Life Sciences 168 (2017) 38–46

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Levosimendan exerts anticonvulsant properties against PTZ-induced seizures in mice through activation of nNOS/NO pathway: Role for K_{ATP} channel

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ARTICLE INFO

Article history:
Received 18 September 2016
Received in revised form 7 November 2016
Accepted 8 November 2016
Available online 14 November 2016

Keywords:
Seizure
Levosimendan
PTZ
K_{ATP} channels
Nitrergic system

ABSTRACT

Aims: Although approving new anticonvulsants was a major breakthrough in the field of epilepsy control, so far we have met limited success in almost one third of patients suffering from epilepsy and a definite and reliable method is yet to be found. Levosimendan demonstrated neuroprotective effects and reduced mortality in conditions in which seizure can be an etiology of death; however, the underlying neuroprotective mechanisms of levosimendan still eludes us. In the light of evidence suggesting levosimendan can be a K_{ATP} channel opener and nitrergic pathway activator, levosimendan may exert antiseizure effects through K_{ATP} channels and nitrergic pathway.
Main methods: In this study, the effects of levosimendan on seizure susceptibility was studied by PTZ-induced seizures model in mice.
Key findings: Administration of a single effective dose of levosimendan significantly increased seizures threshold and the nitrite level in the hippocampus and temporal cortex. Pretreatment with noneffective doses of glibenclamide (a K_{ATP} channel blocker) and L-NAME (a non-selective NOS inhibitor) neutralize the anticonvulsant and nitrite elevating effects of levosimendan. While 7-NI (a neural NOS inhibitor) blocked the anticonvulsant effect of levosimendan, Aminoguanidine (an inducible NOS inhibitor) failed to affect the anticonvulsant effects of levosimendan. Cromakalim (a K_{ATP} channel opener) or L-arginine (an NO precursor) augmented the anticonvulsant effects of a subeffective dose of levosimendan. Moreover, co-administration of noneffective doses of Glibenclamide and L-NAME demonstrated a synergistic effect in blocking the anticonvulsant effects of levosimendan.
Significance: Levosimendan has anticonvulsant effects possibly via K_{ATP}/nNOS/NO pathway activation in the hippocampus and temporal cortex.

1. Introduction

With a prevalence of 1–2%, epilepsies undoubtedly impose a major burden upon patients and societies [1]. Despite major breakthroughs in the field of epilepsy research, anti-seizure medications do not provide sufficient seizure control in almost one-third of patients suffering from epilepsy [2,3]. Thus, investigation on new evidence-based therapeutical strategies is mandatory, and neuropharmacology can be a promising field for this purpose.

Levosimendan is a novel pyridazinone-dinitrile derivate, well-known for positive inotropic effects through enhancing the sensitivity between Ca^{+2} and myofilaments and inhibiting the phosphodiesterase III activity [4]. Some studies suggest possible impact of calcium sensitizers including levosimendan on CNS, demonstrated as central symptoms such as headaches, vertigo, flushing, and nausea [5]. Recent studies demonstrated that levosimendan can improve the survival rates in calcium channel blockers (CCBs) toxicity, which partly might be through seizure reduction as a common consequence of CCB toxicity [6–8].

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http://dx.doi.org/10.1016/j.lfs.2016.11.006

Several lines of evidence confirmed that levosimendan, in addition to its inotropic effects, exerts cardio-protection, vasodilation, and antioxidant activity through modulation of mitochondrial adenosine triphosphate-sensitive potassium (K_{ATP}) channels and nitric oxide (NO) production [9–12].

K_{ATP} channels are non-voltage dependent, inward-rectifier potassium channels, regulated by intracellular ATP/ADP ratio as a marker of the cellular energy supply. Besides, various pharmacologic agents (e.g., cabergoline and glibenclamide) can modulate the activity of these channels without affecting cellular ATP concentration [13–15]. Recent studies have found that the K_{ATP} channels have antiepileptic effects and help to control neuronal excitability and seizure propagation [16–20].

NO is a gaseous neuronal messenger and neurotransmitter modulator in the brain, synthesized by NO synthase (NOS) either constitutive (eNOS, and nNOS) or inducible isoforms (iNOS) from L-arginine [21]. There are several studies reporting a controversial anticonvulsant or pro-convulsant but, at the same time, a fundamental role for the nitrergic system in the modulation of seizures [22–26].

The pivotal role of K_{ATP} channels and NO in both mechanism of action of levosimendan and modulation of seizure thresholds raise the possibility that levosimendan may affect the seizure susceptibility through K_{ATP} and NO-dependent mechanisms. To this end, this study aims to shed light on the effects of levosimendan on PTZ-induced seizure threshold in mice as a model of generalized clonic seizures (GCS) and then ascertain the nature of this effect by delineating the molecular pathways involved in the exertion of this effect.

2. Methods and materials

2.1. Subjects

Swiss male mice at 12–16 weeks were used throughout this study. The mice were housed at a constant temperature (23 °C) and relative humidity (60%) in groups of 6–8 with free access to food and water and a fixed 12 h light/dark cycle. Procedures involving mice and their care were conducted in conformity with the institutional guidelines that are in compliance with the national and international laws and policies. Each mouse was used only once, and each treatment group comprised of 6–8 animals. Additionally, all efforts were made to minimize animal suffering and to use only the minimal number of animals required to produce reliable scientific data.

2.2. Pharmacological treatments

The following drugs were used in the study: levosimendan [a calcium sensitizer (Sigma, St Louis, MO, USA)], pentylenetetrazole (PTZ) [$GABA_A$ receptor antagonist; (Sigma, UK)], L-arginine (L-Arg) [NO precursor; (Sigma, St Louis, MO, USA)], N(G)-nitro-L-arginine methyl ester (L-NAME) [a non-selective NOS inhibitor; (Sigma, St Louis, MO, USA)], 7-nitroindazole (7-NI) [a selective nNOS inhibitor; (Sigma, St Louis, MO, USA)], aminoguanidine (AG) [an iNOS inhibitor; (Sigma, St Louis, MO, USA)], Cromakalim [a K_{ATP} channel opener; (Sigma, St Louis, MO, USA)], Glibenclamide [a K_{ATP} channel blocker; (Sigma, St Louis, MO, USA)].

All drugs were freshly prepared prior to use, and injection volume (10 $mL \cdot kg^{-1}$) was kept constant for in vivo experiments. Levosimendan, L-NAME, 7- NI, AG, L-ARG, Cromakalim and glibenclamide were administered intraperitoneally (i.p.). 7-NI was suspended in a 1% aqueous solution of Tween 80 and all other drugs were dissolved in normal saline. To assess clonic seizure, PTZ was administered intravenously in the tail vein (0.5%, i.v.). The dosage selections, route of drug administration, and injection time of different compounds were based on our previously published data, preliminary experiments and pharmacokinetic considerations [20,22,24]. Control mice were injected with the corresponding volume of vehicles before PTZ-induced seizures. Pharmacological experiments were carried between 9.00 am and 2.00 pm.

2.3. Study design

309 mice were randomized into two categories. The first category, consisting of 35 groups, was used in seizure paradigm to assess the PTZ-induced seizure threshold. Ten groups of mice were randomly assigned to produce scientific and reliable data regarding levosimendan effects on PTZ-induced seizures dose response and time course. In order to delineate the role of K_{ATP} channel and nitrergic system on the anticonvulsant properties of levosimendan mice were randomly assigned to the following groups: 1) glibenclamide (0.5 $mg \cdot kg^{-1}$; i.p.); 2) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.); 3) glibenclamide (0.5 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 4) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 5) Cromakalim (0.5 $mg \cdot kg^{-1}$; i.p.); 6) Cromakalim (1.0 $mg \cdot kg^{-1}$; i.p.); 7) Cromakalim (0.5 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 8) Cromakalim (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 9) L-NAME (1.0 $mg \cdot kg^{-1}$; i.p.); 10) L-NAME (5.0 $mg \cdot kg^{-1}$; i.p.); 11) L-NAME (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 12) L-NAME (5.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 13) L-ARG (30.0 $mg \cdot kg^{-1}$; i.p.); 14) L-ARG (60.0 $mg \cdot kg^{-1}$; i.p.) 15) L-ARG (30.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 16) L-ARG (60.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 17) 7-NI (15.0 $mg \cdot kg^{-1}$; i.p.); 18) 7-NI (30.0 $mg \cdot kg^{-1}$; i.p.); 19) 7-NI (15.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 20) 7-NI (30.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 21) AG (30.0 $mg \cdot kg^{-1}$; i.p.); 22) AG (100.0 $mg \cdot kg^{-1}$; i.p.); 23) AG (30.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 24) AG (100.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 25) L-NAME (1.0 $mg \cdot kg^{-1}$; i.p.) + glibenclamide (0.5 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.) (Table 1).

The second, consisting of 6 groups, was sacrificed to harvest brain tissues for nitrite assays. To study the effects of levosimendan on NO content in brain tissue mice were randomly assigned to the following groups: 1) saline; 2) levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 3) L-NAME 5.0 $mg \cdot kg^{-1}$; i.p.); 4) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.); 5) L-NAME (5.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 6) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.).

2.4. Seizure paradigm

In order to analyze the seizure susceptibility of mouse, we recruited PTZ-induced seizure threshold as a model of GCS. The seizure paradigm test was performed by researchers blinded to experimental conditions using standardized test according to a protocol used previously in our laboratory [22,27]. Following i.v. PTZ administration, eventually a sequence of seizure signs beginning with twitch and progressing to clonus and tonic limb extension is observed. In the present study, forelimb clonus followed by full clonus of the body was used as the endpoint. In preliminary experiments, forelimb clonus was found to be more sensitive to levosimendan than other seizure phases. A 30-gauge, 3/4 in. butterfly needle was inserted into the tail vein of unrestrained freely moving animals, and the needle was secured with a piece of adhesive tape. The needle was connected by a polyethylene tube to a 1 mL syringe mounted on an infusion pump (NE 1000, New Era Pump System, Inc.). PTZ solution (5 $mg \cdot mL^{-1}$) was infused at a constant rate of 1 $mL \cdot min^{-1}$. Infusion was halted at 1 min or when forelimb clonus followed by full clonus of the body was observed, whichever occurred first. The animals were anesthetized by increasing carbon dioxide concentrations and killed by cervical displacement. The PTZ-induced seizure threshold ($mg \cdot kg^{-1}$) was determined according to the following formula: [infusion duration (min) $*$ infusion rate ($mL \cdot min^{-1}$) $*$ PTZ concentration ($mg \cdot mL^{-1}$) $*$ 1000] / [weight of mouse (g)].

Table 1
Seizure paradigm groups: To assess the PTZ-induced seizure threshold in seizure paradigm 280 mice were randomized into 35 categories. Ten groups of mice were randomly assigned to produce scientific and reliable data regarding levosimendan effects on PTZ-induced seizures dose response and time course. In order to delineate the role of K_{ATP} channel and nitrergic system on the anticonvulsant properties of levosimendan mice were randomly assigned to the 25 groups. K_{ATP}: adenosine triphosphate dependent potassium channels, PTZ: pentylenetetrazole, L-NAME: Nω-nitro-L-arginine methyl ester hydrochloride, L-ARG: L-arginine, 7-NI: 7-nitroindazole, AG: aminoguanidine.

Group numbers	Aim	Drug 1 (dose, administered time before PTZ injection)	Drug 2 (dose; administered time before PTZ injection)
1–7	Dose-response relationship	–	Levosimendan (0.0, 0.1, 0.2, 0.5, 1, 2, 5 $mg\cdot kg^{-1}$; 90 min)
8–10	Time course	–	Levosimendan (2 $mg\cdot kg^{-1}$; 30, 60, 120 min)
11–14	Assess the role of K_{ATP} channels on the role of Levosimendan on seizures	1. Glibenclamide (0.5 $mg\cdot kg^{-1}$; 120 min)	–
		2. Glibenclamide (1 $mg\cdot kg^{-1}$; 120 min)	–
		3. Glibenclamide (0.5 $mg\cdot kg^{-1}$; 120 min)	3. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
		4. Glibenclamide (1 $mg\cdot kg^{-1}$; 120 min)	4. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
15–18	Assess the role of K_{ATP} channels on the role of Levosimendan on seizures	1. Cromakalim (0.5 $\mu g\cdot kg^{-1}$; 120 min)	–
		2. Cromakalim (1 $\mu g\cdot kg^{-1}$; 120 min)	–
		3. Cromakalim (0.5 $\mu g\cdot kg^{-1}$; 120 min)	3. Levosimendan (0.2 $mg\cdot kg^{-1}$; 90 min)
		2. Cromakalim (1.0 $\mu g\cdot kg^{-1}$; 120 min)	4. Levosimendan (0.2 $mg\cdot kg^{-1}$; 90 min)
19–22	Assess the role of nitric oxide on the role of Levosimendan on seizures	1. L-NAME (1 $mg\cdot kg^{-1}$; 135 min)	–
		2. L-NAME (5 $mg\cdot kg^{-1}$; 135 min)	–
		3. L-NAME (1 $mg\cdot kg^{-1}$; 135 min)	3. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
		4. L-NAME (5 $mg\cdot kg^{-1}$; 135 min)	4. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
23–26	Assess the role of nitric oxide on the role of Levosimendan on seizures	1. L-ARG (30.0 $mg\cdot kg^{-1}$; 135 min)	–
		2. L-ARG (60.0 $mg\cdot kg^{-1}$; 135 min)	–
		3. L-ARG (30.0 $mg\cdot kg^{-1}$; 135 min)	3. Levosimendan (0.2 $mg\cdot kg^{-1}$; 90 min)
		4. L-ARG (60.0 $mg\cdot kg^{-1}$; 135 min)	4. Levosimendan (0.2 $mg\cdot kg^{-1}$; 90 min)
27–30	Assess the role of nNOS/NO on the role of Levosimendan on seizures	1. 7-NI (15 $mg\cdot kg^{-1}$; 135 min)	–
		2. 7-NI (30 $mg\cdot kg^{-1}$; 135 min)	–
		3. 7-NI (15 $mg\cdot kg^{-1}$; 135 min)	3. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
		4. 7-NI (30 $mg\cdot kg^{-1}$; 135 min)	4. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
21–34	Assess the role of iNOS/NO on the role of Levosimendan on seizures	1. AG (30 $mg\cdot kg^{-1}$; 135 min)	–
		2. AG (100 $mg\cdot kg^{-1}$; 135 min)	–
		3. AG (30 $mg\cdot kg^{-1}$; 135 min)	3. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
		4. AG (100 $mg\cdot kg^{-1}$; 135 min)	4. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)
35	The interplay between nitrergic pathway and K_{ATP} channels on the role of Levosimendan on seizures	1. L-NAME (1.0 $mg\cdot kg^{-1}$; 135 min) + glibenclamide (0.5 $mg\cdot kg^{-1}$; 120 min)	1. Levosimendan (2 $mg\cdot kg^{-1}$; 90 min)

2.5. Spectrophotometry

The NO content in brain tissue was measured in the form of nitrite, a major stable product of NO using the Griess reagent, as previously described [28]. Briefly, brain homogenates were centrifuged and aliquots of supernatants reacted with the same volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H3PO4; [Sigma, St. Louis, MO, USA]); each experimental mouse hippocampus was lysed separately. Nitrite concentrations were quantified spectrophotometrically at 540 nm using a standard curve plotted for known concentrations of sodium nitrite. Each experimental group consisted of 6–8 samples.

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.) in each experimental group. One-way ANOVA followed by Tukey's post hoc multiple comparisons were used to analyze the data where appropriate. In all experimental groups, differences were considered statistically significant in case the probability of type I error (p value) was <0.05.

3. Results

3.1. Levosimendan affects PTZ-induced clonic seizure threshold

Our preliminary experiments revealed levosimendan dose-dependent anticonvulsant effects on PTZ-induced seizures (Fig. 1.a). Levosimendan demonstrated maximal *anti*-convulsive effects at the dose of 2 $mg\cdot kg^{-1}$ ($p < 0.001$ in comparison to the saline-treated control group). Besides, based on our data, levosimendan exerts its maximal effects on PTZ-induced seizure threshold within 90 min of administration ($p < 0.001$ in comparison to the saline-treated control group) (Fig. 1.b).

3.2. The role of K_{ATP} channels in modulation of the activity of levosimendan on seizure threshold

In order to examine the role of K_{ATP} channels in modulation of the activity of levosimendan on seizure threshold, the mice were treated with glibenclamide (0.5 and 1 $mg\cdot kg^{-1}$; a K_{ATP} channel blocker), independently and 30 min before administration of levosimendan (2 $mg\cdot kg^{-1}$). Determination of PTZ-induced seizure threshold 120 min after independent injection of glibenclamide revealed no significant decrease in seizure threshold in comparison to the saline-treated control group. However, pre-treatment with glibenclamide (1 $mg\cdot kg^{-1}$) 30 min before administration of levosimendan (2 $mg\cdot kg^{-1}$) and 120 min before determination of PTZ-induced seizure threshold reversed seizure threshold down to the saline-treated control group (Fig. 2).

Additionally, the mice were treated with Cromakalim (0.5 and 1 $\mu g\cdot kg^{-1}$; a K_{ATP} channel opener), independently and 30 min before administration of levosimendan (0.2 $mg\cdot kg^{-1}$). As shown in Fig. 3, independent administration of Cromakalim (0.5 and 1 $\mu g\cdot kg^{-1}$) 120 min before determination of PTZ-induced seizure threshold did not change the seizure threshold in comparison to the saline-treated control group. However, pre-treatment with Cromakalim (0.5 and 1 $\mu g\cdot kg^{-1}$) 30 min before administration of sub-effective dose of levosimendan (0.2 $mg\cdot kg^{-1}$) and 120 min before determination of

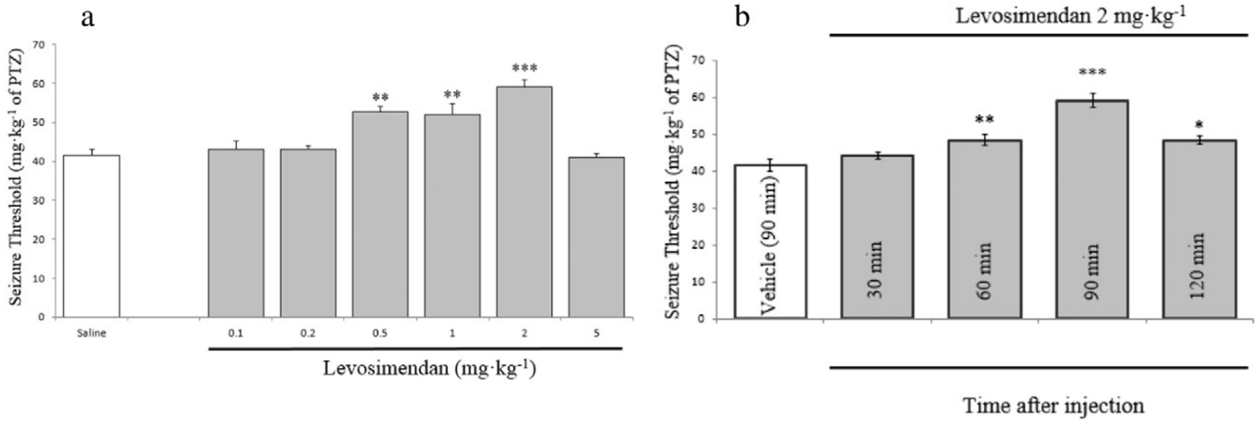

Fig. 1. a. Effects of different doses of levosimendan (0.1, 0.2, 0.5, 1, 2, and 5 mg·kg^{-1}) on PTZ-induced seizure threshold in mice. Levosimendan was administered 90 min before determination of PTZ-induced seizure threshold. Data are expressed as the mean ± SEM of seizure threshold in each group. Each group consisted of 6–8 mice. **$P < 0.01$, ***$P < 0.001$, compared with the saline-treated control group. b. Time course effects of levosimendan (2 mg·kg^{-1}) on PTZ-induced seizure threshold in mice. Levosimendan was administered 30, 60, 90, or 120 min before determination of PTZ-induced seizure threshold, and its anticonvulsant effects were compared with the vehicle-treated control group (90 min before PTZ-induced seizure). Data are expressed as mean ± SEM of seizure threshold in each group. Each group consisted of 6–8 mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in comparison to the vehicle-treated control group.

PTZ-induced seizure threshold significantly increased the anticonvulsant effects of sub-effective dose of levosimendan (0.2 mg·kg^{-1}) in comparison to the saline-treated control group ($p < 0.01$ for 0.5 μg·kg^{-1} of Cromakalim and $p < 0.001$ for 1 μg·kg^{-1} of Cromakalim).

3.3. nNOS/NO pathway mediates the anticonvulsant effects of levosimendan

In order to examine the role of nitrergic pathway in the anticonvulsant effects of levosimendan, nitrite levels were measured in brain homogenates, frontal, temporal, parietal and occipital cortices, the hippocampus and the cerebellum of the mice treated with levosimendan (2 mg·kg^{-1}) (Table 2). Our data revealed that nitrite levels were significantly higher in the hippocampus ($p < 0.001$) and temporal cortex ($p < 0.001$) of the mice treated with levosimendan (2 mg·kg^{-1}) in comparison to the corresponding saline-treated control groups.

Moreover, independent administration of L-NAME (1 and 5 mg·kg^{-1}; a non-selective NOS inhibitor) 135 min before determination of PTZ-induced seizure threshold showed no significant decrease in seizure threshold in comparison to the saline-treated control group. However, pre-treatment with L-NAME (5 mg·kg^{-1}) 45 min before administration of levosimendan (2 mg·kg^{-1}) and 135 min before determination of PTZ-induced seizure threshold reversed seizure threshold down to the saline-treated control group (Fig. 4). Also, independent administration of L-NAME (5 mg·kg^{-1}) showed no significant change in nitrite levels of hippocampus and temporal cortex of the mice in comparison to the corresponding saline-treated control groups. However, pre-treatment with L-NAME (5 mg·kg^{-1}) 45 min before administration of levosimendan (2 mg·kg^{-1}) reversed the increase of nitrite levels in

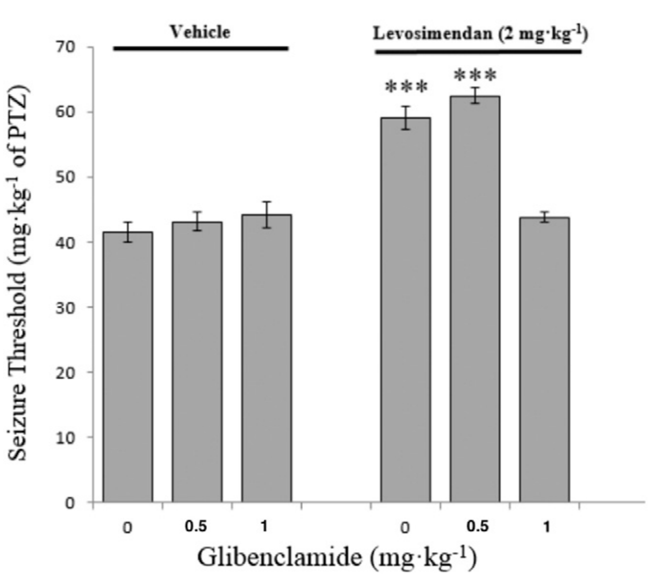

Fig. 2. Graded doses of glibenclamide (0.5 and 1 mg·kg^{-1}; a K_{ATP} channel blocker), which independently did not alter PTZ-induced seizure threshold, decreased anticonvulsant effects of levosimendan (2 mg·kg^{-1}) down to the saline-treated control group. Glibenclamide was administered 30 min before i.p. injection of levosimendan and 120 min before determination of PTZ-induced seizure threshold. Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. ***$P < 0.001$, in comparison to the saline-treated control group.

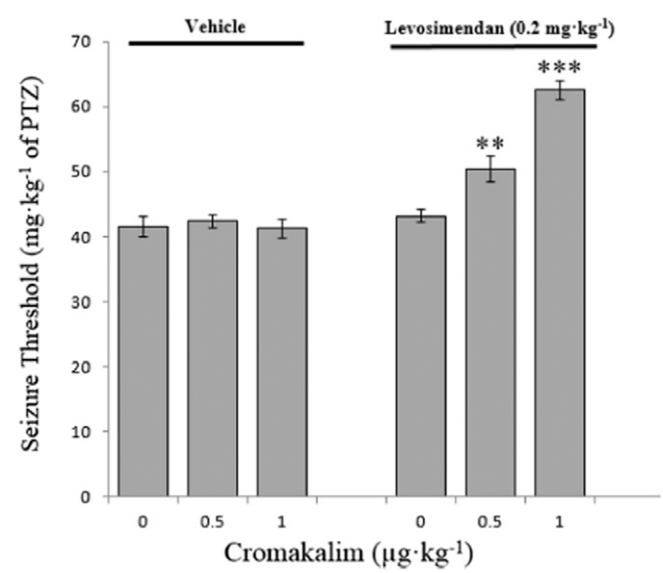

Fig. 3. Graded doses of Cromakalim (0.5 and 1 μg·kg^{-1}; a K_{ATP} channel opener), which independently did not alter PTZ-induced seizure threshold, potentiated anticonvulsant effects of sub-effective dose of levosimendan (0.2 mg·kg^{-1}) in comparison to the saline-treated control group. Cromakalim was administered 30 min before i.p. injection of levosimendan and 120 min before determination of PTZ-induced seizure threshold. Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. **$P < 0.01$, ***$P < 0.001$, in comparison to the saline-treated control group.

the hippocampus and temporal cortex of the mice to the corresponding saline-treated control groups (Table 3).

Furthermore, independent administration of L-Arg (30 and 60 mg·kg^{-1}; NO precursor) 135 min before determination of PTZ-induced seizure threshold did not change seizure threshold in comparison to the saline-treated control group. However, pre-treatment with L-Arg (30 and 60 mg·kg^{-1}) 45 min before administration of sub-effective dose of levosimendan (0.5 mg·kg^{-1}) and 135 min before determination of PTZ-induced seizure threshold significantly potentiated anticonvulsant effects of sub-effective dose of levosimendan (0.5 mg·kg^{-1}) in comparison to the saline-treated control group ($p < 0.05$ for 30 mg·kg^{-1} of L-Arg and $p < 0.001$ for 60 mg·kg^{-1} of L-Arg) (Fig. 5).

To distinguish the nitrergic pathway responsible for the anticonvulsant effects of levosimendan, the mice were treated with 7-NI (15 and 30 mg·kg^{-1}; a selective nNOS inhibitor) and AG (30 and 100 mg·kg^{-1}; an iNOS inhibitor), alone and 45 min before administration of levosimendan (2 mg·kg^{-1}). Administration of 7-NI and AG alone 135 min before determination of PTZ-induced seizure threshold did not change the seizure threshold in comparison to the saline-treated control group (Figs. 6 and 7, respectively). Pre-treatment with 7-NI (30 mg·kg^{-1}) 45 min before administration of levosimendan (2 mg·kg^{-1}) and 135 min before determination of PTZ-induced seizure threshold abolished the anticonvulsant effects of levosimendan to the saline-treated control group (Fig. 6). However, pre-treatment with AG (30 and 100 mg·kg^{-1}) 45 min before administration of levosimendan (2 mg·kg^{-1}) and 135 min before determination of PTZ-induced seizure threshold did not alter the anticonvulsant effects of levosimendan (Fig. 7). Therefore, it seems the nNOS/NO pathway to be responsible for the anticonvulsant effects of levosimendan.

3.4. The interplay between nitrergic pathway and K_{ATP} channels mediates the anticonvulsant effects of levosimendan

As illustrated in Fig. 8, independent administration of sub-effective doses of L-NAME (1 mg·kg^{-1}; a non-selective NOS inhibitor) and glibenclamide (0.5 mg·kg^{-1};a K_{ATP} channel blocker) 135 min and 120 min, respectively, before determination of PTZ-induced seizure threshold did not affect seizure threshold in the mice in comparison to the saline-treated control group. Neither pre-treatment with sub-effective dose of L-NAME (1 mg·kg^{-1}) 45 min before administration of levosimendan (2 mg·kg^{-1}) and 135 min before determination of PTZ-induced seizure threshold nor pre-treatment with sub-effective dose of glibenclamide (0.5 mg·kg^{-1}) 30 min before administration of levosimendan (2 mg·kg^{-1}) and 120 min before determination of PTZ-induced seizure threshold could reduce anticonvulsant effects of levosimendan. However, co-administration of sub-effective doses of L-NAME (1 mg·kg^{-1}) and glibenclamide (0.5 mg·kg^{-1}) 45 min and 30 min before administration of levosimendan(2 mg·kg^{-1}), respectively and 135 min and 120 min before determination of PTZ-induced seizure threshold respectively, decreased seizure threshold down to the saline-treated control group. L-NAME and glibenclamide biological interaction in diminishing the anticonvulsant effects of levosimendan can indicate the interplay between nitrergic pathway and K_{ATP} channels in mediating the anticonvulsant effects of levosimendan.

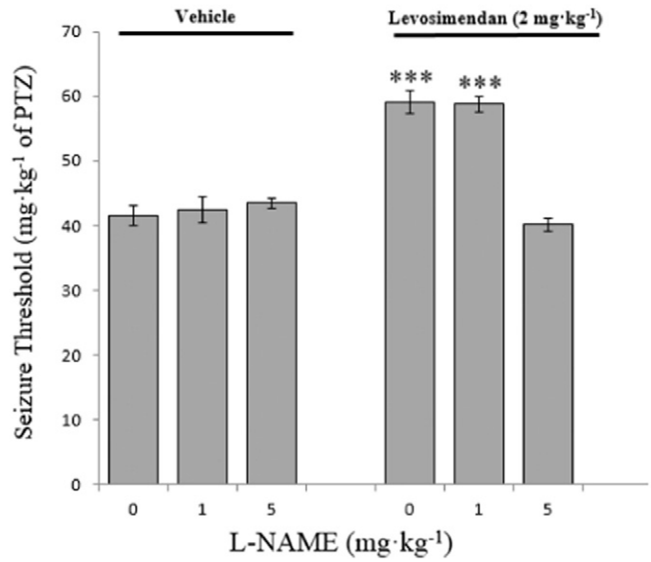

Fig. 4. Graded doses of L-NAME (1 and 5 mg·kg^{-1}; a non-selective NOS inhibitor), which independently did not alter PTZ-induced seizure threshold, decreased anticonvulsant effects of levosimendan (2 mg·kg^{-1}) down to the saline-treated control group. L-NAME was administered 45 min before i.p. injection of levosimendan and 135 min before determination of PTZ-induced seizure threshold. Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. ***$P < 0.001$, in comparison to the saline-treated control group.

In order to determine the underlying interaction between nitrergic pathway and K_{ATP} channels, resulting in anticonvulsant effects of levosimendan, nitrite levels were measured in the hippocampus and temporal cortex of the mice after having been treated with glibenclamide (1 mg·kg^{-1}; a K_{ATP} channel blocker), independently and 30 min before administration of levosimendan (2 mg·kg^{-1}). Nitrite assay showed no significant change in nitrite levels in the hippocampus and temporal cortex of the mice treated with glibenclamide (1 mg·kg^{-1}) alone in comparison to the corresponding saline-treated control groups. However, pre-treatment of the mice with glibenclamide (1 mg·kg^{-1}) 30 min before administration of levosimendan caused nitrite levels in the hippocampus and temporal cortex of the mice to decrease down to the corresponding saline-treated control groups (Table 3).

4. Discussion

The main results of this study are two-fold. First, levosimendan has a dose- and time-dependent anticonvulsant effects on PTZ-induced clonic seizures. Second, levosimendan exerts its anticonvulsant effects on PTZ-induced clonic seizures through activation of K_{ATP} channels, which

Table 2
Nitrite levels (nM·mg^{-1}) in brain homogenates, frontal, temporal, parietal and occipital cortices, the hippocampus and the cerebellum of the mice treated with saline and levosimendan (2 mg·kg^{-1}). Nitrite levels were significantly higher in the hippocampus and temporal cortex of the mice treated with levosimendan (2 mg·kg^{-1}) in comparison to nitrite levels in the corresponding brain regions of the saline-treated control group. Each group consisted of 6–8 mice.

	Cerebral cortex						
	Frontal	Temporal	Parietal	Occipital	Hippocampus	Cerebellum	Brain homogenates
Saline	44.116 ± 1.993 (nM·mg^{-1})	75.018 ± 2.649 (nM·mg^{-1})	45.889 ± 2.386 (nM·mg^{-1})	68.339 ± 1.749 (nM·mg^{-1})	56.497 ± 1.694 (nM·mg^{-1})	33.229 ± 1.387 (nM·mg^{-1})	42.497 ± 2.486 (nM·mg^{-1})
Levosimendan (2 mg·kg^{-1})	43.489 ± 2.497 (nM·mg^{-1})	98.486 ± 1.296 (nM·mg^{-1})[a]	41.396 ± 3.958 (nM·mg^{-1})	63.622 ± 2.795 (nM·mg^{-1})	73.497 ± 1.738 (nM·mg^{-1})[b]	35.259 ± 3.628 (nM·mg^{-1})	54.397 ± 1.694 (nM·mg^{-1})[c]

[a] $P < 0.001$ in comparison to nitrite levels in the temporal cortex of the saline-treated control group.
[b] $P < 0.001$ in comparison to nitrite levels in the hippocampus of the saline-treated control group.
[c] $P < 0.01$ in comparison to nitrite levels in brain homogenates of the saline-treated control group.

Table 3
Nitrite levels (nM·mg^{-1}) in the temporal cortex and hippocampus of the mice treated with saline, levosimendan (2 mg·kg^{-1}), L-NAME (5 mg·kg^{-1}; a non-selective NO inhibitor), glibenclamide (1 mg·kg^{-1}; a K_{ATP} channel blocker), L-NAME (5 mg·kg^{-1}) and levosimendan (2 mg·kg^{-1}), glibenclamide (1 mg·kg^{-1}) and levosimendan (2 mg·kg^{-1}). Independent administration of L-NAME (5 mg·kg^{-1}) and glibenclamide (1 mg·kg^{-1}) did not change nitrite levels in the temporal cortex and hippocampus of the mice in comparison to nitrite levels in the corresponding brain regions of the saline-treated control group. However, pre-treatment with L-NAME (5 mg·kg^{-1}) 45 min before administration of levosimendan (2 mg·kg^{-1}) and also pre-treatment with glibenclamide (1 mg·kg^{-1}) 30 min before administration of levosimendan (2 mg·kg^{-1}) reversed nitrite levels of the temporal cortex and hippocampus of the mice down to the nitrite levels in the corresponding brain regions of the saline-treated control group. Each group consisted of 6–8 mice.

	Saline	Levosimendan (2 mg·kg^{-1})	L-NAME (5 mg·kg^{-1})	Glibenclamide (1 mg·kg^{-1})	L-NAME (5 mg·kg^{-1}) + Levosimendan (2 mg·kg^{-1})	Glibenclamide (1 mg·kg^{-1}) + Levosimendan (2 mg·kg^{-1})
Temporal cortex	75.018 ± 2.649 (nM·mg^{-1})	98.486 ± 1.296 (nM·mg^{-1})[a]	73.592 ± 1.285 (nM·mg^{-1})	71.385 ± 2.958 (nM·mg^{-1})	77.012 ± 0.947 (nM·mg^{-1})	78.379 ± 3.226 (nM·mg^{-1})
Hippocampus	56.497 ± 1.694 (nM·mg^{-1})	73.497 ± 1.738 (nM·mg^{-1})[b]	58.147 ± 3.519 (nM·mg^{-1})	56.594 ± 1.481 (nM·mg^{-1})	59.883 ± 0.439 (nM·mg^{-1})	52.297 ± 2.559 (nM·mg^{-1})

[a] $P < 0.001$ in comparison to nitrite levels in the temporal cortex of the saline-treated control group.
[b] $P < 0.001$ in comparison to nitrite levels in the hippocampus of the saline-treated control group.

further activates nNOS/NO pathway most likely in the hippocampus and temporal cortex.

PTZ-induced clonic seizure threshold used in this study is a well-established paradigm to assess forebrain regulated seizures susceptibility, representing an animal model of clonic seizures in humans. PTZ Binding to the $GABA_A$-gated chloride ionophore results in dis-inhibition of GABAergic transmission and consequent general dis-inhibitory state [29–32]. The dose- and time-dependent effects of levosimendan in increasing this threshold implies potential clinical benefit and is in accordance with suggestions that levosimendan may act as a modulator in some pathways in the brain [33]. The acute systemic effects of levosimendan at comparable doses have been reported to be neuro-protective effects against various neuronal injury including global transient ischaemia/hypoxia, reperfusion injury and traumatic brain injury [34–36]. The mechanisms of reported neuro-protective effects of levosimendan still elude us and require further investigations.

There are several studies reporting levosimendan as a K_{ATP} opener, especially in cardiovascular system such as cardiac myocytes [10,37], isolated mesenteric artery myocytes [38], coronary arteries [39,40] and human portal and saphenous veins [41,42].

In line with these findings, there are mountains of evidence suggesting the important role of K_{ATP} channels in the modulation of neural excitability and consequently seizure threshold in several in vivo and in vitro studies. K_{ATP} channel openers have been shown to decrease neurons excitability in hippocampus [16,18] and have anti-epileptic effects in drug induced epilepsy [43]. In this regards, knockout studies have revealed that Kir6.2 (a subunit of K_{ATP} channels)-deficient mice are more prone to generalized seizures after brief hypoxia [44]. Moreover, it has been reported that lacks in expression of either the SUR1 (a subunit of K_{ATP} channels) or the Kir6.1 (a subunit of K_{ATP} channels) results in more susceptibility to kainic acid-induced seizures in mice [18]. On the other hand, over-expression of SUR1 subunit of K_{ATP} channels in the forebrain of mice can reduce the susceptibility to kainic acid-induced seizures [45]. It has been suggested that expression of functional Kir6.1/SUR1 channels can inhibit neuronal hyper-excitability possibly by limiting excitatory release of glutamate in CA3 of hippocampus [46]. Indeed, there are some reports suggesting that activation of K_{ATP} channels through K_{ATP} channel openers such as Cromakalim could exert anticonvulsant properties in the PTZ-induced seizures, while blocking K_{ATP} channels by glibenclamide could exert pro-convulsant properties [19,20].

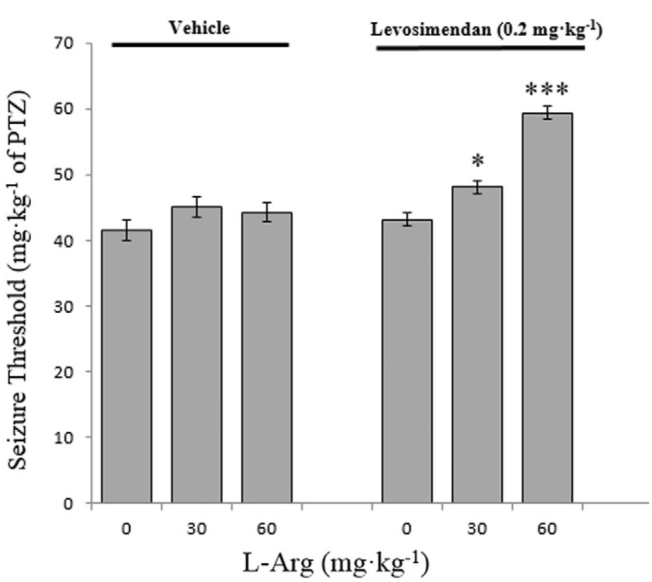

Fig. 5. Graded doses of L-Arg (30 and 60 mg·kg^{-1}; NO precursor), which independently did not alter PTZ-induced seizure threshold, potentiated anticonvulsant effects of sub-effective dose of levosimendan (0.2 mg·kg^{-1}) in comparison to the saline-treated control group. L-Arg was administered 45 min before i.p. injection of levosimendan and 135 min before determination of PTZ-induced seizure threshold. Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. *$P < 0.05$, ***$P < 0.001$, in comparison to the saline-treated control group.

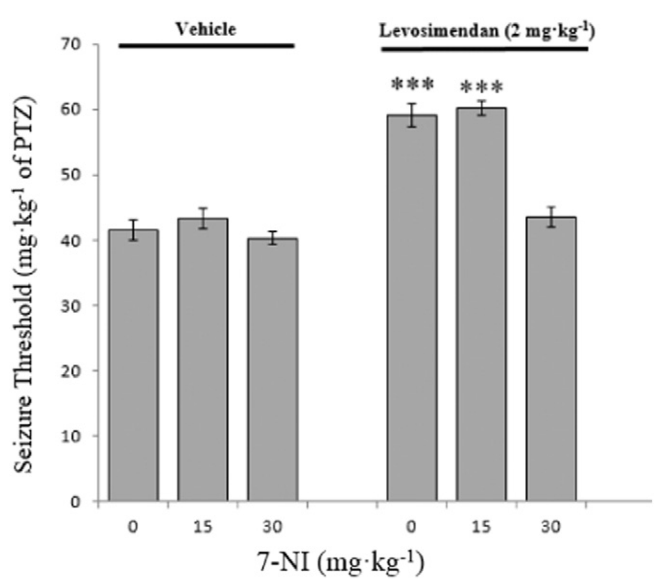

Fig. 6. Graded doses of 7-NI (15 and 30 mg·kg^{-1}; a selective nNOS inhibitor), which independently did not alter PTZ-induced seizure threshold, reversed anticonvulsant effects of levosimendan (2 mg·kg^{-1}) to the saline-treated control group. 7-NI was administered 45 min before i.p. injection of levosimendan and 135 min before determination of PTZ-induced seizure threshold. Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. ***$P < 0.001$, in comparison to the saline-treated control group.

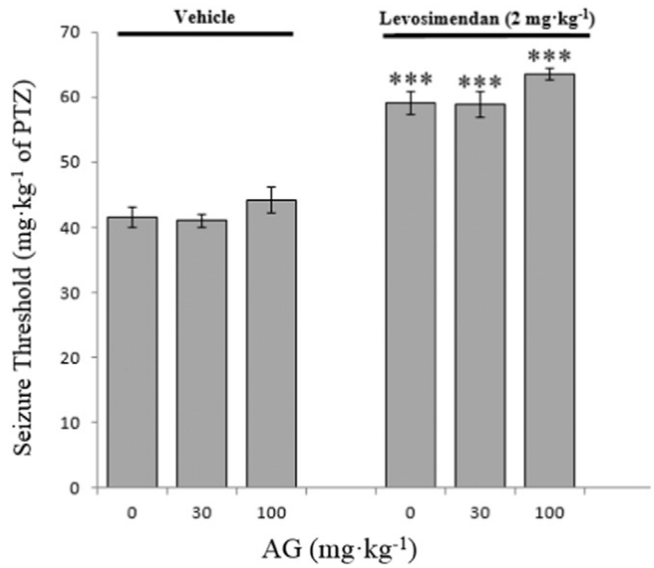

Fig. 7. Independent administration of graded doses of AG (30 and 100 mg·kg^{-1}; a selective iNOS inhibitor) did not alter PTZ-induced seizure threshold in comparison to the saline-treated control group. Also, Pre-treatment with different doses of AG (30 and 100 mg·kg^{-1}) 45 min before i.p. injection of levosimendan and 135 min before determination of PTZ-induced seizure threshold could not alter anticonvulsant effects of levosimendan (2 mg·kg^{-1}). Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. ***$P < 0.001$, in comparison to the saline-treated control group.

Altogether our results indicate levosimendan to have anticonvulsant effects through activation of K_{ATP} channels; its anticonvulsant effects being abolished by glibenclamide (K_{ATP} inhibitor) down to the control group as well as its sub-effective dose being potentiated by Cromakalim (K_{ATP} opener) to a significant rise in seizure threshold in comparison to the control group.

There are several lines of evidence suggesting the modulatory role of nitrergic system in regulation of seizure threshold. The direction of its effect is perpendicular to NO production source and region, other neurotransmitters involved, and seizure types or models [22–26]. There are some reports indicating activation of nitrergic system either by pharmacological agents or pathological conditions could exert anticonvulsant properties in PTZ-induced seizures [24,47]. Conversely, there are ample evidence highlighting NO over-production caused by activation of NOS enzymes or administration of maximal doses of NO precursor L-Arg could exert epileptogenic effects in seizure paradigms induced by GABA inhibition, such as PTZ-induced seizures [22,48].

To further elucidate the downstream events involved in the anticonvulsant effects of levosimendan, in the first step we investigated the possible involvement of nitrergic pathway by measuring nitrite levels in the brain homogenates of the mice and in specific regions including cortical regions (frontal, parietal, temporal and occipital), the hippocampus, and the cerebellum following i.p. injection of levosimendan. Our data indicated significant increased levels of nitrite in temporal cortical region and the hippocampus of the mice, which received a single dose of levosimendan in comparison to control. There are constant endeavors to clearly determine the role of brain structures in the generation of GCS. In a recent study, it has been demonstrated that the NO levels in all brain regions especially temporal cortex contribute to PTZ-induced seizures threshold [49].

Furthermore, we demonstrated that anticonvulsant effects of levosimendan could be completely reversed down to seizure threshold of control group by the means of L-NAME, or 7-NI pretreatments, whereas AG did not alter the seizure threshold significantly. Moreover, L-arginine pre-treatment augments the sub-effective dose of levosimendan to significantly increase the seizure threshold. Indeed, nitrite assays in temporal cortical region and the hippocampus of the mice revealed that nitrite level increase followed by anticonvulsant dose of levosimendan is abolished by pre-treatment with L-NAME, or 7-NI. On the other hand, administration of L-Arg before a sub-effective dose of levosimendan evoked a significant rise in nitrite levels.

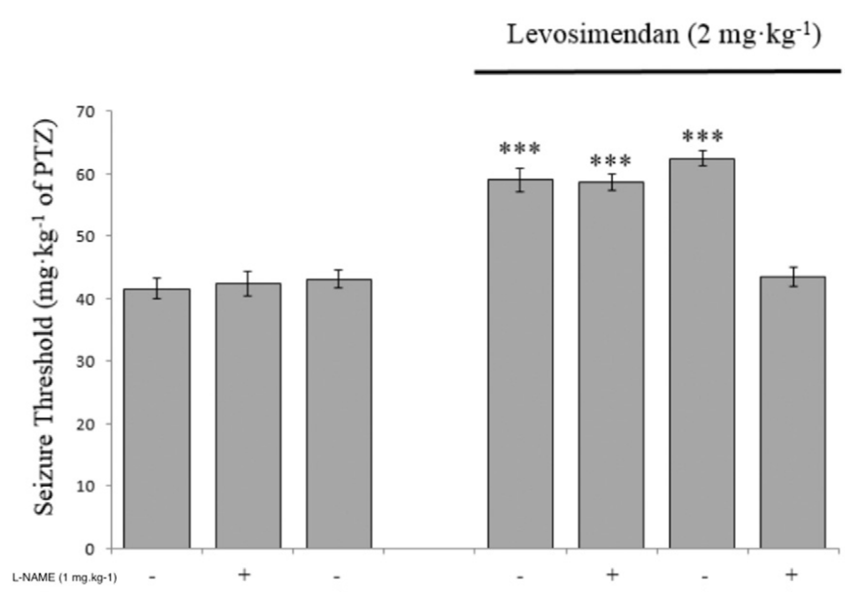

Fig. 8. Independent administration of sub-effective doses of L-NAME (1 mg·kg^{-1}; a non-selective NOS inhibitor) and glibenclamide (0.5 mg·kg^{-1}; a K_{ATP} channel blocker) 45 min and 30 min respectively before determination of PTZ-induced seizure threshold did not alter seizure threshold in comparison to the saline-treated control group. Pre-treatment with sub-effective doses of L-NAME (1 mg·kg^{-1}) or glibenclamide (0.5 mg·kg^{-1}) 45 min and 30 min before i.p. injection of levosimendan (2 mg·kg^{-1}) respectively and 135 min and 120 min respectively min before determination of PTZ-induced seizure threshold respectively did not alter anticonvulsant effects of levosimendan. However, co-administration of sub-effective doses of L-NAME (1 mg·kg^{-1}) and glibenclamide (0.5 mg·kg^{-1}) 54 min and 30 min respectively before i.p. injection of levosimendan (2 mg·kg^{-1}) and 135 min and 120 min respectively before determination of PTZ-induced seizure threshold reversed anticonvulsant effects of levosimendan down to the saline-treated control group. Each group consisted of 6–8 mice. Data are expressed as the mean ± SEM seizure threshold in each group. ***$P < 0.001$, in comparison to the saline-treated control group.

Finally, our results demonstrated that sub-effective doses of these compounds neither changed the seizure threshold, nor the anticonvulsant effects of levosimendan; however, their co-administration blocked the anticonvulsant effects of levosimendan. These results may imply on a biological interaction between K_{ATP} channels and nitrergic pathway can modulate the anticonvulsant properties of levosimendan.

Some of previous studies claim that NO is a downstream product of signaling pathways secondary to K_{ATP} channels [50,51], while others propose that NO can induce hyperpolarization of mitochondrial or cell membrane through opening the K_{ATP} channels [52,53]. Nitrite assay reveals that administration of glibenclamide (a K_{ATP} channel antagonist) before levosimendan prevented the significant rise of nitrite levels in temporal cortex and hippocampus. Hence, our results support the idea that activation of K_{ATP} channels by levosimendan may stimulate nitrergic pathway.

Summing up, our findings for the first time elucidated the role of levosimendan in modulation of seizure threshold as a calcium sensitizer drug. We revealed the novel modulatory role of K_{ATP}/nNOS/NO activation in the hippocampus and temporal cortex in exertion of the anticonvulsant effects of levosimendan. Although the present findings need further investigation by experimental studies on other models of seizures and epilepsies, these findings add an important insight into the mechanism of neuroprotective effects of levosimendan and shall pave the way for innovative strategies in management of patients inflicted by comorbidity of seizures and heart failures (it has been suggested some of people have treatment resistant seizures, could have undiagnosed heart disease [54]; on the other hand heart failure can be a common etiology of sudden death after seizures [55] or drug toxicities, such as CCBs. It also remains an open question, whether K_{ATP} channels openers and levosimendan in specific, are appropriate choices for treatment of epilepsy or can be an adjunctive therapy alongside other antiepileptic drugs.

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