹.

Estimation of PGF_{2 α} levels in striatum. The quantitative determination of PGF_{2 α} levels was done by using the PGF_{2 α} EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA). The assay is carried out as per the procedure given by the manufacturer. The absorbance of the colour development due to the enzymatic reaction is read at 412 nm. The intensity of the colour is inversely proportional to the free amount of PGF_{2 α} present in the sample during incubation. The protein concentration of the nuclear extract was 1 mg·mL⁻¹.

Statistical analysis

The data were analysed by one-way and two-way ANOVA, followed by Tukey's test and two-way ANOVA followed by Fisher's least significant difference test, respectively (Sigmatat 2.0 version, Cranes Software International Ltd., Bangalore, India). All the values are expressed as mean \pm SEM. In all the tests, the criterion for statistical significance was $P < 0.05$.

Results

Effect of licofelone on body weight, locomotor activity and motor coordination in 3-NP-treated rats

Systemic treatment with 3-NP (10 mg·kg⁻¹) significantly reduced body weight (Figure 1) and impaired locomotor (Figure 2) and motor co-ordination (rotarod) (Figure 3) on the 15th day as compared with the vehicle-treated group. The diet restriction followed by vehicle treatment did not significantly affect body weight, locomotor and rotarod activities of the animals as compared with vehicle alone-treated groups. Furthermore, licofelone (2.5, 5 and 10 mg·kg⁻¹, p.o.) treatment attenuated the reduction in body weight, impairment in locomotor and motor co-ordination (rotarod) as compared with 3-NP-treated rats ($P < 0.05$). Licofelone (10 mg·kg⁻¹, p.o.) *per se* did not show any significant effect on the behavioural parameters as compared with vehicle-treated group.

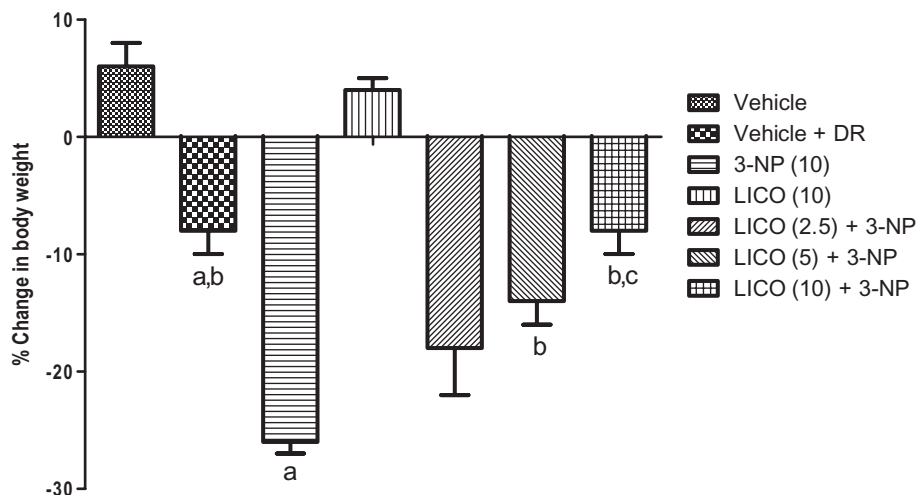


Figure 1

Effect of licofelone (LICO) on body weight in 3-NP-treated rats. Group 1, vehicle ($n = 15$); groups 2, vehicle + DR ($n = 10$); group 3, 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 4, LICO ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 12$); group 5, LICO ($2.5 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 6, LICO ($5 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 7, LICO ($10 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$). ^a $P < 0.05$ versus vehicle-treated group, ^b $P < 0.05$ versus 3-NP, ^c $P < 0.05$ versus [LICO (2.5) + 3-NP]-treated group (one-way ANOVA followed by Tukey's test). Data presented are the percentage of the vehicle-treated group (mean \pm SEM). DR, diet restriction.

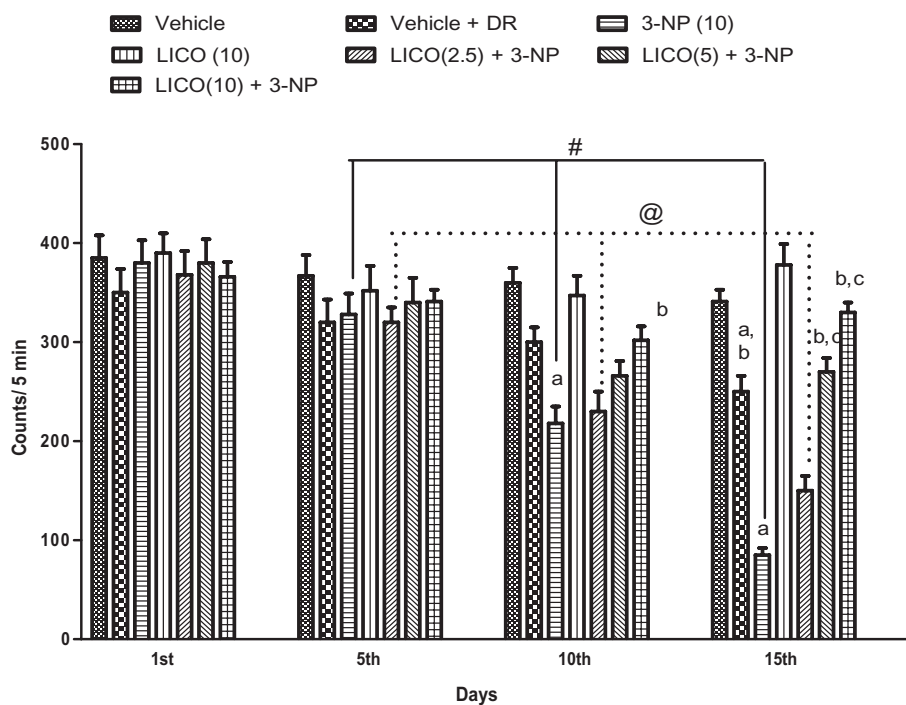


Figure 2

Effect of licofelone (LICO) on locomotor activity in 3-NP-treated rats. Group 1, vehicle ($n = 15$); group 2, vehicle + DR ($n = 10$); group 3, 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 4, LICO ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 12$); group 5, LICO ($2.5 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 6, LICO ($5 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 7, LICO ($10 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$). ^a $P < 0.05$ versus vehicle-treated group, ^b $P < 0.05$ versus 3-NP, [#] $P < 0.05$ versus 3-NP treatment on 5th, 10th and 15th day, [@] $P < 0.05$ versus [LICO(2.5) + 3-NP] treated group on 5th, 10th and 15th days, ^c $P < 0.05$ versus [LICO(2.5) + 3-NP] treated group (two-way ANOVA followed by Fisher's least significant difference test). Data presented are the percentage of the vehicle-treated group (mean \pm SEM). DR, diet restriction.

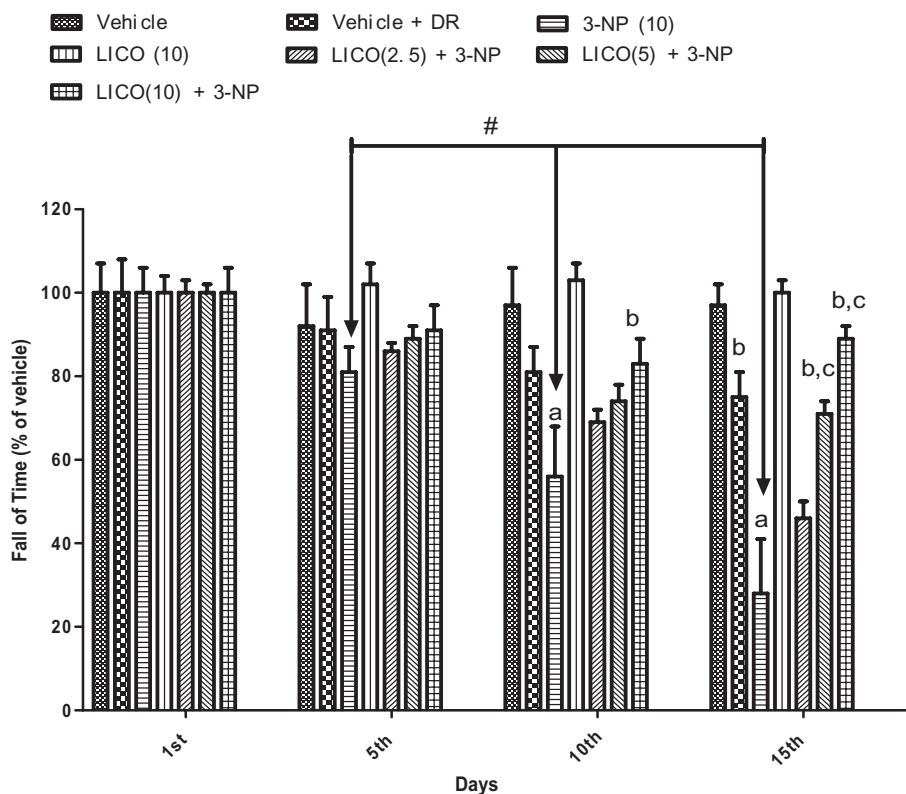


Figure 3

Effect of licofelone (LICO) on rotarod activity in 3-NP-treated rats. Group 1, vehicle ($n = 15$); group 2, vehicle + DR ($n = 10$); group 3, 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 4, LICO ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 12$); group 5, LICO ($2.5 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 6, LICO ($5 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$); group 7, LICO ($10 \text{ mg}\cdot\text{kg}^{-1}$) + 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) ($n = 15$). ^a $P < 0.05$ versus vehicle-treated group, ^b $P < 0.05$ versus 3-NP, [#] $P < 0.05$ versus 3-NP treatment on 5th, 10th and 15th days. ^c $P < 0.05$ versus [LICO (2.5) + 3-NP] treated group (two-way ANOVA followed by Fisher's least significant difference test). Data presented are the percentage of the vehicle-treated group (mean \pm SEM). DR, diet restriction.

Effect of licofelone on lipid peroxidation, nitrite, SOD and catalase in striatum after 3-NP treatment

Systemic administration of 3-NP significantly increased lipid peroxidation, nitrite concentration and depleted SOD and catalase enzyme activity in the striatum as compared with the vehicle-treated group. Licofelone (2.5 , 5 and $10 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) significantly attenuated lipid peroxidation, nitrite concentration and restored endogenous antioxidant enzyme (SOD and catalase) activities as compared with the 3-NP-treated animals ($P < 0.05$) (Table 1). Furthermore, licofelone ($10 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) *per se* did not have a significant effect on lipid peroxidation, nitrite concentration, SOD and catalase enzyme activity as compared with the vehicle-treated group (Table 1).

Effect of licofelone on striatum mitochondrial enzyme complex levels in 3-NP-treated rats

Systemic 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) administration significantly impaired all the mitochondrial enzyme complexes (I, II and IV) and MTT (mitochondrial redox) activity as compared with the vehicle-treated group. Licofelone (2.5 , 5 and $10 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) treatment significantly restored mitochondrial enzyme complex (I, II and IV) and MTT activities as compared to the 3-NP-treated group ($P < 0.05$) (Figure 4).

Licofelone ($10 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) *per se* did not have a significant effect on mitochondrial enzyme complexes as compared with the vehicle-treated group.

Effect of licofelone on NF- κ B p65, TNF- α , IL-6 and caspase-3 activities in 3-NP-treated rats

Systemic 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) treatment for 14 days significantly increased the levels of pro-inflammatory markers (NF- κ B, p65, TNF- α , IL-6) as well as apoptosis (caspase-3) (Figure 5) as compared with vehicle-treated group. Licofelone (5 and $10 \text{ mg}\cdot\text{kg}^{-1}$) pretreatment significantly attenuated the levels of NF- κ B, p65, TNF- α , IL-6 and caspase-3 levels in the striatum as compared with 3-NP-treated group (Figure 5). The lower dose of licofelone ($2.5 \text{ mg}\cdot\text{kg}^{-1}$) failed to modify the increased levels of these neuroinflammatory markers (NF- κ B, p65, TNF- α , IL-6) as well as (caspase-3) activity in the striatum induced by 3-NP treatment.

Effect of licofelone on PGE₂ and PGF_{2 α} activities in 3-NP-treated rats

Systemic 3-NP ($10 \text{ mg}\cdot\text{kg}^{-1}$) treatment for 14 days significantly increased the levels of pro-inflammatory mediators (PGE₂ and PGF_{2 α}) levels (Figure 6) as compared with vehicle-

Table 1

Effect of licofelone on 3-NP treatment-induced biochemical changes in the striatum

| Treatment (mg·kg ⁻¹) | MDA, nmol mg ⁻¹ protein (% of vehicle) | Nitrite level, μmol mg ⁻¹ protein (% of vehicle) | SOD, U mg ⁻¹ protein (% of vehicle) | Catalase, μmol of H ₂ O ₂ decomposed min ⁻¹ mg ⁻¹ protein (% of vehicle) |
|----------------------------------|---|---|--|--|
| Vehicle | 100 ± 5.6 | 100 ± 6.3 | 100 ± 8.9 | 100 ± 6.6 |
| 3-NP (10) | 253 ± 5.5 ^a | 195 ± 3.4 ^a | 49 ± 4.0 ^a | 55 ± 4.0 ^a |
| LICO (10) | 93 ± 9.2 | 95 ± 5.7 | 101 ± 5.8 | 101 ± 6.9 |
| LICO (2.5) + 3-NP | 213 ± 3.3 ^b | 177 ± 4.3 | 62 ± 4.3 | 64 ± 4.5 |
| LICO (5) + 3-NP | 173 ± 6.5 ^{b,c} | 145 ± 6.8 ^b | 78 ± 4.4 ^b | 85 ± 7.5 ^b |
| LICO (10) + 3-NP | 113 ± 10.3 ^{b,c,d} | 114 ± 6.0 ^{b,c} | 97 ± 6.8 ^{b,c} | 94 ± 4.2 ^b |

Values expressed as % of vehicle-treated group.

^a*P* < 0.05 versus vehicle, ^b*P* < 0.05 versus 3-NP, ^c*P* < 0.05 versus [LICO (2.5) + 3-NP], ^d*P* < 0.05 versus [LICO (5) + 3-NP].

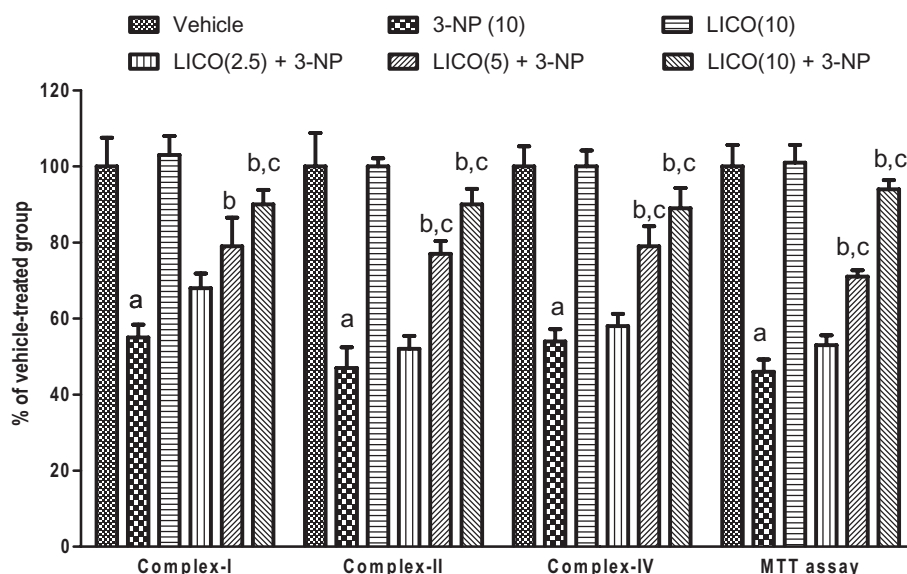


Figure 4

Effect of licofelone (LICO) on mitochondrial complexes I, II, IV and MTT assay in striatum in 3-NP-treated rats. ^a*P* < 0.05 versus vehicle-treated group, ^b*P* < 0.05 versus 3-NP, ^c*P* < 0.05 versus [LICO(2.5) + 3-NP] treated group (one-way ANOVA followed by Tukey's test). Data presented are the percentage of the vehicle-treated group (mean ± SEM).

treated group. Licofelone (10 mg·kg⁻¹) pretreatment significantly attenuated the levels of PGE₂ and PGF_{2α} in the striatum as compared with the 3-NP-treated group (Figure 6).

Discussion

Clinical use of COX inhibitors has been limited due to their side effects, mainly on the cardiovascular system (Fosslien, 2005). Researchers are now focusing on the alteration of another new class of anti-inflammatory drugs named dual COX/LOX inhibitors, and in particular dual COX/5-LOX inhibitors may represent an excellent substitute (Bishnoi *et al.*, 2005; 2006; 2007; Kalonia *et al.*, 2009a,b,c). Based on the above reports, the present study is an attempt to explore

the neuroprotective mechanism of licofelone (dual COX/5-LOX inhibitors) against 3-NP-induced behavioural, biochemical and cellular alterations in rats.

Systemic administration of 3-NP in rats and non-human primates produces homogeneous blockade of complex II within the brain (Kumar and Kumar, 2009a,b,c,d,e), leading to preferential excitotoxic striatal degeneration associated with behavioural abnormalities that are highly reminiscent of HD (Brouillet *et al.*, 1999; 1993; Bizat *et al.*, 2003). The 3-NP experiment model mimics both the hyperkinetic and hypokinetic symptoms of HD, depending upon the duration of administration (Borlongan *et al.*, 1997). In the present study, systemic administration of 3-NP for 14 days significantly reduced locomotor activity (hypokinetic movements), body weight and motor in coordination (grip strength per-

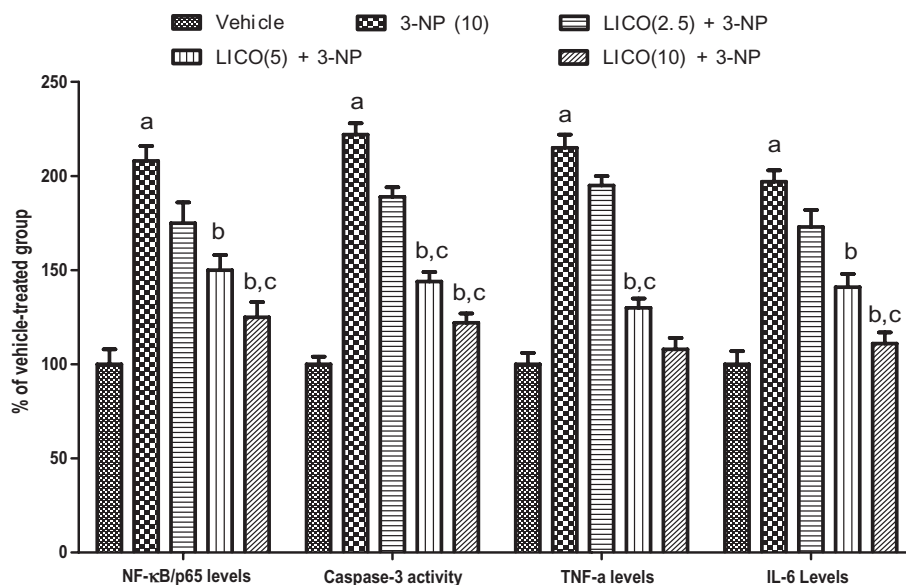


Figure 5

Effect of licofelone (LICO) on NF-κB/p65, IL-6, caspase-3 and TNF-α in striatum in 3-NP-treated rats. ^a $P < 0.05$ versus vehicle-treated group, ^b $P < 0.05$ versus 3-NP, ^c $P < 0.05$ versus [LICO(2.5) + 3-NP] treated group (one-way ANOVA followed by Tukey's test). Data presented are the percentage of the vehicle-treated group (mean ± SEM).

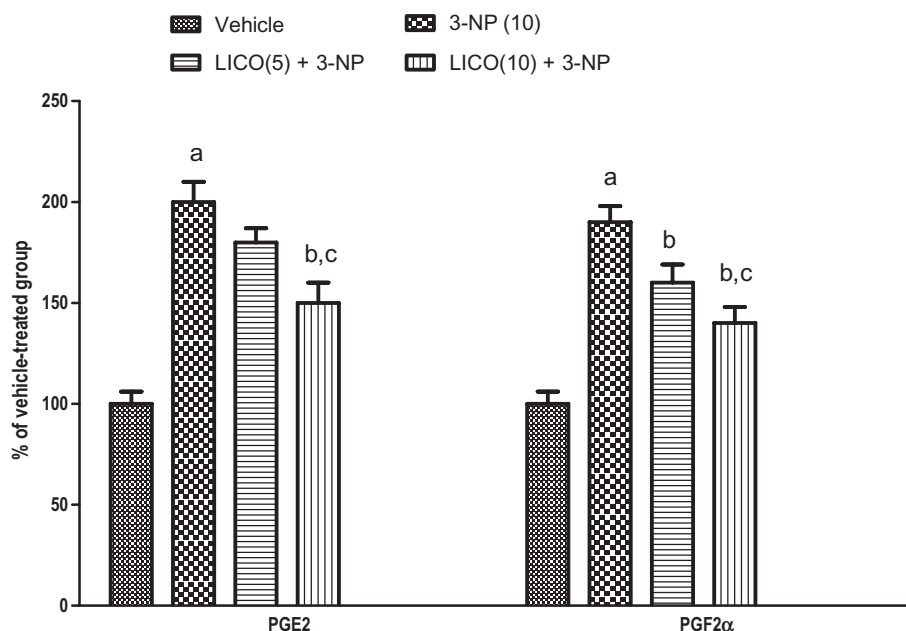


Figure 6

Effect of licofelone (LICO) on PGE₂ and PGF_{2α} in striatum after 3-NP treatment. ^a $P < 0.05$ versus vehicle-treated group, ^b $P < 0.05$ versus 3-NP, ^c $P < 0.05$ versus [LICO(2.5) + 3-NP] treated group (one-way ANOVA followed by Tukey's test). Data presented are the percentage of the vehicle-treated group (mean ± SEM).

formance) in rats, suggesting that the effects of 3-NP most probably mimic either the juvenile onset or late stages of HD-like behaviour. The diet restriction with vehicle group also showed alterations in the locomotor and rotarod activity compared with the control group. However, these were less

marked than those produced in the 3-NP-treated group. Earlier reports from our laboratory had confirmed that 3-NP caused impairment in motor and cognitive functions, oxidative defence and mitochondrial enzyme complex activities in rats (Kumar *et al.*, 2006; 2007; Kumar and Kumar, 2008;

2009a,b,c,d,e). Observations using the this mitochondrial toxin (3-NP) also support the so-called 'indirect excitotoxic' hypothesis for HD (Kumar *et al.*, 2006; 2007; Kumar and Kumar, 2008). Hyperstimulation of NMDA receptors leading to a massive calcium influx activates calcium-dependent PLA2 cascades (Arzberger *et al.*, 1997). Furthermore, PLA2 cleaves membrane phospholipids to yield arachidonic acid (AA), which is converted into PGs by COX (Hurley *et al.*, 2002). Licofelone treatment significantly attenuated the alterations in body weight, locomotor and grip strength performances, suggesting its therapeutic potential against HD-like behaviour. These findings are comparable with those from an earlier report involving a combination of COX-2 and 5-LOX inhibitors, indicating efficacy of COX-2/5-LOX inhibitors in quinolinic acid induced HD-like conditions (Kalonja *et al.* 2009a,b,c).

There is substantial evidence that oxidative damage significantly contributes to the pathogenesis of several neurodegenerative diseases including HD (Kumar *et al.*, 2007; Kumar and Kumar, 2009a,b,c,d,e). In the present study, 3-NP significantly increased oxidative damage as evidenced by the increase in MDA, nitrite and the depletion of endogenous antioxidant defence enzyme (SOD and catalase) activities in the striatum. Disruption of the mitochondrial enzyme complex activity is associated with reactive oxygen species (ROS). In the present study, 3-NP significantly impaired mitochondrial enzyme complex activities (complexes I, II, IV) and mitochondrial redox activity in the striatum. Licofelone pretreatment significantly ameliorated the levels of oxidative stress parameters and restored the mitochondrial enzyme complexes activities in the striatum, suggesting it has antioxidant-like properties in addition to its inhibitory effects on the actions of COX/LOX. Several studies have also highlighted the possibility that individual COX-2 and 5-LOX inhibitors improve the activities of mitochondrial enzyme complexes, and that this could be one of the possible reasons for its beneficial effects against neurotoxin-induced neuronal degeneration (Bishnoi *et al.*, 2005; 2006; 2007; Kalonja *et al.*, 2009a,b,c). Previous reports from our laboratory have also documented the therapeutic potential of COX-2/5-LOX inhibitors in HD-like conditions (Kalonja *et al.*, 2009a; 2010). Evidence has been obtained indicating that the expression of LOX is enhanced during a stimulus-evoked neurodegeneration (Bishnoi *et al.*, 2007; Kalonja *et al.*, 2009a,b). The 5-LOX translocation and subsequent production of LTs increase in neurodegenerative processes (Qu *et al.*, 2000), and an 5-LOX inhibitor can reduce oedema as well as ameliorate the traumatic and excitotoxic events associated with brain injury (Arai *et al.*, 2001). Previous reports have also demonstrated that 5-LOX pathways are activated during excitotoxic brain injury and that licofelone has a protective effect in animal models of this condition (Kulkarni and Singh, 2007; 2008).

Caspase-3 is considered to be a key protease responsible for many of the biological and morphological features of apoptosis (Tuney *et al.*, 2006). Bizat and coworkers examined the involvement of death proteases of the caspase family and Ca²⁺-activated neutral protease calpains in 3-NP toxicity in rats (Bizat *et al.*, 2003). In the present study, systemic 3-NP treatment for 14 days significantly increased caspase-3 activity, indicating the activation of apoptosis in the striatum.

Licofelone pretreatment significantly suppressed caspase-3 activity suggesting it has an anti-apoptotic effect.

Members of the NF- κ B family of transcription factors are activated within the CNS in the pathological settings of apoptosis and neurological diseases (Meffert and Baltimore, 2005). A variety of stimuli are known to activate NF- κ B, which includes cytokines (e.g. TNF and IL-1) and oxidative stress (Meffert and Baltimore, 2005), but evidence for the involvement of NF- κ B p65, TNF- α and IL-6 in the pathogenesis of 3-NP-induced neurotoxicity is lacking. In the present study 3-NP was found to significantly increase these neuroinflammatory markers (NF- κ B, p65, TNF- α) and activate the apoptosis pathways (caspase-3) in the striatum. Furthermore, prostaglandin (PGE₂ and PGF_{2 α}) levels were also found to be increased by 3-NP in the striatum. This study is the first of its kind to report an increased level of prostaglandins, which corresponds to increased COX activity, in the brains of 3-NP-treated rats. These findings highlight the involvement of COX early in the disease, which provides a reasonable and prudent target for therapeutics. These results further showed that there is an association between the behavioural and cellular alterations involved in the pathogenesis of 3-NP-induced HD-like symptoms in animals. Licofelone treatment significantly attenuated the levels of caspase-3, NF- κ B, p65, TNF- α , IL-6 and prostaglandins in the striatum, indicating its action on these cellular cascades. Licofelone also had an inhibitory effect on COX-1, COX-2 and 5-LOX protein expression and directly decreased the production of neuroinflammatory markers by inhibiting the production of the PGs and LTs. Previous reports have also shown that licofelone has a protective effect against pain in animal models (Singh *et al.*, 2005a,b; 2006). In another study, Vidal and coworkers reported that the inhibitory effect of licofelone on 5-LOX expression was associated with the attenuation of the activation of NF- κ B observed in vascular atheroma in rabbits (Vidal *et al.*, 2007).

In summary, in the present study licofelone (dual 5-LOX/COX-2) was shown to have a neuroprotective effect against 3-NP-induced HD-like symptoms in rats. It was further demonstrated that the beneficial effects of licofelone are probably due to its ability to suppress various neuroinflammatory and apoptosis pathways. These findings indicate that licofelone could be used in the management of HD-like symptoms.

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Conflict of interest

All authors do not have conflict of interest.

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