Seizure 19 (2010) 102–108

Contents lists available at ScienceDirect

Seizure

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

Endothelial nitric oxide synthase activity involves in the protective effect of ascorbic acid against penicillin-induced epileptiform activity

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ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form 18 November 2009
Accepted 17 December 2009

Keywords:
Ascorbic acid
ECoG
Experimental epilepsy
L-Arginine
L-NAME
7-NI

ABSTRACT

Ascorbic acid and nitric oxide are known to play important roles in epilepsy. The aim of present study was to identify the involvement of nitric oxide (NO) in the anticonvulsant effects of ascorbic acid on penicillin-induced epileptiform activity in rats. Intracortical injection of penicillin (500, International Units (IU)) into the left sensorimotor cortex induced epileptiform activity within 2–5 min. Thirty minutes after penicillin injection, nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg), neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI, 40 mg/kg), NO substrate, L-arginine (500 mg/kg) were administered with the most effective dose of ascorbic acid (100 mg/kg) intraperitoneally (i.p.). The administration of L-arginine significantly decreased the frequency of epileptiform activity while administration of L-NAME did not influence the mean frequency of epileptiform activity. Injection of 7-NI decreased the mean frequency of epileptiform activity but did not influence amplitude. Ascorbic acid decreased both the mean frequency and amplitude of penicillin-induced epileptiform activity in rats. The application of L-NAME partially and temporarily reversed the anticonvulsant effects of ascorbic acid. The results support the hypothesis of neuro-protective role for NO and ascorbic acid. The protective effect of ascorbic acid against epileptiform activity was partially and temporarily reversed by nonspecific nitric oxide synthase inhibitor L-NAME, but not selective neuronal nitric oxide synthase inhibitor 7-NI, indicating that ascorbic acid needs endothelial-NOS/NO route to decrease penicillin-induced epileptiform activity.

1. Introduction

Epilepsy is a common chronic neurological disorder characterized by recurrent spontaneous seizures that is caused by episodic abnormal electrical activity in the brain.[1] Most epileptic seizures are due to discharges generated in cortical and hippocampal structures, although subcortical structures are also involved in some seizure types.[2] It has been proposed that active oxygen free radicals participate in the mechanisms of epileptic discharges.[3,4] Further support for a role of free radicals in seizures, comes from the successful use of exogenously administered antioxidants in protecting the brain against seizure-induced brain damage.[5,6] Moreover, Arzimanoglou et al.[7] suggested that anticonvulsant treatment of epilepsy has been related to neuro-protection, since it aims to reduce the duration or totally suppress seizures. Ascorbic acid is effective antioxidant and it has been shown that ascorbic acid exert both anticonvulsant and proconvulsant effects in different models of experimental seizures.[8–11] Ascorbate, at the high dose, produced either no effect, or an opposing effect on these behaviors constitutes further evidence for a biphasic effect of ascorbate on central nervous system (CNS) functions.[12,13] In animals, ascorbic acid was found to be effective against ferrous chloride seizures, pentylenetetrazol (PTZ)-induced seizures, and penicillin-induced seizures.[8,9,11] Wilson et al.[14] also recorded that systemic administration of ascorbic acid may change neuronal firing rates during single unit recording.

On the other hand, it has been suggested that nitric oxide plays a role in variety of physiological processes in the brain.[15,16] NO is an atypical regulatory molecule, which acts both as a second messenger and as a neurotransmitter.[17] NO has unique property as it can diffuse through cell membranes without using transporters.[18] NO is synthesized from L-arginine by activation of nitric oxide synthase (NOS) to produce NO through the formation of citrulline.[19] Several studies support the possibility that NO in the CNS is involved in the pathogenesis of epilepsy.[19–22] Although several reports have suggested the involvement of NO in various models of epilepsy[21,23–25] by using various NOS inhibitors and NO donors, the results were inconsistent. While some researchers demonstrate that NO may be an endogenous anticonvulsant,[20,21,26–29] and the others suggest a proconvulsant role for NO.[30–32] In addition, it was reported that N^G-nitro-L-arginine methyl ester (L-NAME), NOS inhibitor, is able to reduce the

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doi:10.1016/j.seizure.2009.12.005

protective activity of some conventional antiepileptics.[33] Homayoun et al.[34] also reported that pretreatment with L-NAME (1 and 10 mg/kg, 4 days) dose-dependently inhibited both anticonvulsant and proconvulsant effects of morphine. 7-NI is a selective neuronal nitric oxide synthase (nNOS) inhibitor. Although many reports have supported an anticonvulsant role for 7-NI in different models of experimental epilepsy,[31,35–37] some studies have demonstrated proconvulsant effects of 7-NI.[28,29,38] The effect of 7-NI on epilepsy are unclear and controversial. However, there are no published data available about on the role of NO pathway in the anticonvulsant effects of ascorbic acid on penicillin-induced epileptiform ECoG activity in rats. Thus, we decided for the first time, to investigate the possible involvement of NO in the anticonvulsant effect of ascorbic acid on penicillin-induced epileptiform activity in the rat by using nitric oxide synthase inhibitors, L-NAME and 7-nitroindazole as well as NO substrates, L-arginine.

2. Methods

2.1. Animals

Ninety-one male Wistar rats weighing 225–275 g were used. They were maintained on a 12-h light/dark cycle, with free access to tap water and standart laboratory food. All experimental protocols were performed in accordance with governmental approval according to local guidelines for the care and use of laboratory animals. Each animal group was composed of seven rats. Rats were assigned to the following experiments and groups:

(1) Artificial cerebrospinal fluid (2.5 μl, intracortical (i.c.)) (aCSF, containing (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; HEPES, 10; pH 7.4 when saturated with 95% O_2 and 5% CO_2);
(2) 100 mg/kg ascorbic acid (i.p.);
(3) 500 international units (IU) penicillin (2.5 μl, i.c.) + physiological saline (i.p.);
(4) 500 IU penicillin (2.5 μl, i.c.) + 100 mg/kg L-NAME (i.p.);
(5) 500 IU penicillin (2.5 μl, i.c.) + 40 mg/kg 7-nitroindazole (i.p.);
(6) 500 IU penicillin (2.5 μl, i.c.) + 500 mg/kg L-arginine (i.p.);
(7) 500 IU penicillin (2.5 μl, i.c.) + 500 mg/kg D-arginine (i.p.);
(8) 500 IU penicillin (2.5 μl, i.c.) + 100 mg/kg ascorbic acid (i.p.);
(9) 500 IU penicillin (2.5 μl, i.c.) + 100 mg/kg L-NAME (i.p.) + 100 mg/kg ascorbic acid (i.p.);
(10) 500 IU penicillin (2.5 μl, i.c.) + 100 mg/kg D-NAME (i.p.) + 100 mg/kg ascorbic acid (i.p.);
(11) 500 IU penicillin (2.5 μl, i.c.) + 40 mg/kg 7-nitroindazole (i.p.) + 100 mg/kg ascorbic acid (i.p.);
(12) 500 IU penicillin (2.5 μl, i.c.) + 500 mg/kg L-arginine (i.p.) + 100 mg/kg ascorbic acid (i.p.);
(13) 500 IU penicillin (2.5 μl, i.c.) + 500 mg/kg L-arginine (i.p.) + 40 mg/kg 7-nitroindazole (i.p.) + 100 mg/kg ascorbic acid (i.p.).

2.2. Induction of epileptiform activity

The animals were anesthetized with urethane (1.25 g kg^{-1}, i.p.). The left cerebral cortex was carefully exposed by craniotomy. After incision of the skull, the head of the animal was placed in a stereotaxic apparatus (Harvard Instruments, South Natick, MA, USA). Four different corners of the scalp were stitched by surgical threads and stretched in order to form a liquid vaseline pool (37 °C). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system (Homeothermic Blanket Control Unit, Harvard Apparatus, MA, USA). A polyethylene cannula was introduced into the right femoral artery to monitor blood pressure, which was kept above 100 mmHg during the experiments (mean 115 ± 5 mmHg) by drop infusion of dextran 40 (rheomacrodex) via femoral vein. All contact and incision points were infiltrated with procaine hydrochloride to minimize possible sources of pain.

The epileptic focus was produced by 500 international units penicillin G potassium injection (acute experimental model of focal epilepsy; 1 mm beneath the brain surface by a Hamilton microsyringe type 701RN; infusion rate 0.5 μl/min).[39,40] Penicillin was prepared in sterile apyrogen distilled water and administered intracortically in a volume of 2.5 μl into the left sensorimotor cortex.

2.3. Drug and drug administration

L-Ascorbic acid, L-arginine, D-arginine, L-NAME, 7-NI and D-NAME, urethane (Sigma-Aldrich Co. USA), dimethylsulfoxide (Merck, Germany), Penicillin G potassium (I.E. Ulagay, Turkey) were used in the experiments. All solutions were prepared freshly before experiments. L-Arginine, D-arginine, L-NAME and D-NAME were dissolved in sterile physiological saline solution to such concentrations that requisite doses were administrated intraperitoneally in a volume of 5 ml/kg. 7-NI was dissolved initially in dimethylsulfoxide to which was added sterile physiological saline (final solution DMSO/saline 3:7, v/v, respectively) and administered intraperitoneally in a volume of 10 ml/kg body weight. Ascorbic acid, in a dose of 100 mg/kg, was dissolved in sterile physiological saline solution and administered (i.p.) 30 min after penicillin (i.c.) application.

The coordinates used for i.c. injection, with the bregma point as the reference, were AP = − 2 mm, L = 3 mm. In the first set of experiments, an effective dose of ascorbic acid, L-arginine, D-arginine, L-NAME and 7-NI was intraperitoneally administered 30 min after penicillin (i.c.) application. In the second set of experiments, animals received the effective dose of L-arginine, L-NAME, D-NAME, 7-NI and L-arginine + 7-NI 10 min before ascorbic acid administration.[11,21,33,35,41]

2.4. Electrocorticographical (ECoG) recordings

ECoG recordings were made in urethane anesthesized animals. Two Ag–AgCl ball electrodes were placed over the left neocortex (electrode coordinates: first electrode; 2 mm lateral to sagittal suture and 1 mm anterior to bregma; (primary motor cortex), second electrode; 2 mm lateral to sagittal suture 5 mm posterior to bregma (secondary visual cortex mediomedial area). These recording electrodes were stabilized on the cortex surface by two different electrode holders. The common reference electrode was fixed on the right pinna. The ECoG activity was continuously monitored using a four-channel data acquisition system (PowerLab, 4/SP, AD Instruments, Australia). All recordings were stored on a computer. The frequency and amplitude of epileptiform activity was analyzed off line. Spikes were automatically detected and counted using amplitude threshold detector and counting module of Chart software (AD Instruments, Australia) with a variety of filter options available for EEG signals. It was counted only the number of spikes with amplitudes greater than three-fold baseline activity. Spike amplitude was measured automatically as the voltage change from peak to peak.

2.5. Statistical analysis

All statistical procedures were performed using SPSS (12.0, SPSS Inc., USA) statistical software package. Statistical analyses were

carried out by one-way analysis of variance (ANOVA), followed by post hoc Tamhane test to correct for multiple comparisons of treatments. Data are expressed as the means ± SEM. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of nonspecific nitric oxide synthase inhibitor, L-NAME, selective neuronal nitric oxide synthase inhibitor, 7-NI and NO substrate, L-arginine on penicillin-induced epileptiform activity

Baseline activities of each animal were recorded before the administration of intracortical penicillin (Fig. 1A). Intracortical injection of penicillin (500 IU) induced an epileptiform ECoG activity characterized by bilateral spikes or spike-wave complexes (Fig. 1B). This ECoG activity began 2–5 min after penicillin application and lasted for 3–5 h. It reached a constant level as to frequency and amplitude in maximally 30 min. The mean spike frequency and amplitude of ECoG activity in the control group were 28 ± 2 spike/min, 1005 ± 159 μV in the 90 min after physiological saline injection (i.p.), respectively. Administration of L-NAME (100 mg/kg, i.p.) 30 min after penicillin injection did not influence either the frequency or amplitude of epileptiform ECoG activity (Figs. 1C and 2). The mean frequency and amplitude of epileptiform ECoG activity was 29 ± 2 spike/min, 998 ± 213 μV in the 90 min after L-NAME (100 mg/kg) injection, respectively. Administration of 7-NI (40 mg/kg, i.p.) significantly decreased the mean frequency of epileptiform ECoG activity but did not influence amplitude. The mean frequency and amplitude of epileptiform ECoG activity was 11 ± 2 spike/min, 865 ± 90 μV in the 90 min after 7-NI (40 mg/kg) injection, respectively (Figs. 1D and 2). The frequency of epileptiform ECoG activity was decreased to 15 ± 2 spike/min in the 90 min after L-arginine (500 mg/kg) administration (Figs. 1E and 2). The significant effects appeared 120 min after L-arginine administration and lasted for 60 min (Fig. 2). The mean amplitude of epileptiform ECoG activity did not change after L-arginine administration. The administration of D-arginine (500 mg/kg, i.p.), which is not a substrate for NO production, 30 min after penicillin injection did not influence either the frequency or amplitude of epileptiform ECoG activity compared with penicillin injected group (Figs. 1F and 2).

3.2. Effects of nonspecific nitric oxide synthase inhibitor, L-NAME, selective neuronal nitric oxide synthase inhibitor, 7-NI and NO substrate, L-arginine on anticonvulsant activity of ascorbic acid in penicillin-induced epileptiform activity

The dose of 100 mg/kg ascorbic acid was administered 30 min after penicillin injection. Ascorbic acid, in a dose of 100 mg/kg, significantly decreased both the mean frequency and amplitude of epileptiform ECoG activity to 8 ± 1, spike/min, 556 ± 110 μV in the

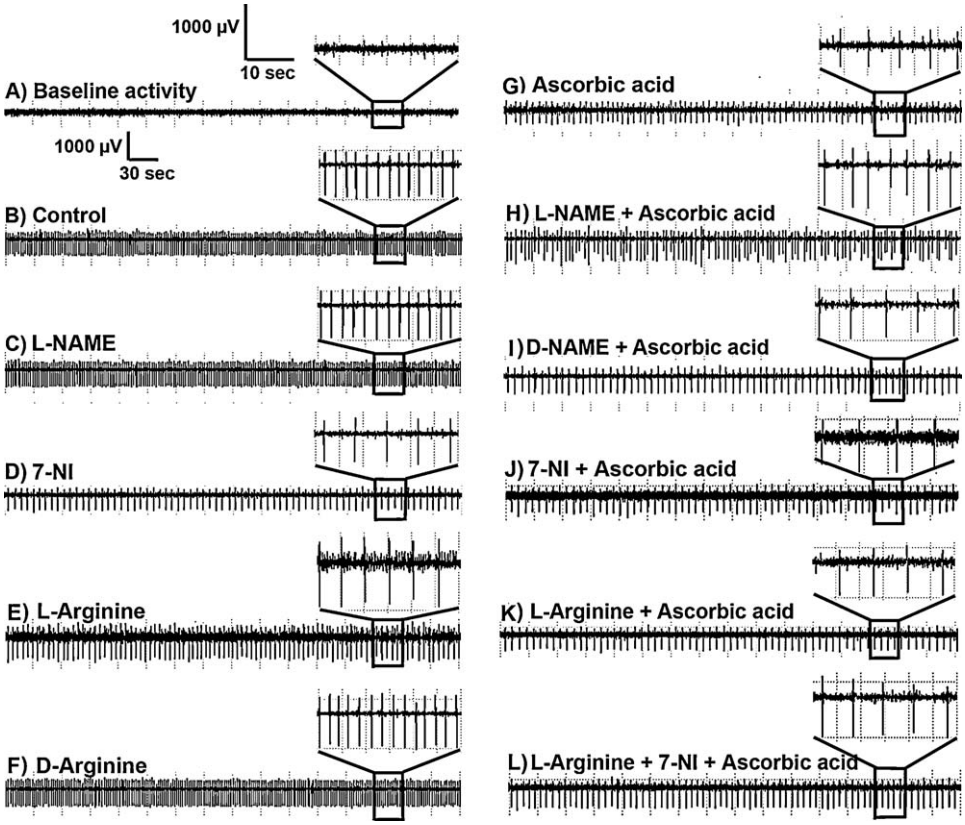

Fig. 1. (A) Baseline ECoG activity before penicillin or the injection of other substances. (B) Intracortical injection of penicillin (500 IU) induced epileptiform activity on ECoG. (C) Nitric oxide synthase inhibitor, L-NAME (100 mg/kg, i.p.) did not influence both of the mean frequency or amplitude of penicillin-induced epileptiform ECoG activity. (D) Specific neuronal nitric oxide synthase inhibitor, 7-NI (40 mg/kg, i.p.) decreased the frequency of penicillin-induced epileptiform activity. (E) Precursor of nitric oxide, L-arginine (500 mg/kg, i.p.) decreased the mean frequency of penicillin-induced epileptiform ECoG activity without changing amplitude. (F) The inactive enantiomer D-arginine (500 mg/kg, i.p.) did not influence both of the mean frequency or amplitude of penicillin-induced epileptiform ECoG activity. (G) Ascorbic acid, in a dose of 100 mg/kg (i.p.), decreased both the frequency and amplitude of penicillin-induced epileptiform activity. (H) The administration of L-NAME (100 mg/kg, i.p.) 10 min before ascorbic acid (100 mg/kg, i.p.) injection partially and temporarily reversed the anticonvulsant activity of ascorbic acid (I) The administration of D-NAME (100 mg/kg, i.p.), inactive antipode on NOS, 10 min before ascorbic acid (100 mg/kg) injection failed to reverse the anticonvulsant activity of ascorbic acid. (J) The administration of 7-NI (40 mg/kg, i.p.) 10 min before ascorbic acid (100 mg/kg, i.p.) injection did not change the anticonvulsant activity of ascorbic acid. (K) The administration of L-arginine (500 mg/kg, i.p.) 10 min before ascorbic acid (100 mg/kg, i.p.) injection caused an earlier anticonvulsant activity. (L) The administration of L-arginine (500 mg/kg, i.p.) 10 min before 7-NI (40 mg/kg, i.p.) injection did not change the anticonvulsant activity of both 7-NI (40 mg/kg, i.p.) and ascorbic acid (100 mg/kg, i.p.). Representative ECoGs are presented for the 90 min after the administration of above mentioned substances.

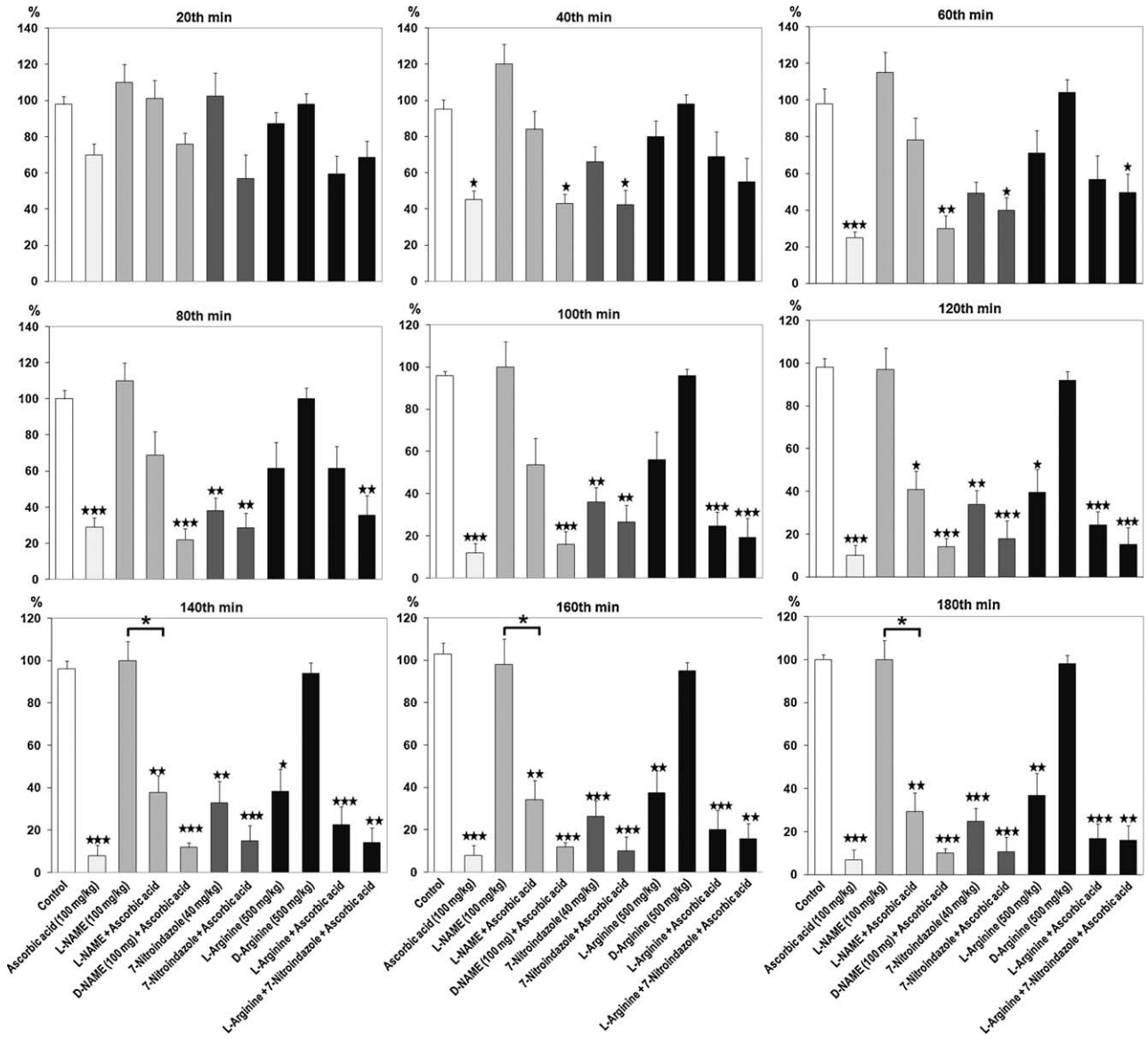

Fig. 2. The effects of nonspecific nitric oxide synthase inhibitor, L-NAME (100 mg/kg, i.p.), selective neuronal nitric oxide synthase inhibitor, 7-NI (40 mg/kg, i.p.) as well as NO substrate, L-arginine (500 mg/kg, i.p.) and its inactive enantiomer D-arginine (500 mg/kg, i.p.) on the mean spike frequency of penicillin-induced epileptiform ECoG activity in rat. L-NAME (100 mg/kg, i.p.) and D-arginine (500 mg/kg, i.p.) did not influence the mean frequency of penicillin-induced epileptiform ECoG activity. 7-NI (40 mg/kg, i.p.) decreased the frequency of penicillin-induced epileptiform activity. The frequency of epileptiform ECoG activity was decreased in the 120 min after L-arginine (500 mg/kg, i.p.) administration. The administration of L-NAME (100 mg/kg, i.p.) 10 min before ascorbic acid injection partially and temporarily reversed the anticonvulsant activity of ascorbic acid. The same dose of its inactive enantiomer D-NAME failed to reverse the anticonvulsant activity of ascorbic acid. The earlier effect was seen in the L-arginine (500 mg/kg, i.p.) + ascorbic acid (100 mg/kg, i.p.) administered group compared with L-arginine (500 mg/kg, i.p.) administered group alone. The administration of 7-NI (40 mg/kg, i.p.), did not change the anticonvulsant activity of ascorbic acid. The administration of L-arginine (500 mg/kg, i.p.) 10 min before 7-NI failed to change the anticonvulsant activity of both 7-NI (40 mg/kg) and ascorbic acid (100 mg/kg, i.p.). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (when compared with control group). The percentage frequency of epileptiform ECoG activity value depends on both the frequency of epileptiform ECoG activity before and after the substance administered as it is defined as:Frequency value $\% = \frac{\text{The mean of spike frequency after substance administered}}{\text{The mean of spike frequency before substance administered}} \times 100$

90 min after ascorbic acid injection (i.p.), respectively (Figs. 1G and 2). The significant effects appeared 40 min after 100 mg/kg ascorbic acid injection and lasted for 140 min (Fig. 2). The administration of L-NAME (100 mg/kg, i.p.) 10 min before ascorbic acid injection partially and temporarily reversed the anticonvulsant activity of ascorbic acid whereas the inactive enantiomer D-NAME (100 mg/kg, i.p.) failed to affect the anticonvulsant activity of ascorbic acid (Figs. 1H, I and 2). The frequency of epileptiform ECoG activity became significantly higher in the 20 min after L-NAME administration compared with ascorbic acid administered group (Fig. 2). However, the reversal effects of L-NAME disappeared in the 120 min after L-NAME injection. L-NAME did not influence the amplitude of epileptiform ECoG activity during experiments (Fig. 1H). The mean frequency and amplitude of epileptiform ECoG activity were 18 ± 2, 7 ± 1 spike/min, 754 ± 192, 589 ± 144 μV in the L-NAME and D-NAME groups in the 90 min after injection, respectively (Fig. 1H and I). Administration of 7-NI (40 mg/kg, i.p.) did not affect the frequency and amplitude of epileptiform ECoG activity compared with ascorbic acid administered group. The mean frequency and amplitude of epileptiform ECoG activity were 8 ± 1 spike/min, 482 ± 170, respectively in the 7-NI + ascorbic acid group (Figs. 1J and 2). A dose of 500 mg/kg L-arginine (i.p.) was administered 10 min before ascorbic acid (100 mg/kg, i.p.) injection. The significant effects appeared 90 min after L-arginine injection and lasted for 90 min in the L-arginine + ascorbic acid group (Fig. 2).

Earlier anticonvulsant effect was seen in the L-arginine + ascorbic acid group compared to L-arginine group alone (Fig. 2). The mean frequency and amplitude of epileptiform ECoG activity were 10 ± 2 spike/min and 944 ± 220 μV in the 90 min after L-arginine administration, respectively (Fig. 1K). The administration of L-arginine (500 mg/kg, i.p.) 10 min before 7-NI (40 mg/kg, i.p.) did not affect the frequency and amplitude of epileptiform ECoG activity compared ascorbic acid and 7-NI administered groups. The mean frequency and amplitude of epileptiform ECoG activity were 10 ± 2 spike/min, 569 ± 206, respectively (Figs. 1L and 2). Intraperitoneal injection of 100 mg/kg ascorbic acid did not cause any change in the frequency or amplitude of ECoG activity with respect to control base line in non-penicillin injected animals. There was also no change in the mean of frequency and amplitude in aCSF injected animals.

4. Discussion

A widely used method for inducing epileptiform activity in rats is application of penicillin to the cerebral cortex.[42] Application of penicillin to the neocortex results in synchronous discharge of neurones, which bears an electrophysiological resemblance to human focal interictal epileptic discharges.[43] Interictal epileptiform discharges can occur in various forms, such as spikes, polyspikes and sharp waves and are believed to be the result of summated membrane events from abnormally hypersynchronous neurons within epileptic tissue.[44] Therefore, we used penicillin-induced epilepsy model to determine the role of nitric oxide in the anticonvulsant effects of ascorbic acid in rats in the present study.

It has been suggested that ascorbic acid has neuro-protective properties in some experimental epilepsy models such as iron,[8] pentylenetetrazol[9] and penicillin-induced.[11] Oliveira et al.[9] reported that ascorbate, at a high dose (300 mg/kg), protected against PTZ-induced convulsions whereas ascorbate, at a low dose (30 mg/kg) did not alter PTZ-induced convulsions. They also indicated that ascorbate, at an intermediate dose (100 mg/kg), potentiated the duration of convulsive episodes.[9] In our previous study, we clearly showed that ascorbic acid, at doses of 50, 100, 200 and 400 mg/kg, was effective in decreasing the frequency of penicillin-induced epileptiform activity whereas ascorbic acid, at doses of 25 and 800 mg/kg, did not alter the mean frequency of penicillin-induced epileptiform activity in rats.[11] Ascorbic acid, at a dose of 100 mg/kg, was the most effective dose in changing the frequency and amplitude of penicillin-induced epileptiform activity.[11] Therefore we used a dose of 100 mg/kg ascorbic acid to have maximum anticonvulsant effect or neuro-protective activity in this study. It was reported that ascorbate significantly attenuated trimethyltin-induced seizures as well as the initial oxidative stress, impaired glutathione homeostasis.[45] Moreover, Oliveira et al.[9] reported that the effects of ascorbate are complex, and other mechanisms, unrelated to its reactive species scavenger ability, are claimed to explain the neuro-protective actions of ascorbic acid. Finally, the mechanism of ascorbic acid action still remains to be determined.

On the other hand, the functional involvement of NO in epilepsy has been demonstrated by many researchers although data often are contradictory.[19–22,46,47] It has been reported that NO can act as an anticonvulsant or a proconvulsant depending on the seizure stimulus, the cellular form of NO and activation of specific NOS isoforms.[34,48] Akula et al.[36] reported that L-NAME (2.5 mg/kg, i.p.) potentiated the anticonvulsant action of sub-effective dose of adenosine (50 mg/kg, i.p.) in PTZ-induced seizure in mice. Conversely, in another study it was suggested that L-NAME (1 mg/kg) reversed the anticonvulsant property of the combination of melatonin (10 mg/kg) plus morphine (0.5 mg/kg) in mouse model of PTZ-induced clonic seizures.[49] The inhibitors of NO synthase may produce diverse effects upon seizure susceptibility, the results of present study revealed that L-NAME (100 mg/kg) did not influence both of the frequency and amplitude of penicillin-induced epileptiform activity in rats, which is consistent with the results of other studies, concerning the effect of NO synthase inhibition upon the electroconvulsive threshold and kainate-induced toxicity.[33,50,51] On the other hand, L-NAME partially and temporarily reversed the protective activity of ascorbic acid against penicillin-induced epileptiform activity whereas the same dose of D-NAME failed to show significant effects on the protective activity of ascorbic acid in the present study. This result is consistent with other studies, concerning the inhibition of L-NAME upon protective effects of morphine, cyclosporine A, phenobarbital and alpha-tocopherol.[33,34,37,52,53]

A large number of published studies have used 7-NI, an indazole derivative and selective inhibitor of nNOS, to examine the role of nNOS in epilepsy.[36,37,54,55] Proconvulsant effect of 7-NI has been demonstrated in soman-induced convulsions in rats, where 7-NI enhanced the severity of clonic convulsions and increased lethality produced by soman.[54] On the other hand, it was reported that 7-NI inhibited both NOS activity in vivo and glufosinate-induced convulsions in mice.[37] Akula et al.[36] explained that 25 mg/kg 7-NI (i.p.) potentiated the anticonvulsant action of sub-effective dose of adenosine (50 mg/kg, i.p.) against pentylenetetrazol seizure threshold in mice. In addition, recent a study indicate that anticonvulsant effect of levetiracetam was increased when given in combination with 7-nitroindazole in an experimental model of partial complex seizures named maximal dentate gyrus activation in rats.[55] In the present study, we demonstrate that specific nNOS inhibitor 7-NI, at a dose of 40 mg/kg, significantly reduced the frequency of epileptiform activity without changing amplitude. These results are in accordance with previous study by our group that 25 and 50 mg/kg 7-NI has anticonvulsant effect on epileptiform activity in rats.[35] We also found that 40 mg/kg 7-NI injection did not chance the anticonvulsant effect of ascorbic acid.[35] On the other hand, the administration of L-arginine before 7-NI injection did not alter the anticonvulsant effect of 7-NI in the presence of ascorbic acid. The administration of L-arginine (500 mg/kg, i.p.) 10 min before 7-NI (50 mg/kg) failed to change the anticonvulsant activity of either ascorbic acid or 7-NI. These results are consistent with previously reported findings.[22,31,35,56] The mechanism of these contradictory effects of L-NAME and 7-NI on penicillin-induced epileptiform activity is still unclear. However, these apparently divergent data do not exclude either NO might play a different role in various models of epileptic disorders or it may act as an anticonvulsant or as a proconvulsant agent depending on the experimental procedures and particular brain structures involved as suggested by several reports.[19–23,46–48]

It was demonstrated that L-arginine, as a precursor of NO has an efficacious action in decreasing the susceptibility to seizure, comparable to the antiepileptic drugs, suggesting a potential involvement of NO as an anticonvulsant.[24,46] L-Arginine (500 mg/kg) decreased the frequency of epileptiform activity in penicillin-treated rats without changing amplitude whereas the administration D-arginine (500 mg/kg), inactive enantiomer, did not influence either the frequency or amplitude of epileptiform ECoG activity in the present study. The significant effects appeared in the 90 and at 120 min after L-arginine administration. The present results clearly demonstrate that NO may exert anticonvulsant effects in epileptic seizures which confirm previous studies which show that L-arginine, at high dose (500 mg/kg, i.p.), has an anticonvulsant effect in the different model of experimental epilepsy.[31,53,56] In contrast, Czuczwar et al.[57] reported that L-arginine, at a dose of 500 mg/kg, did not affect the convulsive threshold for the clonic phase of PTZ-induced seizures. Although, the earlier effect was seen in the L-arginine + ascorbic acid administered group compared with L-arginine administered group, we may conclude that L-arginine

(500 mg/kg, i.p.) did not provide an additional anticonvulsant activity for ascorbic acid in the present study. It would be logical not to expect an additional anticonvulsant activity in the L-arginine + ascorbic acid group if ascorbic acid and L-arginine use the same molecular mechanisms to affect the frequency of epileptiform activity.

According to GABA hypothesis of epilepsy, both a decrease in GABAergic inhibition and an increase in glutamatergic excitation suggested as one of the reasons for the initiation and spread of epileptic seizures.[58–60] Furthermore, Tsuda et al.[61] suggested that penicillin exerts its proconvulsant effect by inhibiting GABA-gated chloride ion influx. Previous studies revealed a link between NO and potentiation of synaptic GABA release, which has been proposed to explain the aggravation of seizure induced by NOS inhibitors in the experimental model of epilepsy.[61–63] Taken together, the results of present study are consistent with the hypothesis that the antiaconvulsant effects of ascorbic acid are, probably, due to the inhibition of GABA reuptake.[64] L-NAME, a nonspecific NOS inhibitor, partially and temporarily reversed the anticonvulsant effects of ascorbic acid whereas 7-NI, a specific neuronal NOS inhibitor, failed to influence anticonvulsant activity of ascorbic acid. The mechanism of these contradictory effects of L-NAME and 7-NI on the anticonvulsant action of ascorbic acid in penicillin-induced epileptiform activity is unclear. However, the possibility of a pharmacokinetic interaction between NO and anticonvulsant in the present study cannot be ruled out. Further studies are needed to assess possible molecular mechanisms for these findings.

In summary, we confirmed that ascorbic acid, in a dose of 100 mg/kg, decreased the mean frequency and amplitude of penicillin-induced epileptiform ECoG activity in rat. The administration of L-arginine resulted in the inhibition of epileptiform activity whereas D-arginine, inactive enantiomer, did not influence either the frequency or amplitude of epileptiform activity. Moreover, a non-effective dose of L-NAME (non-specific NOS inhibitor) partially and temporarily diminished the anticonvulsant effects of ascorbic acid. The administration of D-NAME, the inactive antipode on NOS, failed to show significant effect on the anticonvulsant activity of ascorbic acid. Specific nNOS inhibitory, 7-NI significantly decreased the frequency of epileptiform activity but did not alter the anticonvulsant effect of ascorbic acid implying that nNOS activity does not involve in protective effect of ascorbic acid against penicillin-induced epileptiform activity. Therefore, it can be concluded that either the endothelial-NOS activity participate in the anticonvulsant activity of ascorbic acid or partially and temporarily inhibitory effect of L-NAME on the anticonvulsant activity of ascorbic acid might be its own nonspecific effect, which is unrelated to endothelial-NOS activity in the brain.

References

1. Dichter MA. The epilepsies and convulsive disorders. In: Isselbacher KJ, editor. *Harrison's principles of internal medicine*. New York: McGraw-Hill; 1994. p. 2223–33.
2. Avanzini G, Franceschetti S. Cellular biology of epileptogenesis. *Lancet* 2003;**2**:33–42.
3. Braugher JM, Hall ED. Central nervous system trauma and stroke: I. Biochemical considerations for oxygen radical formation and lipid peroxidation. *Free Rad Biol Med* 1989;**6**:289–301.
4. Mori A, Hiramatsu M, Yokoi I, Edamatsu R. Biochemical pathogenesis of post-traumatic epilepsy. *Pav J Biol Sci* 1990;**25**:54–62.
5. Lapin IP, Mirzaev SM, Ryzov IV, Oxenkrug GF. Anticonvulsant activity of melatonin against seizures induced by quinolinate, kainate, glutamate, NMDA, and pentylenetetrazole in mice. *J Pineal Res* 1998;**24**:215–8.
6. Murashima YL, Kasamo K, Suzuki J. Antiepileptic effects of allopurinol on EL mice associated with changes in SOD isoenzyme activities. *Epilepsy Res* 1998;**32**:254–65.
7. Arzimanoglou A, Hirsch E, Nehlig A, Castelnau P, Gressens P, De Vasconcelos AP. Epilepsy and neuroprotection: an illustrated review. *Epileptic Disord* 2002;**3**:173–82.
8. Yamamoto N, Kabuto H, Matsumoto S, Ogawa N, Yokoi I. α-Tocopheryl-L-ascorbate-2-O-phosphate diester, a hydroxyl radical scavenger, prevents the occurrence of epileptic foci in a rat model of post-traumatic epilepsy. *Pathophysiology* 2002;**8**:205–14.
9. Oliveira MS, Furian AF, Royes LFF, Fighera MR, De Carvalho J, Fiorenza NG, et al. Ascorbate modulates pentylenetetrazol-induced convulsions biphasically. *Neuroscience* 2004;**128**:721–8.
10. Foksinski M, Gackowski D, Rozaski R, Siomek A, Guz J, Szpila A, et al. Effects of basal level of antioxidants on oxidative DNA damage in humans. *Eur J Nutr* 2007;**46**(3):174–80.
11. Ayyildiz M, Coskun S, Yildirim M, Agar E. The effect of ascorbic acid on penicillin-induced epileptiform activity in rats. *Epilepsia* 2007;**48**(7):1388–95.
12. Tolbert LC, Thomas TN, Middaugh LD, Zemp JW. Effect of ascorbic acid on neurochemical, behavioral, and physiological systems mediated by catecholamines. *Life Sci* 1979;**25**:2189–95.
13. Pierce RC, Rowlett JK, Rebec GV, Bardo MT. Ascorbate potentiates amphetamine-induced conditioned place preference and forebrain dopamine release in rats. *Brain Res* 1995;**688**:21–6.
14. Wilson RL, Kamata K, Wightman RM, Rebec GV. Unilateral, intranigral infusions of amphetamine produce differential changes in unit activity and extracellular levels of ascorbate in the neostriatum of the rat. *Brain Res* 1986;**384**:342–7.
15. Moncada S, Palmer RMJ, Higgs EA. Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol* 1989;**38**:1709–15.
16. Garthwaite J. Glutamate, nitric oxide and cell–cell signalling in the nervous system. *Trends Neurosci* 1991;**14**:60–7.
17. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 1991;**43**:109–42.
18. Kaneko K, Itoh K, Berliner LJ, Miyasaka K, Fujii H. Consequences of nitric oxide generation in epileptic-seizure rodent models as studied by in vivo EPR. *Magn Reson Med* 2002;**48**:1051–6.
19. Ferraro G, Sardo P. Nitric oxide and brain hyperexcitability. *In Vivo* 2004;**18**:357–66.
20. Buisson A, Lackhmeche N, Verrecia C, Plotkine M, Bolou RG. Nitric oxide: an endogenous anticonvulsant substance. *Neuroreport* 1993;**4**:444–6.
21. Marangoz C, Ayyildiz M, Agar E. Evidence that sodium nitroprusside possesses anticonvulsant effects mediated through nitric oxide. *Neuroreport* 1994;**5**: 2454–6.
22. De Sarro GB, Gareri P, Falconi U, De Sarro A. 7-Nitroindazole potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. *Eur J Pharmacol* 2000;**394**:275–88.
23. De Sarro GB, Donato Di Paola E, De Sarro A, Vidal MJ. Role of nitric oxide in the genesis of excitatory amino acid-induced seizures from the deep prepiriform cortex. *Fundam Clin Pharmacol* 1991;**5**:503–11.
24. Herberg LJ, Grottick A, Rose IC. Nitric oxide synthesis, epileptic seizures and kindling. *Psychopharmacol* 1995;**119**:115–23.
25. Milatovic D, Gupta RC, Dettbarn W-D. Involvement of nitric oxide in kainic acid-induced excitotoxicity in rat brain. *Brain Res* 2002;**957**:330–7.
26. Penix LP, Davis W, Subramaniam S. Inhibition of NO synthase increases the severity of kainic acid-induced seizures in rodents. *Epilepsy Res* 1994;**18**:177–84.
27. Ferraro G, Montalbano ME, La Grutta V. NO and hippocampal epilepsy: an electrophysiological evidence of a new modulatory system. *Epilepsia* 1997;**38**:190–1.
28. Noh HS, Kim DW, Cho GJ, Choi WS, Kang SS. Increased nitric oxide caused by the ketogenic diet reduces the onset time of kainic acid-induced seizures in ICR mice. *Brain Res* 2006;**1075**:193–200.
29. Royes LF, Fighera MR, Furian AF, Oliveira MS, Fiorenza NG, Petry JC, et al. The role of nitric oxide on the convulsive behavior and oxidative stress induced by methylmalonate: an electroencephalographic and neurochemical study. *Epilepsy Res* 2007;**73**(3):228–37.
30. Mulsch A, Busse R, Mordvintcev PI, Vanin AF, Nielsen EO, Scheel-Kruger J, et al. Nitric oxide promotes seizure activity in kainate-treated rats. *Neuroreport* 1994;**5**:2325–8.
31. Smith SE, Man CM, Yip PK, Tang E, Chapman AG, Meldrum BS. Anticonvulsant effects of 7-nitroindazole in rodents with reflex epilepsy may result from L-arginine accumulation or a reduction in nitric oxide or L-citrulline formation. *Br J Pharmacol* 1996;**119**:165–73.
32. Yasuda H, Fuji M, Fujisawa H, Ito H, Suzuki M. Changes in nitric oxide synthesis and epileptic activity in the contralateral hippocampus of rats following intrahippocampal kainate injection. *Epilepsia* 2001;**42**:13–20.
33. Borowicz KK, Starownik R, Kleinrok Z, Czuczwar SJ. The influence of L-N^G-nitroarginine methyl ester, an inhibitor of nitric oxide synthase, upon the anticonvulsive activity of conventional antiepileptic drugs against maximal electroshock in mice. *J Neural Transm* 1998;**105**:1–12.
34. Homayoun H, Khavandgar S, Namiranian K, Gaskari SA, Dehpour AR. The role of nitric oxide in anticonvulsant and proconvulsant effects of morphine in mice. *Epilepsy Res* 2002;**48**:33–41.
35. Bosnak M, Ayyildiz M, Yildirim M, Agar E. The role of nitric oxide in the anticonvulsant effects of pyridoxine on penicillin-induced epileptiform activity in rats. *Epilepsy Res* 2007;**76**:49–59.
36. Akula KK, Dhir A, Kulkarni SK. Nitric oxide signaling pathway in the anticonvulsant effect of adenosine against pentylenetetrazol-induced seizure threshold in mice. *Eur J Pharmacol* 2008;**587**:129–34.
37. Matsumura N, Kikuchi-Utsumi K, Nakaki T. Activities of 7-nitroindazole and 1-(2-(trifluoromethylphenyl)-imidazole independent of neuronal nitric-oxide synthase inhibition. *J Pharmacol Exp Ther* 2008;**325**:357–62.

38. Yahyavi-Firouz-Abadi N, Tahsili-Fahadan P, Riazi K, Ghahremani MH, Dehpour AR. Involvement of nitric oxide pathway in the acute anticonvulsant effect of melatonin in mice. *Epilepsy Res* 2006;**68**:103–13.
39. Wyler AR, Ward Jr AA. Epileptic neurons. In: Lockard J, Ward Jr AA, editors. *Epilepsy: a window to brain mechanisms*. New York: Raven Press; 1980 p. 51–68.
40. Ayyildiz M, Yildirim M, Agar E. The effect of vitamin E on penicillin-induced epileptiform activity in rats. *Exp Brain Res* 2006;**174**:109–13.
41. Chavko M, Auker CR, McCarron RM. Relationship between protein nitration and oxidation and development of hyperoxic seizures. *Nitric Oxide* 2003;**9**:18–23.
42. Holmes O, Wallace MN, Campbell AM. Comparison of penicillin epileptogenesis in rat somatosensory and motor cortex. *Q J Exp Physiol* 1987;**72**:439–52.
43. Prince DA. Topical convulsant drugs and metabolic antagonists. In: Purpura DP, Penry JK, Tower DB, Woodbury DM, Walter RD, editors. *Experimental models of epilepsy*. New York: Raven Press; 1972. p. 51–83.
44. Matsumoto H, Ajmonemarsan C. Cellular mechanisms in experimental epileptic seizures. *Science* 1964;**10**(144):193–4.
45. Shin EJ, Suh SK, Lim YK, Jhoo WK, Hjelle OP, Ottersen OP, et al. Ascorbate attenuates trimethyltin-induced oxidative burden and neuronal degeneration in the rat hippocampus by maintaining glutathione homeostasis. *Neuroscience* 2005;**133**:715–27.
46. Przegalinski E, Baran L, Siwanowicz J. The role of nitric oxide in chemically- and electrically-induced seziures in mice. *Neurosci Lett* 1996;**217**:145–8.
47. Kabuto H, Yokoi I, Habu H, Willmore LJ, Mori A, Ogawa N. Reduction in nitric oxide synthase activity with development of an epileptogenic focus induced by ferric chloride in the rat brain. *Epilepsy Res* 1996;**25**:65–8.
48. Itoh K, Watanabe M. Paradoxical facilitation of pentylenetetrazole-induced convulsion susceptibility in mice lacking neuronal nitric oxide synthase. *Neuroscience* 2009;**159**:735–43.
49. Yahyavi-Firouz-Abadi N, Tahsili-Fahadan P, Riazi K, Ghahremani MH, Dehpour AR. Melatonin enhances the anticonvulsant and proconvulsant effects of morphine in mice: role for nitric oxide signaling pathway. *Epilepsy Res* 2007;**75**:138–44.
50. Tutka P, Klonowski P, Dziecivch J, Kleinork Z, Czucwar SJ. N^G-nitro-L-arginine differentially affects glutamate or kainate-induced seizures. *Neuroreport* 1996;**7**:1605–8.
51. Jones PA, Smith RA, Stone TW. Nitric oxide synthase inhibitors L-NAME and 7-nitroindazole protect rat hippocampus against kainate-induced toxicity. *Neurosci Lett* 1998;**249**:75–8.
52. Homayoun H, Khavandgar S, Dehpour AR. Anticonvulsant effects of cyclosporin A on pentylenetetrazole-induced seizure and kindling: modulation by nitricoxidergic system. *Brain Res* 2002;**939**:1–10.
53. Ayyildiz M, Yildirim M, Agar E. The involvement of nitric oxide in the anticonvulsant effects of α-tocopherol on penicillin-induced epileptiform activity in rats. *Epilepsy Res* 2007;**73**:166–72.
54. Lallement G, Shih TM, Pernot-Marino I, Baubichon D, Foquin A, McDonough JH. The role of nitric oxide in soman-induced seizures, neuropathology, and lethality. *Pharmacol Biochem Behav* 1996;**54**:731–7.
55. Sardo P, D'Agostino S, Rizzo V, Carletti F, Lonobile G, Ferraro G. In the rat maximal dentate activation model of partial complex epilepsy, the anticonvulsant activity of levetiracetam is modulated by nitric oxide-active drugs. *J Neural Transm* 2009;**116**(7):831–9.
56. Borowicz KK, Kleinrok Z, Czuczwar SJ. 7-Nitroindazole, differentially affects the anticonvulsant activity of antiepileptic drugs against amygdala-kindled seizures in rats. *Epilepsia* 2000;**41**:1112–8.
57. Czuczwar SJ, Tutka P, Klonowski P, Kleinrok Z. N^G-nitro-L-arginine impairs the anticonvulsive action of ethosuximide against pentylenetetrazol. *Eur J Pharmacol* 1999;**366**:137–42.
58. Corda M, Orlandi M, Lecca D, Carboni G, Frau V, Giorgi O. Pentylenetetrazol-induced kindling in rats: effect of GABA function inhibitors. *Pharmacol Biochem Behav* 1991;**40**:329–33.
59. Löscher W. Basic aspects of epilepsy. *Curr Opin Neurol Neurosurg* 1993;**6**(2):223–32.
60. Meldrum BS. Excitatory amino acid receptors and their role in epilepsy and cerebral ischemia. *Ann N Y Acad Sci* 1995;**757**:492–505.
61. Tsuda A, Ito M, Kishi K, Shiraishi H, Tsuda H, Mori C. Effect of penicillin on GABA-gated chloride ion influx. *Neurochem Res* 1994;**19**(1):1–4.
62. Jayakumar AR, Sujatha R, Paul V, Asokan C, Govindasamy S, Jayakumar R. Role of nitric oxide on GABA, glutamic acid, activities of GABA-T and GAD in rat brain cerebral cortex. *Brain Res* 1999;**837**(1-2):229–35.
63. Li DP, Chen SR, Finnegan TF, Pan HL. Signalling pathway of nitric oxide in synaptic GABA release in the rat paraventricular nucleus. *J Physiol* 2004;**554**(Pt 1):100–10.
64. Sershen H, Debler EA, Lajtha A. Effect of ascorbic acid on the synaptosomal uptake of [3H]MPP+, [3H]dopamine, and [14C]GABA. *J Neurosci Res* 1987;**17**(3):298–301.