Anticonvulsive Effects of Licofelone on Status Epilepticus Induced by Lithium-pilocarpine in Wistar Rats: a Role for Inducible Nitric Oxide Synthase

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Background and Purpose: Status epilepticus (SE) is a neurological disorder with high prevalence and mortality rates, requiring immediate intervention. Licofelone is a cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) inhibitor, which its effectiveness to treat osteoarthritis has been approved. Increasing evidence suggests an involvement of COX and LOX enzymes in epileptic disorders. Thus, in the present study we investigate possible effects of licofelone on prevention and termination of SE. We also evaluated whether the nitrergic system could participate in this effect of licofelone.

Methods: We have utilized lithium-pilocarpine model of SE in adult Wistar rats to assess the potential effect of licofelone on seizure susceptibility. Licofelone was administered 1 h before pilocarpine. To evaluate probable role of nitric oxide (NO) system, L-arginine (60 mg/kg, i.p.), as a NO precursor; L-NAME (15 mg/kg, i.p.), as a non-selective nitric oxide synthase (NOS) inhibitor; aminoguanidine (100 mg/kg, i.p.), as an inducible NOS (iNOS) inhibitor and 7-nitroindazole (60 mg/kg, i.p.), as a neuronal NOS inhibitor were injected 15 min before licofelone. Also, licofelone and diazepam 10 mg/kg were administered 30 minutes after onset of SE.

Results: Pre-treatment with licofelone at the dosage of 10 mg/kg, significantly prevented the onset of SE in all subjects ($p < 0.001$). L-arginine significantly inverted this anticonvulsant effect ($p < 0.05$). However, L-NAME and aminoguanidine, potentiated the anticonvulsant effect of licofelone ($p < 0.05$, $p < 0.01$). Licofelone could not terminate seizures after onset which was terminated by diazepam.

Conclusions: Our findings showed that anticonvulsive effects of licofelone on SE could be mediated by iNOS. Also, we suggest that COX/5-LOX activation is possibly required in the initial stage of onset but SE recruits extra excitatory pathways with prolongation. **(2016;6:53-60)**

Key words: Status epilepticus, Licofelone, Cyclooxygenase, 5-lipoxygenase, Nitric oxide synthase

Introduction

Status epilepticus (SE), a life-threatening emergency condition, is a prolonged self-perpetuating seizure which requires prompt intervention to prevent its injury and mortality.[1] Benzodiazepines, such as diazepam and lorazepam, are recommended as the first choice anti-epileptic medication to terminate seizure, but unfortunately if SE lasts more than 30-40 minutes, it becomes progressively more refractory to these agents.[2] Therefore, applying rapid therapies for treating SE, enhances neuroprotection and suppresses the long-term sequels such as epileptogenesis, neuronal damage and cognitive deficits.[3]

Licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2, 3,dihy-dro-1H-pyrrolizine-5-yl]-acetic acid; previously named ML3000) is a substrate analogue of arachidonic acid which inhibits cyclooxygenase type 1 and 2 (COX1 and COX2) and 5-lipoxygenase (5-LOX), decreasing prostaglandins and leukotrienes production.[4] Licofelone inhibits these enzymes at the equal concentration.[5] Licofelone was developed in order to find compounds with anti-inflammatory activity to obviate the gastrointestinal side-effects related to non-steroidal anti-inflammatory drugs and its safety profile compared to these agents has been authenticated in healthy individuals.[6] Recent experimental studies have shown that licofelone exerts anti-inflammatory properties,[7] anti-asthmatic[8] and neuroprotective effects in the central nervous system.[9] Licofelone can also modulate neuroinflammation and diminish mechanical hypersen-

sitivity.[10] Anticancer effects of this agent in lung,[11] colon and small intestine[12] have been proved.

It is well established that prostaglandins and leukotrienes possess regulatory effect in the pathophysiology of neurological disorders.[13] For instance, potential therapeutic effects of licofelone on neurodegeneration and cognitive impairment in animal models of Alzheimer's disease has been reported.[14] Licofelone also exerted anticonvulsive effects in some animal models of seizure such as pentylenetetrazol-induced seizures in mice.[15] Celecoxib, a selective COX-2 inhibitor, could attenuate neuronal death in the hippocampus.[16] In another study, COX-2 inhibition ameliorated brain injury and oxidative stress in kainic acid model of SE.[17] However, the possible anticonvulsive effects of licofelone on SE has not been yet investigated.

Among the proposed mechanisms for protective effects of COX and LOX inhibitors in the central nervous system (CNS), nitric oxide (NO) pathways has been an interest.[18] Nitric oxide synthases (NOSs) are the group of enzymes, synthesizing NO from L-arginine (L-Arg). Depending on the source of NO, seizure models and involving neurotransmitter systems, NO represents either proconvulsive or anticonvulsive effects.[15,19] In respect of remarkable role of COX and LOX enzymes with NO signaling pathways in CNS disorders, we aimed to investigate whether licofelone as a dual COX/LOX inhibitor prevent or terminate SE induced by lithium-pilocarpine model in rat and examine which isoform of NOS could potentially mediate this effect.

Methods

Chemicals

In our study following drugs were used: L-arginine (L-Arg), a NO precursor; L-N^G-Nitroarginine methyl ester (L-NAME), a non-specific NOS inhibitor; aminoguanidine (AG), a specific iNOS inhibitor and 7-Nitroindazole (7-NI), a specific nNOS inhibitor; pilocarpine and lithium chloride (Sigma, St Louis, MO, USA). Licofelone ([2,2 dimethyl-6-(4-chlorophenyl-7-phenyl-2,3-dihydro-1H pyrrazoline-5-yl] acetic acid) was a dedicated as a gift from Tofigh daru (Tehran, Iran); Scopolamine methyl bromide, a cholinergic muscarinic antagonist from Osvah.

Licofelone was freshly dissolved in slightly alkaline water and 7-NI in a 1% aqueous solution of DMSO then followed by sonication. Other drugs were dissolved in normal saline solution (0.9%). Solutions and suspensions of drugs were always prepared in the day of experiment. On the basis of our previous study, dosages and time of administrations were chosen.[15]

Animals

Male adult Wistar rats weighing 200-250 g were selected from our center of breeding facilities. The animals were kept in a standard temperature-controlled (22 ± 3°C) environment under a 12-h light/12-h dark cycle schedule. They had free accessibility to tap water and food except for experimental procedure. Each group consisted of 8 animals. All animal maintenance and procedures were established in agreement with institutional guidelines for animal care and use published by national institutes of health and with the approval of the Ethics Committee on Animal Experiments of Tehran University of Medical Sciences and all efforts were made to minimize suffering of the animals.

Induction of SE by lithium-pilocarpine

Animals were injected lithium chloride (127 mg/kg, i.p.). After 20 hours SE was induced with intraperitoneally injection of pilocarpine hydrochloride (60 mg/kg, i.p.). Thirty minutes prior to injection of pilocarpine, scopolamine methyl bromide 2 mg/kg i.p was administered to limit peripheral side effects of pilocarpine (e.g., salivation, diarrhea and lacrimation) which was not achieved with the standard regimen of scopolamine methyl bromide 1 mg/kg. After pilocarpine injection, animals were then closely observed for signs of seizure activity, and seizure severity was ranked using Racine's scale[20]: 1 = seizure consisted of immobility and occasional facial clonus; 2 = head nodding; 3 = bilateral forelimb clonus; 4 = rearing; 5 = rearing and falling. Generally, "the seizure behavior consists of head bobbing with intermittent forelimb and hind limb clonus, hyperextension of tails, loss of posture, falling back, and myoclonic jerks, whereas SE is a condition where these recurrent generalized seizures last for more than 30 minutes in the animals".[21] In consistent with previous studies, SE was defined as consecutive seizures with a score of 3 or above.[20,22-24] In our study, we measured the incidence of SE, 20 minutes after pilocarpine administration in separate experimental groups as explained below.

Experimental procedure

In our first experiment, different doses of licofelone (1, 5 and 10 mg/kg; i.p.) were injected 1 hours before pilocarpine induced SE. Control group was injected with vehicle (slightly alkaline water). In the next experiment, we evaluated the effect of the L-arginine as NO precursor on the effective dose of licofelone. In this regard, rats were administered L-arginine (10, 30 and 60 mg/kg) 15 min before licofelone (5 mg/kg) or 75 min before SE induction by pilocarpine. To ex-

amine the effect of NO inhibition on seizure susceptibility, rats were administered the non-specific NOS inhibitor L-NAME at doses of (1, 5 and 15 mg/kg) 75 min before pilocarpine. To evaluate which isoform of NO could specifically mediate this process, 7-NI (60 mg/kg) as a neuronal NOS inhibitor and AG (100 mg/kg) as an inducible NOS inhibitor were used 75 min prior to pilocarpine administration. To evaluate whether licofelone could terminate seizure, we injected licofelone (10 mg/kg) or diazepam (10 mg/kg, as gold standard) 30 minute after SE.

Statistical analysis

In each experimental group results of seizure incidences are expressed by population proportions of SE occurrence. Data were analyzed by one-way z-score test. In all experiments, p value 0.05 was regarded as significant.

Results

Effect of different doses of licofelone on lithium-pilocarpine induced SE

Fig. 1 elucidates the effect of acute administration of licofelone (1, 5 and 10 mg/kg) on lithium-pilocarpine induced SE. Licofelone was injected 1 h before pilocarpine. One tailed Z-test explains a significant effect for administration of licofelone (10 mg/kg) [The Z-Score was 4 and the p value was <0.0001]. The result is sig-

nificant at $p < 0.001$ as an anticonvulsant factor compared with vehicle control rats.

Effect of L-arginine pre-treatment in combination with licofelone

Fig. 2 shows the effect of pre-treatment with L-arginine (10, 30 and 60 mg/kg) on the anticonvulsive dose of licofelone (5 mg/kg, i.p.) on lithium-pilocarpine induced SE. L-arginine was injected 15 minutes prior to licofelone. One tailed Z-test explains that L-arginine (60 mg/kg) reduced the anticonvulsive effect of licofelone [The Z-Score is 1.9215. The p value was 0.0273. The result was significant at $p < 0.05$].

Effects of L-NAME pre-treatment in combination with licofelone

Fig. 3 shows the effect of pre-treatment with L-NAME (1, 5 and 15 mg/kg) on anticonvulsive effects of licofelone (5 mg/kg, i.p.) on lithium-pilocarpine induced SE. L-NAME was injected 15 minutes prior to licofelone. One tailed Z-test shows that L-NAME (15 mg/kg) increased the anticonvulsive effect of licofelone (5 mg/kg) [The Z-Score was 2.0656. The p value was 0.0194. The result was significant at $p < 0.05$].
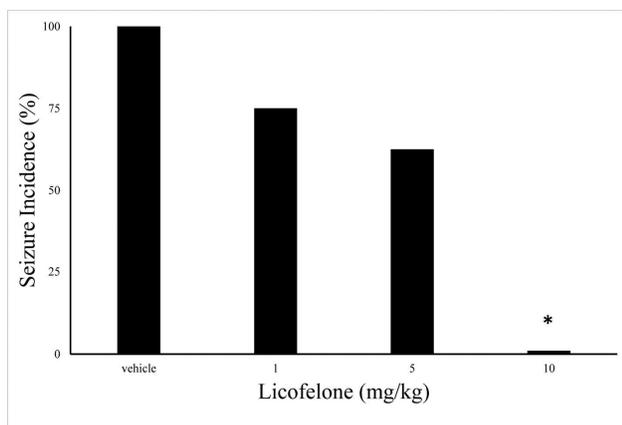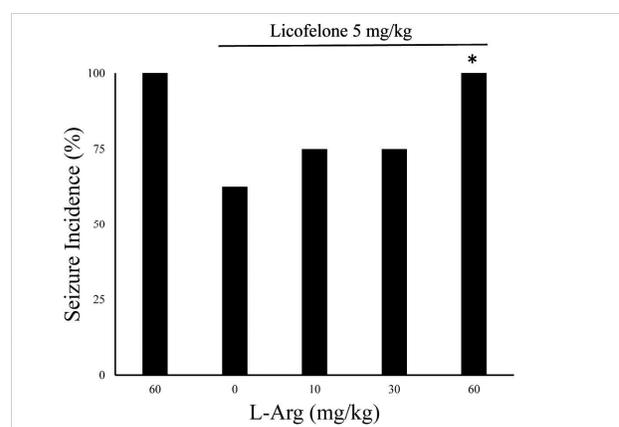

Figure 1. Effect of acute administration of licofelone (1, 5 and 10 mg/kg) on lithium-pilocarpine induced SE. Licofelone was administered 1 h before pilocarpine. One tailed Z-test explain a significant effect for licofelone 10 mg/kg compare with vehicle control group. At the dose of 10 mg/kg the Z-Score is 4. The p-value is < 0.0001. The result is significant at $p < 0.001$. Data are expressed as the percentages of rats that experienced SE. Each group comprised of 8 rats. *$p < 0.001$ compared with vehicle control group.

Figure 2. Effect of pre-treatment with different doses of the NO precursor L-arginine (10, 30 and 60 mg/kg) on the anticonvulsive trait of licofelone (5 mg/kg) in lithium-pilocarpine induced SE. L-arginine was injected 15 minutes prior to licofelone. One tailed Z-test explains that L-arginine (60 mg/kg) reversed the effect of licofelone. In experimental group of 60 mg/kg Z-Score is 1.9215. The p-value is 0.0273. The result is significant at $p < 0.05$. Data are expressed as the percentages of rats that experienced SE. Each group comprised of 8 rats. *$p < 0.05$ compared with the licofelone (5 mg/kg) treated group. NO, nitric oxide; SE, status epilepticus.
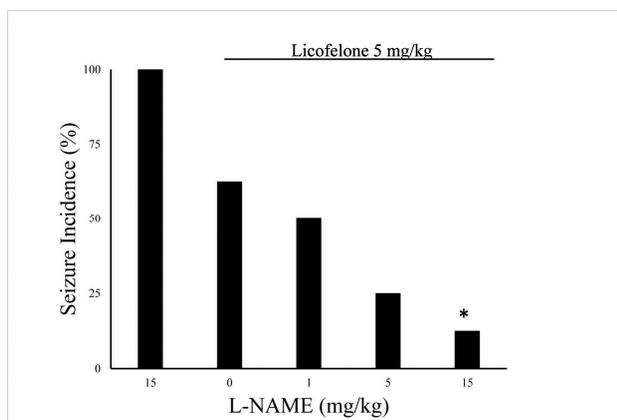
Figure 3. Effect of the non-specific NOS inhibitor L-NAME at different doses (1, 5 and 15 mg/kg) on the anticonvulsive property of licofelone (5 mg/kg) in lithium-pilocarpine induced SE. L-NAME was injected 15 minutes prior to licofelone. One tailed Z-test shows that L-NAME increased the anticonvulsive effect of licofelone compared with group receiving saline and licofelone (5 mg/kg). At the dose of L-NAME 15 mg/kg Z-Score is 2.0656. The p-value is 0.0194. The result is significant at $p < 0.05$. Data are expressed as the percentages of rats that experienced SE. Each group comprised of 8 rats. *$p < 0.05$ compared with corresponding licofelone (5 mg/kg) treated group. NOS, nitric oxide synthase.
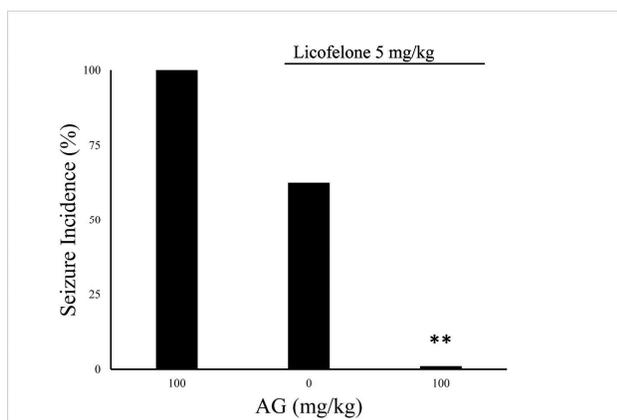

Figure 4. Effect of the selective iNOS inhibitor AG (100 mg/kg) on the anticonvulsive property of licofelone (5 mg/kg) on lithium-pilocarpine induced SE. AG was injected 15 minutes prior to licofelone. One tailed Z-test explains that AG (100 mg/kg) increased the anticonvulsive effect of licofelone compared with group receiving saline and licofelone (5 mg/kg). The Z-Score is 2.6968. The p-value is 0.00347. The result is significant at $p < 0.01$. Data are expressed as the percentages of rats that experienced SE. Each group comprised of 8 rats. ** $p < 0.01$ compared with the vehicle control group. iNOS, inducible nitric oxide synthase, SE, status epilepticus; AG, aminoguanidine.

Effects of pre-treatment with AG in combination with licofelone

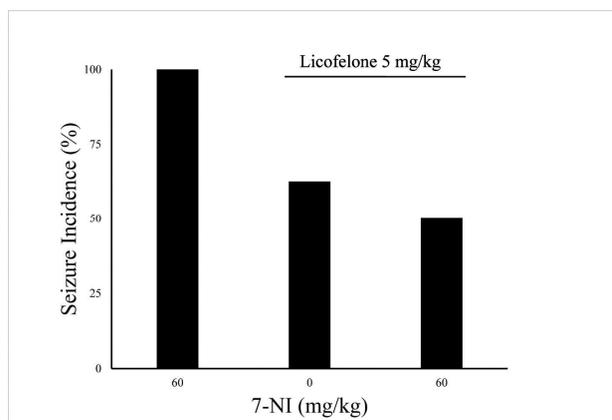Fig. 4 shows the effect of pre-treatment with AG (100 mg/kg) on

Figure 5. Effect of the neuronal NOS inhibitor 7-NI (60 mg/kg) on the anticonvulsive property of licofelone (5 mg/kg) on lithium-pilocarpine induced SE. 7-NI was injected 15 minutes prior to licofelone. In the administration of 7-NI (60 mg/kg) our data shows that it does not have significant involvement on the effect of licofelone. Data are expressed as the percentages of rats that experienced SE. Each group comprised of 8 rats. NOS, nitric oxide synthase; SE, status epilepticus.

the anticonvulsant trait of licofelone (5 mg/kg) on lithium-pilocarpine induced SE. AG was injected 15 minutes prior to licofelone. One tailed Z-test explains that AG (100 mg/kg) increased the anticonvulsant effect of licofelone (5 mg/kg) [The Z-Score is 2.6968. The p value was 0.00347. The result was significant at $p < 0.01$].

Effects of pre-treatment with 7-NI in combination with licofelone

Fig. 5 shows the effect of pre-treatment with 7-NI (60 mg/kg) on the anticonvulsant trait of licofelone (5 mg/kg, i.p.) on lithium-pilocarpine induced SE. 7-NI was injected 15 minutes prior to licofelone. Our data shows that 7-NI did not have significant involvement on the effect of licofelone.

Effects of treatment with Licofelone and diazepam after SE

Fig. 6 shows the response of subjects to licofelone and diazepam administration 30 minutes after onset of SE. Our Data clarifies that licofelone (10 mg/kg) could not terminate SE which was terminated by diazepam (10 mg/kg).

Discussion

In the present study, we demonstrated that licofelone exerted anticonvulsive effects against SE induced by lithium-pilocarpine in male Wistar rats in a dose dependent manner. Pre-treatment with the NO
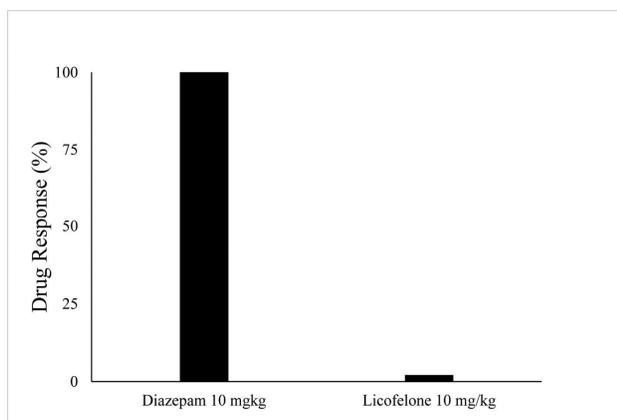
Figure 6. Response to administrating of diazepam (10 mg/kg) as a central nervous system depressant and licofelone (10 mg/kg) 30 minutes after onset of SE that made by lithium-pilocarpine. Experiments shows that diazepam (10 mg/kg) terminated SE in all subjects but licofelone (10/mg) (that worked properly as prophylactive anticonvulsive agent [Fig. 1]) could not terminate SE. Data are expressed as the percentages of rats that experienced SE. Each group comprised of 8 rats. SE, status epilepticus.

precursor L-arginine decreased the anticonvulsive effects of licofelone, whereas the non-selective NOS inhibitor L-NAME potentiated the protective effects of licofelone. To clarify which isoform of NOS is involved in this effect of licofelone, the selective nNOS inhibitor 7-NI and the selective iNOS inhibitor AG were injected in combination with 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 the COX/5-LOX inhibitor licofelone on SE induced by lithium-pilocarpine in male Wistar rats. Despite its protective effect as prophylaxis, it could not terminate SE, indicating that after onset, COX/5-LOX blockage are not enough to terminate SE.

There is evidence that inflammatory processes could participate in the pathophysiology of SE. Accordingly, Marchi et al. reported that in pilocarpine induced SE, pilocarpine causes acute peripheral inflammation mostly through interleukin 1 beta (IL-1β), leading to blood-brain barrier (BBB) leakage before the onset of SE[25] and increasing BBB permeability may promote entry of cofactors (e. g. K$^+$) into the brain leading to pilocarpine-induced SE. More investigation revealed that administration of the muscarinic acetylcholine receptor antagonist, atropine, blocked the seizure while it is not affected when SE is established, representing that activation of muscarinic receptors are required in the initial phase but recruits noncholinergic pathways with prolongation.[26] These pathways include excitotoxic

transmission system including *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors which open ionic channels permeable to calcium (Ca^{2+}).[27] During SE, gamma-aminobutyric acid (GABA) biosynthesis in substantia nigra fails and there will be a reduction in available GABA$_A$ postsynaptic receptors.[28] These losses of inhibitory neurotransmitter and its receptors are possible etiologies to become pharmacoresistance to benzodiazepines.

Recent studies also reported that COX/LOX inhibition could have protective effects in CNS disorders via its property to modulate inflammation, apoptosis and oxidative stress.[29,30] BW755C, a dual LOX/COX inhibitor, has also been shown to protect brain damage in kainate-induced seizure.[31] Cyclooxygenases (COX) are the enzymes existing in two types of COX-1 and COX-2, metabolizing arachidonic acid to biologically active molecules.[32] COX-1 is continually expressed in most tissue, producing prostaglandins (PGs) at low concentration to maintain the physiological functions.[33] COX-2 is the inducible form of COX which plays a critical part in inflammation.[34] However, COX-2 is continuously expressed in stomach and kidney which is not associated with inflammation.[35] Several studies revealed the anticonvulsive and neuroprotective effect of selective and non-selective COX inhibitors in various models of seizure.[36,37] A selective COX-2 inhibitor, celecoxib, and a non-selective COX inhibitor, aspirin, could attenuate hippocampal neuronal loss.[16,38] Some studies showed that NO activates COX enzymes[39] and mutually it is hypothesized that COX-2 activation causes NO upregulation.[40] However it is proposed that NO activates COX-1 derived prostaglandin while inhibits COX-2 production.[41] Interaction between iNOS and COX-2 have also been shown to be involved in IL-1β induced activation of rat primary hippocampal culture.[42]

5-LOX is an enzyme that inserts oxygen in arachidonic acid, turning to leukotrienes, which are potent mediators in inflammatory processes. LOX mostly exists in brain tissue and neurons and may participate in neurodegeneration.[43] 5-LOX is upregulated in various CNS disorders such as multiple sclerosis, stroke, and Alzheimer's disease.[44-46] Accordingly, 5-LOX inhibitors have been considered to be neuroprotective. Additionally, the end products of 5-LOX, leukotrienes, can induce NO synthesis,[47] while it has been reported that NO-donor compounds inhibits LOX activity.[48] Bhujade and colleagues reported that a plant extracts inhibited inflammatory mediators, COX and 5-LOX through down regulation of iNOS.[49]

Many molecular signaling pathways are triggered during epileptogenesis. Among these proposed mechanisms, activation of the AMPA, kainite and NMDA receptors has been reported to play a key

role in neuronal excitability in seizures.[50] Increment of hippocampal nitrite content after SE, suggests a role for NO system in SE.[51] Glutamate release due to NOS activation might be the underlying mechanism.[52] In one study, median convulsive dose of ciprofloxacin was increased by AG and decreased with L-arginine, suggesting that elevation of brain glutamate is the consequence of iNOS activation.[53] In consistent with the above evidence, our data also revealed that iNOS inhibition could potentiate the anticonvulsive effects of the COX/5LOX inhibitor licofelone on SE induced by lithium-pilocarpine. These results are in line with previous study that showed the involvement of iNOS in the anticonvulsive effect of licofelone against pentylenetetrazol-induced seizures in mice.[15] Additionally, previous studies have shown that licofelone is able to decrease NO production or decrease iNOS activation in cartilage chondrocytes.[54,55] It is also shown that activation of LOX and COX causes oxidative stress leading to apoptosis of GABAergic neurons[56]; and neuronal damage due to seizures is associated with upregulation of iNOS in hippocampus.[57] It seems that variant models of seizure, source of NO, and other neurotransmitter involving during epileptogenesis are the underlying reasons for these differences.

In conclusion, in the present study, we demonstrated that anticonvulsive effect of licofelone, as a COX/5-LOX inhibitor, on lithium-pilocarpine induced SE in rats, is potentiated by both the non-selective NOS inhibitor L-NAME and the selective iNOS-inhibitor AG, representing that NO pathway, especially iNOS, mediates this protective effect of licofelone.

Conflict of Interest

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