

Licofelone Attenuates LPS-induced Depressive-like Behavior in Mice: A Possible Role for Nitric Oxide

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ABSTRACT - PURPOSE: Licofelone, a dual cyclooxygenase/5-lipoxygenase inhibitor, possesses antioxidant, antiapoptotic, neuroprotective, and anti-inflammatory properties. The aim of the present study was to investigate the effect of licofelone on lipopolysaccharide (LPS)-induced depression in a mouse model and also a possible role for NO. **METHODS:** To elucidate role of NO on this effect of licofelone (5 and 20 mg/kg, i.p.), L-NAME, a non-specific NO synthase (NOS) inhibitor; aminoguanidine (AG), a specific inducible NOS (iNOS) inhibitor; 7-nitroindazole (7-NI) a preferential neuronal NOS inhibitor (nNOS) and; L-arginine (L-Arg), as a NO donor, were used. The animal's behaviors were evaluated employing forced swimming test (FST), tail suspension test (TST) and open field test (OFT). **RESULTS:** LPS (0.83 mg/kg, i.p.) induced depressive-like behavior increasing immobility time in FST and TST. Conversely, licofelone (20 mg/kg i.p.) reversed the depressive effect of LPS and lowered the immobility time in FST and TST. On the other hand, pretreatment with L-Arg also reversed the antidepressant-like effect of licofelone (20 mg/kg) in FST and TST. On the other hand, L-NAME (10 and 30 mg/kg), AG (50 and 100 mg/kg) and 7-NI (60 mg/kg) could potentiate licofelone (5 mg/kg) and lowered the immobility duration. **CONCLUSIONS:** NO down-regulation possibly through iNOS and nNOS inhibition may involve in the antidepressant property of licofelone.

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INTRODUCTION

Oxidative arachidonic acid (AA) metabolism is considered a major feature in neuroinflammation (1). Following its release from the cell membrane by activated neuronal phospholipase A2 (PLA2), AA is metabolized through cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) into the prostaglandins (PGs) and leukotrienes (LTs), respectively (2). Neural and non-neural mammals' cells and tissues express several distinct forms of COX and LOX under normal or stimulated conditions (3). These enzymes are involved in initiation, maintenance, and modulation of inflammatory processes, and also aging, apoptosis, platelet aggregation, oxidative stress and synaptic activity (3). Neuroprotective potential of COX-2 inhibitors and 5-LOX inhibitors has been documented in different experimental model of neurotoxicity (4, 5).

In animal studies, administration of exogenous pro-inflammatory cytokines or a cytokine inducer such as lipopolysaccharide (LPS), triggers depressive-like behavior in rodents, leading to enhanced immobility in the forced swimming (FST) and tail suspension (TST) tests, decreased consumption of a sweetened solution and suppression of sexual behavior, which can be attenuated by antidepressant treatments (6,7). Based on the neuroinflammation hypothesis of depression, LPS-received mice are extensively used to evaluate the underlying mechanisms of depression (8, 9). Licofelone ([2, 2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2, 3, dihydro-1H-pyrrolizine-5-yl]-acetic

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acid) is a substrate analogue of AA and consequently inhibits 5-LOX, COX-1 and COX-2, reducing production of eicosanoid (10). Due to its conformational similarity to AA, licofelone is able to bind the active sites of COX and 5-LOX, therefore blocking their catalytic action (11). Licofelone is a representative of the so-called 'dual inhibitors' of the COX and 5-LOX pathway that have shown to be efficient in several preclinical animal models of inflammation and clinical studies on human subjects (12). Licofelone inhibits prostaglandins synthesis as well as leukotrienes, responsible for the various behavioral (anxiety or depression like behaviors) and biochemical alterations (13).

Licofelone has good oral bioavailability, long half-life (11 h) with no genotoxicity (13, 14). Licofelone has been specifically developed to overcome the gastrointestinal (GI) side-effects associated to non-steroidal anti-inflammatory drugs (NSAIDs) (16). Experimental reports propose that licofelone holds neuroprotective, anti-inflammatory, analgesic, antipyretic, and anti-platelet activities (14, 13, 17), antioxidant and antiapoptotic capacity (18, 19).

It has been shown that licofelone may have a potential therapeutic effect on neurodegenerative disorders correlated with cognitive impairment in the animal model of Alzheimer's disease (AD) by modulation of neuroinflammatory markers (20). Further, chronic licofelone (5 and 10 mg/kg, p.o.) and minocycline treatments significantly attenuated behavioral alterations, oxidative damage and restored mitochondrial enzyme complex activities, whereas combination of licofelone (5 mg/kg) with minocycline (50 mg/kg) profoundly potentiated their protective effect (21). The neuroprotective activities of licofelone has been highlighted (17, 22), which was attributed due to its anti-inflammatory, anti-apoptotic, anti-microglial and anti-oxidant properties (16, 13, 23). Licofelone modulates neuroinflammation and attenuates mechanical hypersensitivity in the chronic phase of spinal cord injury. Long-term treatment of chronically injured rats with chronic licofelone (50 mg/kg, p.o.) elevated levels of endogenous anti-oxidant and anti-inflammatory metabolites within the lesion site (24). Another COX/LOX inhibitor, BW755C, revealed protective effects in an epilepsy model and attenuated the brain damage in rats (25).

Several mechanisms have been suggested for protective effects of COX or LOX inhibitors in the central nervous system (CNS). Among these

pathways, nitric oxide (NO) has always been the focus of many researches (26, 27). NO, a retrograde intracellular second messenger with a very concise half-life is an endogenous modulator of neuronal function (28). NO is synthesized from L-arginine (L-Arg) by specific subtypes of nitric oxide synthases (NOSs). NOSs are classified to distinct isoforms based on the location of expression and cell type. These isoforms include: calcium/calmodulin-dependent which contain endothelial-derived NOS (eNOS) or neuronal-derived NOS (nNOS), and calcium/calmodulin dependent which contain cytokine-inducible NOS (iNOS) (29, 30). Various studies propose that both neuronal and inducible isoforms of NOS involve in various crucial brain processes (31, 32). NO may modulate COX activity and employ COX enzymes as mediators of pathophysiological responses of NO. Additionally, products of the COX pathway may inhibit or enhance NO release (33). The activation of COX-1 by NO may be correlated with the NO effect on glutathione metabolism (34). The NO effect on activation of COX-2 is debated. It has reported that NO activated COX-1, nonetheless; inhibited COX-2- derived prostaglandin production (35), on the other hand; another studies hypothesized that COX-2 activation caused up-regulation of NO (33, 36).

Regarding the neuroprotective activities of licofelone in the nervous system disorders and the functional interactions of COX and/or LOX enzymes with NO signaling pathway, the present study aims to investigate acute effects of licofelone as a dual COX/LOX inhibitor on depression susceptibility via LPS-induced depression-like behavior for the first time in a mouse model and examine the probable role of NO on antidepressant-like effect of licofelone. In this regards, specific and non-specific NOS inhibitors as well as a NO precursor are employed. Thus, the aim of this study was to investigate the role of each NOS isoform and find the corresponding one.

METHODS

The chemicals

The following drugs were used in the study: lipopolysaccharide (LPS), L-arginine (L-Arg), NG-L-arginine methyl ester (L-NAME), aminoguanidine (AG), and 7-nitroindazole (7-NI) were purchased from Sigma Chemical Co. (St Louis, MO). Licofelone were prepared from Tofigh Daru co., Tehran, Iran.

Animals and experimental groups

Male NMRI mice weighing 25 ± 5 g (Tehran University of Medical Sciences, Tehran, Iran) were used throughout the study. The animals were allowed free access to food and water. All the behavioral experiments were conducted during the period between 9:00 and 12:00 A.M. with normal room light (12 h regular light/dark cycle) and temperature (22 ± 1 °C). The mice were handled as indicated in the criteria proposed by the Guide for the Care and Use of Laboratory Animals (NIH US publication, no. 23-86, revised 1985).

All other drugs were administered intraperitoneally (i.p) and the injections were in a volume of 10 ml/kg of the body weight of the mice. LPS, L-NAME, AG and L-Arg were dissolved in sterile normal saline solution (0.9%). Licofelone and 7-NI were suspended in a 1 % aqueous solution of tween 80. Solutions of the drugs and chemicals were always prepared fresh.

The mice were divided into 21 groups of 6. To induce depression-like behavior, they were injected with LPS (0.83 mg/kg, i.p.) (37) and 24 h later the other treatments were done and behavioral tests carried out. Licofelone was administered in doses 5 and 20 (mg/kg, i.p), 75 min prior to the behavioral tests. In experiments investigating the possible role of NO pathway in the licofelone central effects, L-Arg, a NO precursor; L-NAME, a non-specific NOS inhibitor; AG, a specific iNOS inhibitor; 7-NI, a preferential neuronal NO synthase inhibitor, or the vehicles were administered 15 min before licofelone administration. The dosages and timing of drugs administrations in the present study were based upon prior reports (38- 40).

Behavioral tests

Open-field test (OFT)

To ensure that alterations in the duration of immobility do not arise from the changes in motor activity, the locomotor behavior of mice was assessed in an open-field box (41, 42). The apparatus consisted of a Plexiglass box $40 \times 60 \times 50$ cm. The box floor was divided into 12 equal squares. The animals were gently placed in the one corner of the field and the number of squares which was crossed with all paws of the animal counted manually during 6 min.

Forced swimming test (FST)

When the animals are exposed to the FST, they typically adopt an immobile posture, which is

thought to reflect a state of behavioral despair or helplessness (43) and the decrease in immobility time is used as an index of antidepressant activity (44). Briefly, immediately after OFT, the mice were individually placed in an open cylindrical container (diameter 10 cm, height 26 cm) containing 20 cm of water at 23 ± 1 °C. The mice were allowed to swim for 6 min. The total duration of immobility was recorded manually using stopwatches during the last 4 min of the total 6 min duration of the test (45, 46). Each mouse was judged to be immobile when it was ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above the water.

Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al., (47). Briefly, a new group of mice was acoustically and visually isolated then suspended 50 cm above the floor by adhesive tapes placed approximately 1 cm from the tip of the tail. Immobility time was recorded manually using a stopwatch during a 6 min-period (48).

STATISTICAL ANALYSIS

All data were analyzed with two-way analysis of variance for the drug interactions followed by *Tukey's* posttest (Graph pad prism, version 5), except for the main effect of licofelone which was performed with one-way ANOVA. Totally, A value of $P < 0.05$ was considered to be significant.

RESULTS

Figure 1 illustrates effects of licofelone at doses 5 and 20 (mg/kg, i.p.) in FST, TST and locomotor activity on LPS-induced depressive behavior in male mice. As can be observed, LPS at dose 0.83 mg/kg markedly enhanced the immobility measure compared with control group [$F(5, 28) = 7.489$, $P < 0.01$] in FST (Fig. 1a). On the other hand, licofelone at dose 20 mg/kg, i.p. successfully lowered the immobility time of the animals compared with the corresponding LPS group [$F(5, 28) = 7.489$, $P < 0.01$].

In Fig. 1b, effect of licofelone at dose 20 (mg/kg, i.p.) on locomotor activity of LPS-treated animals is shown. As can be understood, no significant change is observed between the groups.

In Fig.1c, as can be observed, LPS at dose 0.83 mg/kg markedly enhanced the immobility measure compared with the control group [$F(5, 28) = 4.904$, $P < 0.05$] in TST. Conversely, licofelone at dose 20 mg/kg, i.p. successfully lowered the immobility time of the animals compared with the corresponding LPS group [$F(5, 28) = 4.904$, $P < 0.05$].

Figure 2 demonstrates effect of L-Arg (750 mg/kg) on antidepressant like effect of licofelone (20 mg/kg) in FST (Fig. 2a), TST (Fig. 2c) and locomotion in OFT (Fig. 2b). As can be observed in Fig. 2a, LPS + licofelone 20 mg/kg is significantly different from LPS group and has lower immobility measure [$F(5, 28) = 7.489$, $P < 0.01$]. On the other hand, LPS + L-Arg 750 mg/kg + licofelone 20 mg/kg has markedly higher immobility time compared with LPS group [$F(5, 28) = 7.489$, $P < 0.01$]. Compared with LPS + licofelone 20 mg/kg, the groups LPS + L-Arg 750 mg/kg, and LPS + L-Arg 750 mg/kg + licofelone 20 mg/kg spend significantly more time immobile [$F(5, 28) = 7.489$, $P < 0.01$] and [$F(5, 28)$

$= 11.385$, $P < 0.001$], respectively. In Fig. 2c, immobility periods of the animals in TST regarding to LPS + licofelone 20 mg/kg is considerably lower compared with LPS group $P < 0.05$. In contrast, the group LPS + L-Arg 750 mg/kg + licofelone 20 mg/kg has markedly higher immobility time compared with LPS + licofelone 20 mg/kg [$F(5, 28) = 4.904$, $P < 0.05$].

Figure 3 demonstrates effect of L-NAME (10 and 30 mg/kg) on licofelone sub-effective dose (5 mg/kg) in FST (Fig. 3a), TST (Fig. 3c) and locomotion in OFT (Fig. 3b). As can be observed in Fig. 3a, the groups LPS + L-NAME 30 mg/kg + licofelone 5 mg/kg spend significantly less time immobile compared with LPS + licofelone 5 mg/kg [$F(5, 28) = 4.904$, $P < 0.05$]. In Fig. 3c, immobility periods of the animals in TST regarding to LPS + licofelone 5 mg/kg is considerably lower in LPS + L-NAME 10 and 30 mg/kg + licofelone 5 mg/kg, [$F(5, 28) = 4.904$, $P < 0.05$].

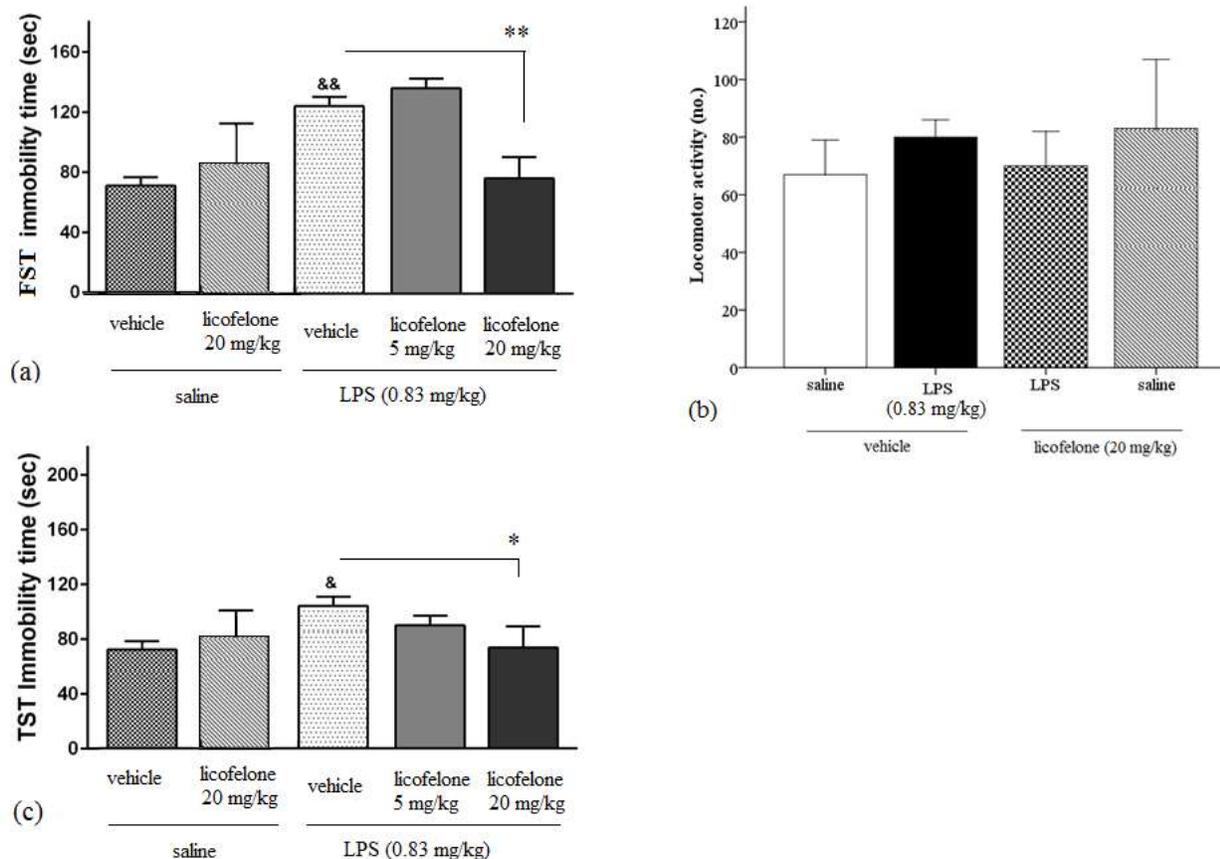


Figure 1. Effects of licofelone at doses 5 and 20 mg/kg, i.p. on LPS-induced depressive effect (0.83 mg/kg) in male mice ($n = 6$) on immobility time in forced-swimming test (FST) (Fig. 1a), tail suspension test (TST) (Fig. 1c) and also locomotor activity (Fig. 1b) compared with the corresponding control groups. & $P < 0.05$ and && $P < 0.01$ significantly different from saline + vehicle group. * $P < 0.05$ and ** $P < 0.01$ significantly different from LPS + vehicle group.

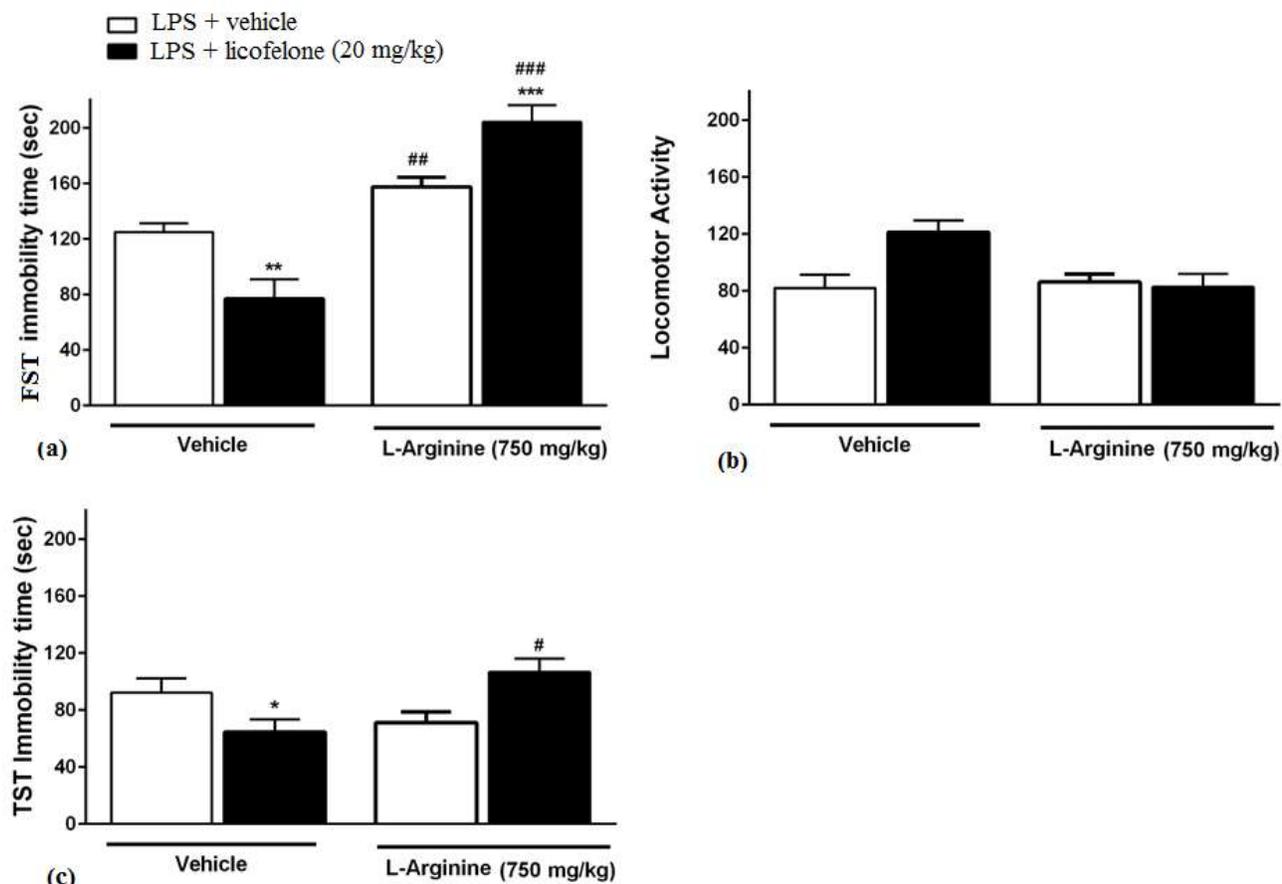


Figure 2. Effects of L-arginine (L-Arg) 750 mg/kg on antidepressant like effect of licofelone (20 mg/kg) in FST (a), TST (c) and locomotion in OFT (b). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ significantly different from LPS group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ significantly different from licofelone (20 mg/kg) group. (n= 6)

Figure 4 demonstrates effect of AG (50 and 100 mg/kg) on sub-effective dose of licofelone (5 mg/kg) in FST (Fig. 4a), TST (Fig. 4c) and locomotion in OFT (Fig. 4b). As can be observed in Fig. 4a, LPS + AG 100 mg/kg is significantly different from LPS group and has lower immobility measure [$F(5, 28) = 4.904, P < 0.05$]. On the other hand, LPS + AG 100 mg/kg + licofelone 5 mg/kg has markedly lower immobility time compared with LPS + licofelone 5 mg/kg group [$F(5, 28) = 4.904, P < 0.05$]. In Fig. 4c, immobility periods of the animals in TST, the groups LPS + AG (50 and 100 mg/kg) + licofelone 5 mg/kg have markedly higher immobility time compared with LPS + licofelone 5 mg/kg [$F(5, 28) = 4.904, P < 0.05$].

Figure 5 demonstrates effects of 7-NI (30 and 60 mg/kg) on sub-effective licofelone (5 mg/kg) in FST (Fig. 5a), TST (Fig. 5c) and locomotion in OFT (Fig. 5b). As can be observed in Fig. 5a, the group LPS + 7-NI 60 mg/kg + licofelone 5 mg/kg is significantly

different from LPS + licofelone 5 mg/kg group and has lower immobility measure [$F(5, 28) = 5.924, P < 0.05$]. In Fig. 5c, immobility periods of the animals in TST regarding to LPS + 7-NI 60 mg/kg + licofelone 5 mg/kg are considerably lower compared with LPS licofelone 5 mg/kg group [$F(5, 28) = 5.924, P < 0.05$].

DISCUSSION

In the current study, we illustrated that licofelone, as a dual COX/5-LOX inhibitor, exerts an antidepressant-like property in the animal mouse model of depression. We used LPS to induce depressant-like behavior and the antidepressant effect of licofelone was demonstrated using animal behavioral tests FST and TST measuring duration of immobility as an index of depressive behavior. In this study, LPS enhanced the immobility measure of the animals, nevertheless; licofelone successfully

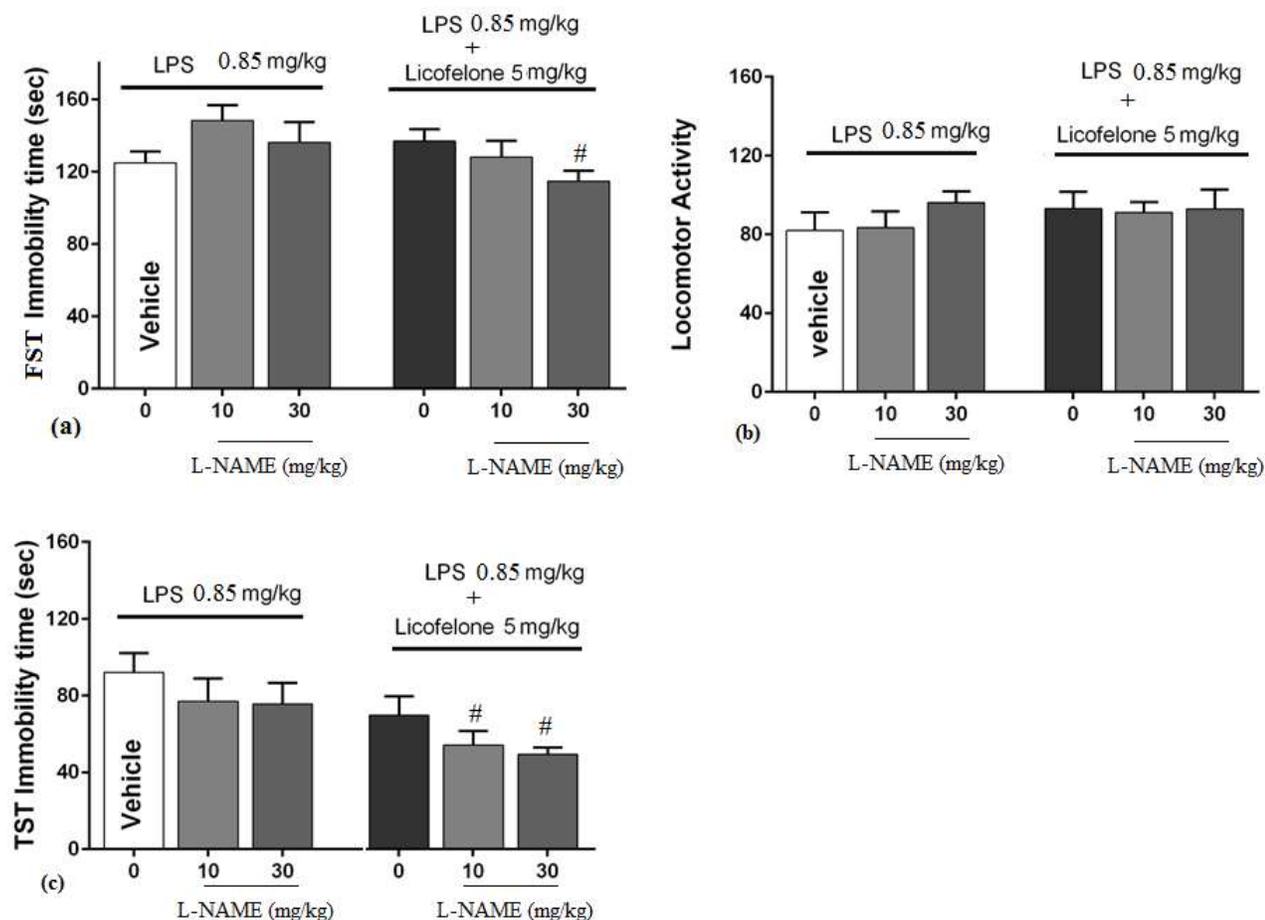


Figure 3. Effects of L-NAME (10 and 30 mg/kg) on antidepressant like effect of licofelone sub-effective dose (5 mg/kg) in FST (Fig. 3a), TST (Fig. 3c) and locomotion in OFT (Fig. 3b). # $P < 0.05$ significantly different from LPS + licofelone (5 mg/kg). (n= 6)

reversed LPS drawback and markedly decreased the immobility figure, consequently, showed antidepressant-like effect. In addition, L-Arg could block antidepressant-like activity of the effective dose of licofelone and increased the immobility time. Contrary to this, the nonselective and selective NOS inhibitors L-NAME, AG and 7-NI efficiently potentiated the sub-effective dose of licofelone and as a consequence, decrease the immobility period considerably.

Practically consistent with our experiment, recent studies revealed that COX/LOX inhibitors such as licofelone could have central protective effects in different settings of CNS disorders (3). Licofelone had a potential therapeutic effect on a neurodegenerative disorder correlated with cognitive impairment and oxidative stress in the animal model of AD induced by intracerebroventricular streptozotocin. Chronic licofelone (2.5, 5, and 10

mg/kg, p.o.) significantly decreased tumor necrosis factor- α (TNF- α) and interleukin 1 beta (IL-1 β) as the oxidative and neuroinflammatory markers in AD (20). In accordance with our study, licofelone possesses a neuroprotective effect against quinolinic acid-induced Huntington disease (HD) like symptoms in rats. Licofelone (2.5, 5 and 10 mg/kg, p.o.) treatment significantly improved body weight, locomotor activity, rotarod performance, balance beam walk performance, oxidative defense, mitochondrial enzyme complex activities and attenuated TNF- α level and striatal lesion (17). Likewise, the neuroprotective mechanism of licofelone (2.5, 5 and 10 mg/kg; p.o.) against 3-nitropropionic acid (3-NP)-induced behavioral, biochemical and cellular alterations was demonstrated in rats which are HD-like symptoms. The beneficial effects of licofelone are probably due to its ability to suppress various neuroinflammatory and apoptosis pathways (49).

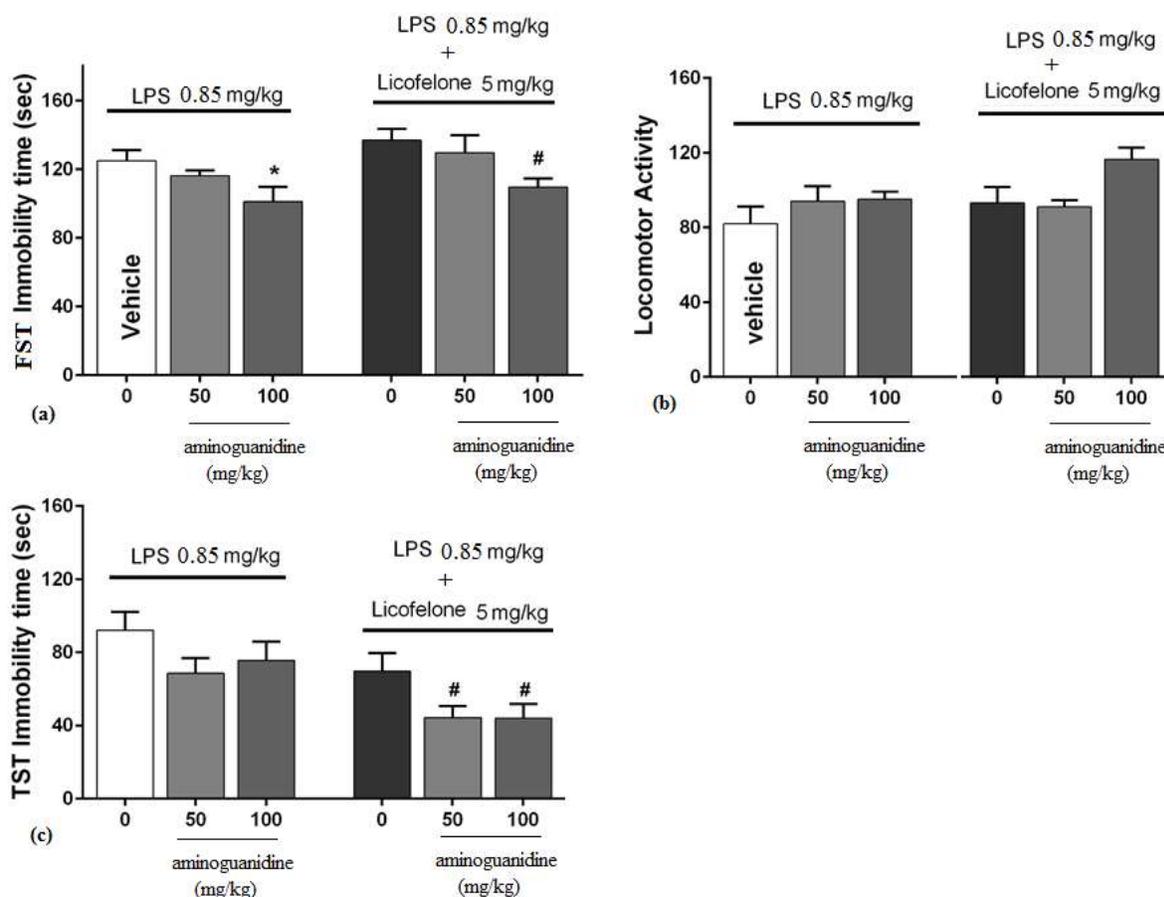


Figure 4. Effects of aminoguanidine (AG) 50 and 100 mg/kg on antidepressant like effect of sub effective dose of licofelone (5 mg/kg) in FST (Fig. 4a), TST (Fig. 4c) and locomotion in OFT (Fig. 4b). # $P < 0.05$ significantly different from LPS + licofelone 5 mg/kg group. (n= 6).

It has shown that LPS activated the nuclear factor- κ B (NF- κ B), iNOS and cyclooxygenase-2 (COX-2) expression in the prefrontal cortex of depressed mice (50). The relation between central effect of licofelone and suppression of NO from iNOS source was demonstrated in a study. Licofelone at the dose of 10 mg/kg (i.p) revealed anticonvulsant properties through iNOS down-regulation in mice. Pre-treatment L-Arg reversed this anticonvulsant effects dose dependently. However, L-NAME potentiated the anticonvulsant effects of licofelone which are completely in line with our relevant results. Nevertheless, 7-NI did not affect seizure threshold alone or in combination with licofelone. 7-NI in our study also potentiated the anticonvulsant effects of licofelone. Using non-effective doses of selective inhibitors of iNOS, AG or 1400W, significantly increased the seizure threshold when were accompanied by licofelone in low doses. These data

support the involvement of NO as an important role player in the central neuro-protective properties of licofelone. Furthermore, it implied that down-regulation of iNOS seems crucial for anticonvulsant properties of this COX/5-LOX inhibitor in seizure susceptibility (51). In agreement with our experiment, licofelone also exerted anticonvulsive effects against status epilepticus (SE) induced by lithium-pilocarpine in male Wistar rats in a dose dependent manner. Pre-treatment with L-Arg decreased the anticonvulsive effects of licofelone, whereas L-NAME potentiated the protective effects of licofelone. Pre-treatment with 7-NI did not change seizure susceptibility significantly, but co-administration of AG and licofelone exerted anticonvulsive effects. These data suggested that the NO pathway especially iNOS contributes to the anticonvulsive effects of licofelone (52).

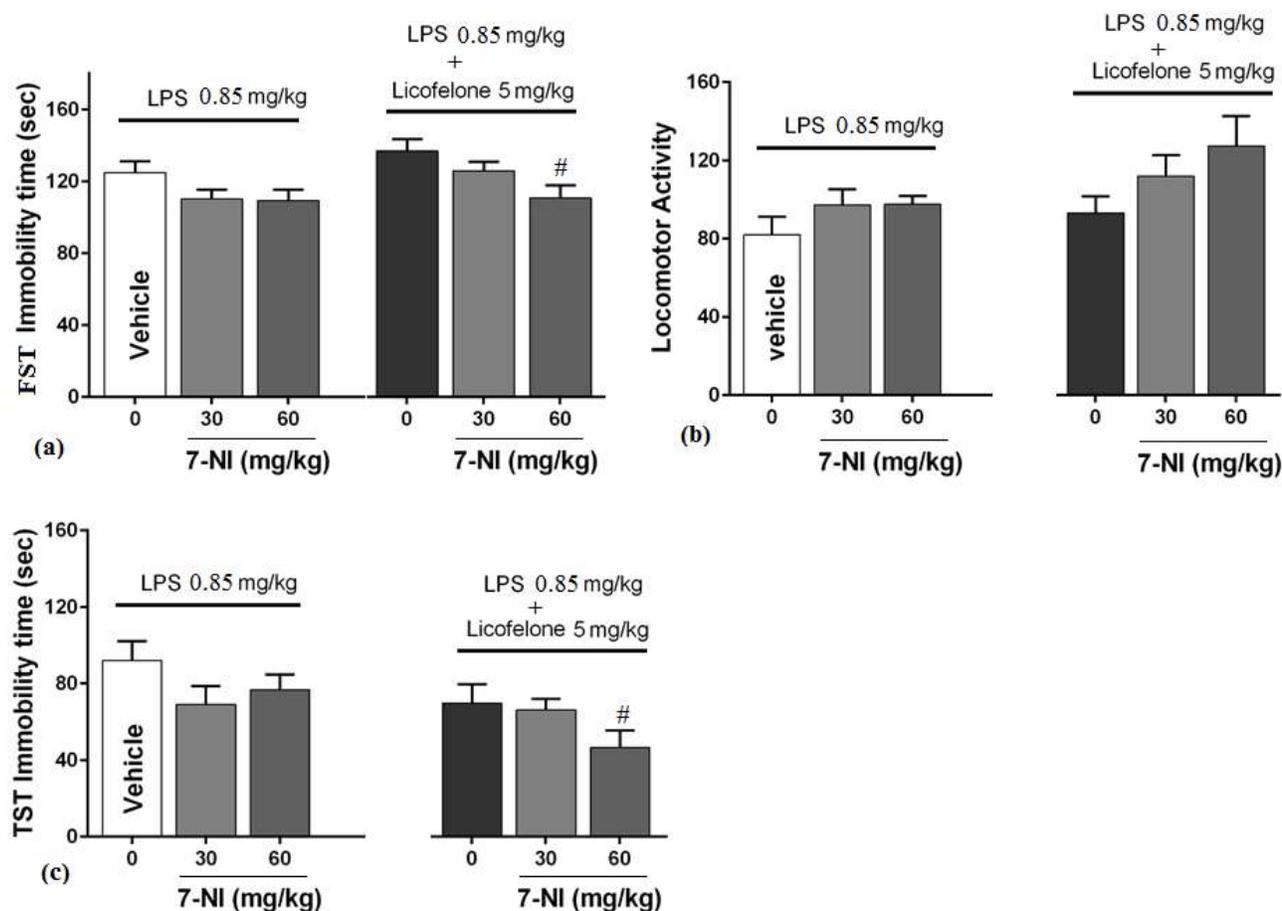


Figure 5. Effects of 7-nitroindazole (7-NI) 30 and 60 mg/kg on antidepressant like effect of licofelone (5 mg/kg) in FST (Fig. 5a), TST (Fig. 5c) and locomotion in OFT (Fig. 5b). # P<0.05 significantly different from LPS + licofelone 5 mg/kg group. (n= 6)

On the whole, our experiments showed that NO system and especially down regulation of iNOS are probably involved in this neuroprotective effect of licofelone in the current inflammatory animal model of depression. These data support excess of NO as an important role player in depressant-like effect of LPS. Furthermore, it implies that down-regulation of NOS especially iNOS appears crucial for development of antidepressant-like property of this COX/5-LOX inhibitor in depression susceptibility. Moreover, licofelone may exert this effect through its anti-inflammatory property as well. Further, these findings may indicate a side effect of licofelone with drugs which modulate NO concentration including sodium nitroprusside (SNP) and organic nitrates. Nevertheless, complimentary studies will be needed to clarify and strengthen the role of NO system pathways in licofelone central protective effects.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests. There is no conflict of interest for any of the authors.

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