

The effect of combined treatment of alpha-tocopherol, ascorbic acid, and pyridoxine with NMDA blocker memantine on penicillin-induced epileptiform activity in rats

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Aim: To evaluate the effects of coadministration of vitamins alpha-tocopherol, ascorbic acid, and pyridoxine with memantine on a penicillin-induced experimental epilepsy model in rats so as to clarify the eventual interaction between these vitamins and the N-methyl-D-aspartate (NMDA) system.

Materials and methods: The epileptic focus was produced by intracortical penicillin G potassium injection. The effects of intraperitoneal injections of memantine, memantine + alpha-tocopherol, memantine + ascorbic acid, and memantine + pyridoxine combinations on epileptiform activity were evaluated in electrocorticogram recordings.

Results: The antiepileptiform effects appeared earlier in all memantine and vitamin coadministrated groups compared to the memantine-alone group, but the difference between the groups was not statistically significant considering the frequency and amplitude of the epileptiform activity.

Conclusion: Coadministration of vitamins does not enhance the antiepileptiform activity of memantine in penicillin-induced epilepsy in rats. However, this coadministration causes the earlier appearance of antiseizure effects. Since moderate doses of these vitamins have no side effects, it might be a good idea to use them with NMDA blockers to provide an earlier antiepileptic effect.

Key words: Experimental epilepsy, NMDA, memantine, ascorbic acid, pyridoxine, alpha-tocopherol

1. Introduction

Epileptogenesis is associated with an imbalance between excitatory and inhibitory control systems in selective regions of the brain. Particularly, excessive activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, manifested as neuronal excitotoxicity, has been linked to several neurological disorders including epilepsy (1). The therapeutic potential of various NMDA antagonists has been investigated in different experimental models of epilepsy (1,2), but none have proven to be both effective and safe. Memantine is a low-affinity, uncompetitive antagonist on NMDA receptors. It is approved for treatment of Alzheimer disease and now has potentially wide-ranging applications, from neuroprotection to the treatment of experimental epilepsy, without the undesirable side effects of many high-affinity NMDA receptor antagonists (3).

On the other hand, it was reported that free radicals could inactivate glutamine synthase and could induce epileptic seizures by abnormal expression of the excitatory neurotransmitter, glutamic acid (4). The biological

effects of free radicals are controlled by a wide range of antioxidants, such as ascorbic acid, alpha-tocopherol, and pyridoxine (5–7). It has also been suggested that such vitamins have neuroprotective properties in some experimental models of epilepsy (7–11). Alpha-tocopherol protects cell membranes against oxidative damage by regulating the production of reactive oxygen species (ROS) and maintaining oxidative phosphorylation in mitochondria, thus accelerating the regeneration of high energy phosphates (6), whereas ascorbic acid scavenges the aqueous ROS by very rapid electron transfer and thus inhibits lipid peroxidation (5). Ascorbate has also been suggested to decrease NMDA-mediated currents by acting at the redox modulatory site of the NMDA receptor. Pyridoxine is the active coenzyme for many enzymes, including glutamic acid decarboxylase, an enzyme that has a role in gamma-aminobutyric acid (GABA) synthesis in mammalian cells (12). GABA is a neurotransmitter with an important role in the pathophysiology and treatment of epilepsy.

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Therefore, we can say that all 3 vitamins chosen for this study are related to 1 or more antiepileptogenic pathways. However, to our knowledge, no published data are available about the effects of these vitamins on the antiepileptic potential of any drugs in experimental models of epilepsy. Consequently, the objectives of the present study were to comparatively study the effects of coadministration of memantine with ascorbic acid, pyridoxine, and alpha-tocopherol on penicillin-induced epileptiform activity in rats in order to clarify their interaction with the NMDA system.

2. Materials and methods

2.1. Subjects

Adult male Wistar rats weighing 200–250 g were used throughout this study. All described procedures were approved by the local ethics committee. The animals were housed in standard cages and were allowed free access to food and water. They were kept in a temperature-controlled (22 ± 1 °C) environment on a 12-h light/dark cycle.

2.2. Experimental groups

The rats were assigned to the following experimental groups and each group comprised 7 rats:

- (1) Penicillin (500 IU, 2.5 μ L, intracortical);
- (2) Penicillin + memantine (5 mg/kg, intraperitoneal [i.p.]);
- (3) Penicillin + memantine + pyridoxine (40 mg/kg, i.p.);
- (4) Penicillin + memantine + alpha-tocopherol (500 mg/kg, i.p.);
- (5) Penicillin + memantine + ascorbic acid (100 mg/kg, i.p.)

2.3. Surgical procedure

The animals were anesthetized with a single injection of urethane (1.25 g/kg, i.p.) and placed in a stereotaxic frame. Rectal temperature was maintained between 36–37 °C using a feedback-controlled heating system. A polyethylene cannula was introduced into the right femoral artery to monitor blood pressure, which was kept above 110 mmHg during the experiments (mean: 120 ± 5 mmHg). The left cerebral cortex was exposed by craniotomy (5 mm posterior to bregma and 3 mm lateral to sagittal sutures). Two Ag-AgCl ball electrodes were placed over the left somatomotor cortex (electrode coordinates: first electrode 2 mm lateral to sagittal suture and 1 mm anterior to bregma, second electrode 2 mm lateral to sagittal suture and 5 mm posterior to bregma). The common reference electrode was fixed on the pinna.

2.4. Drug and drug administration

The epileptic focus was produced by 500 IU of intracortical penicillin G potassium injection (short-term experimental model of focal epilepsy; 1 mm beneath the brain surface by a Hamilton microsyringe, type 701N (Aldrich, Milwaukee,

WI, USA), infusion rate 0.5 μ L/min) (9). Memantine was dissolved in distilled water and administered 30 min after the penicillin injection (5 mg/kg, i.p.). Ascorbic acid, alpha-tocopherol, or pyridoxine were administered intraperitoneally 15 min after the memantine injection.

2.5. Electrocorticography recordings

The electrocorticography (ECoG) activity was continuously monitored on a recorder (PowerLab, 4/SP, AD Instruments, Castle Hill, Australia) and was stored on a computer. The frequency and amplitude of the epileptiform ECoG activities were analyzed offline.

2.6. Data analysis

All statistical procedures were performed using SPSS 12.0. The differences between the groups were analyzed with one-way ANOVA. Significant differences were further evaluated using the Tamhane post hoc test. Data were expressed as mean \pm standard error of the mean (SEM). Statistical significance was set at $P < 0.05$.

3. Results

The epileptiform activity characterized by spikes and spike-wave complexes began 2–5 min after the penicillin injection, reached a constant level of frequency and amplitude in 30 min, and lasted for 3–5 h (Figure 1). We used the most effective doses of memantine (5 mg/kg, i.p.) (9), ascorbic acid (100 mg/kg, i.p.) (2), alpha-tocopherol (500 mg/kg, i.p.) (3), and pyridoxine (40 mg/kg, i.p.) (7) for penicillin-induced epileptiform activity, which were all determined in previous studies. Memantine significantly decreased the frequency of epileptiform activity 50 min after injection (Figure 2). The mean spike frequency of ECoG activity was 14 ± 2 spikes/min in the memantine-administrated group at 60 min after injection. Significant anticonvulsant effects were seen at 40 min, 40 min, and 30 min after memantine injection in the groups administered memantine + ascorbic acid, memantine + alpha-tocopherol, and memantine + pyridoxine, respectively (Figure 2). The mean spike frequencies of epileptiform activities were 13 ± 3 , 13 ± 3 , and 12 ± 2 spikes/min at 60 min after memantine injection in the memantine + ascorbic acid, memantine + alpha-tocopherol, and memantine + pyridoxine groups, respectively. The earliest antiepileptiform effect appeared in the memantine + pyridoxine group as compared to the other groups. No significant difference was found regarding the mean spike amplitude of epileptiform activity in any of the groups (Figure 3). The mean spike amplitudes of ECoG activities were 783 ± 50 μ V, 1014 ± 146 μ V, and 1000 ± 258 μ V in the memantine + ascorbic acid, memantine + alpha-tocopherol, and memantine + pyridoxine groups 60 min after the memantine injection, respectively.

4. Discussion

Previous experiments have shown that memantine, pyridoxine, alpha-tocopherol, and ascorbic acid have

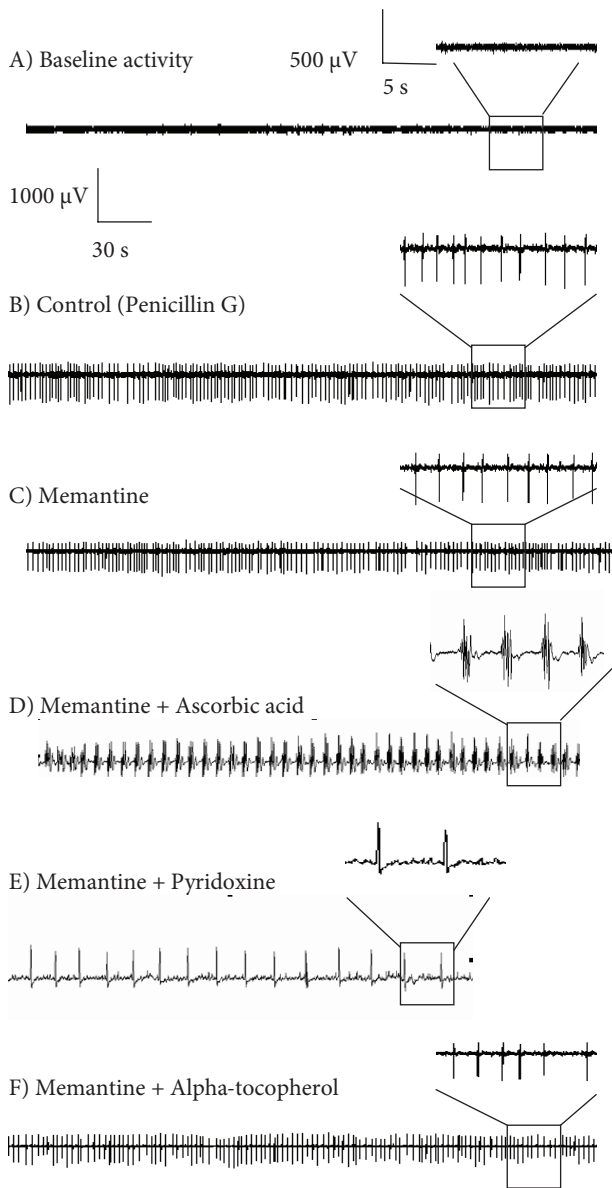


Figure 1. A) The baseline ECoG activities. B) Penicillin (500 IU)-induced epileptiform ECoG activity characterized by spikes and spike-wave complexes. ECoG activities of C) memantine, D) memantine + ascorbic acid, E) memantine + pyridoxine, and F) memantine + alpha-tocopherol at 40 min after memantine injection. Antiepileptogenic activity of memantine has not begun, but we can see decreased spike frequencies in all memantine + vitamin groups.

anticonvulsive effects on penicillin-induced epileptic activity in rats (3,8–10). In this study, we investigated for the first time the effects of coadministration of the NMDA receptor antagonist memantine with pyridoxine, alpha-tocopherol, and ascorbic acid on penicillin-induced epileptiform activity in order to clarify the eventual

relationship between the antiepileptiform effects of these vitamins and the NMDA system.

Penicillin injection to the somatomotor cortex is a widely used method for inducing epileptiform activity in rats. In the anesthetized animal, this activity is characterized by the focal interictal epileptiform discharges (13). Penicillin was reported to increase the excitability of presynaptic nerve terminals and to enhance the excitatory effects of glutamate on the brain (14).

Glutamate is the major excitatory neurotransmitter in the central nervous system. Excessive activation of the NMDA subtype of glutamate receptors causes hyperexcitation neurotoxicity, which is involved in the pathophysiology of neurological disorders including epilepsy. Memantine is an uncompetitive voltage-dependent NMDA receptor antagonist that preserves the normal function of the receptor but protects the brain from NMDA receptor-mediated excitotoxicity. Memantine has potential antiepileptic effects in some experimental models of epilepsy, such as seizures induced by picrotoxin, bicuculline, 3-mercaptopropionic acid (15), and pentylenetetrazol (16) in the absence of serious side effects, suggesting that it might have potential efficiency in the treatment of epileptic seizures. In our previous study we used 5 different doses of memantine (1, 2.5, 5, 10, and 20 mg/kg) on penicillin-induced epilepsy, and we showed that memantine decreased the mean frequency of epileptiform activity at doses of 2.5 and 5 mg/kg (i.p.), with a maximal effect at 5 mg/kg without changing the amplitude (3). Thus, in this study, we used the most effective antiepileptogenic dose of memantine (5 mg/kg) on penicillin-induced epilepsy. However, the present study and most of the research in this field indicate that memantine alone is not able to prevent epileptic activity. Therefore, it would be a good idea to use combinations of memantine with other potential antiepileptogenic agents to obtain stronger antiseizure activity. For this purpose, we chose 3 vitamins with different mechanisms of action but similar potential antiepileptogenic effects. The first vitamin, pyridoxine (vitamin B₆), is an essential nutrient for normal function of multiple organ systems and a precursor of the cofactors, pyridoxal-5-phosphate and pyridoxamine-5-phosphate, that form the active site of many essential enzymes in human tissues (10,17). Pyridoxine deficiency causes convulsions, particularly in infants and also in experimental animals (18). The antiepileptic effects of pyridoxine have also been studied in different experimental epilepsy models. Bosnak et al. (10) indicated that low-dose pyridoxine (40 mg/kg) significantly reduced penicillin-induced epileptiform activity in rats. Furthermore, the intravenous administration of pyridoxine at 50–100 mg doses dramatically attenuated the clinical and electrographic features of seizures (17). On the other hand, high doses of this vitamin were shown to cause damage

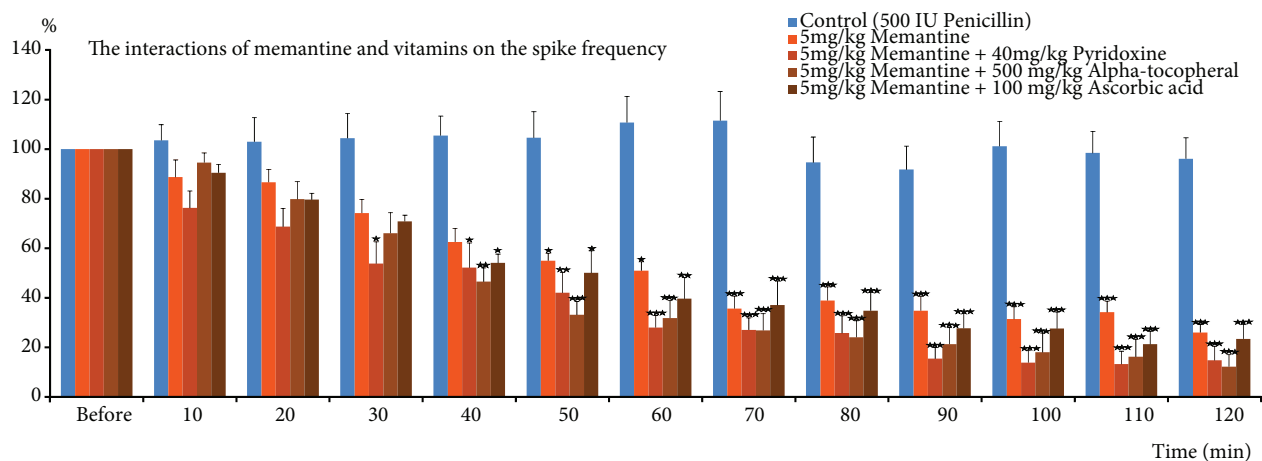


Figure 2. Effects of memantine (5 mg/kg, i.p.), memantine + ascorbic acid (100 mg/kg, i.p.), memantine + alpha-tocopherol (500 mg/kg, i.p.) and memantine + pyridoxine (40 mg/kg, i.p.) on the mean spike frequency of penicillin-induced epileptiform activity in rats. Memantine significantly decreased the frequency of epileptiform activity 50 min after injection. The significant effects of memantine + ascorbic acid, memantine + alpha-tocopherol, and memantine + pyridoxine on spike frequency were seen at 40 min, 40 min, and 30 min after memantine injection, respectively. All memantine + vitamin combinations showed earlier antiepileptic effect compared to memantine applied alone (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The percentage frequency of epileptiform ECoG activity value depends on both the frequency of epileptiform ECoG activity before and after the substance administered and is defined as: frequency value (%) = (the mean of spike frequency after substance administered / the mean of spike frequency before substance administered) \times 100.

in the cerebral cortex in long-term treatment (19). The second vitamin, alpha-tocopherol, was also shown to have preventive effects on oxidative stress in animal studies (20). Alpha-tocopherol was shown to have antiseizure activity in several animal models, including ferrous chloride (21), hyperbaric oxygen (22), kindling, pentylentetrazol (23), and penicillin (11) models. Kryzhanovskii et al. (24) showed that penicillin-induced epileptiform activity increased the lipoperoxidation product levels in the crude synaptosomal fraction from the hyperactive focus, and alpha-tocopherol abolished the effects of lipoperoxidation products and decreased the seizure frequency on ECoG recordings. It was also shown that alpha-tocopherol protects cells from glutamate-induced toxicity by direct antioxidant action in immature primary cortical neuron cultures (25). The third vitamin, ascorbic acid, is highly concentrated in the brain, and the release of ascorbic acid from brain cells is associated with the glutamatergic neuron activity by the glutamate-ascorbate heteroexchange across the neuronal or glial cell membranes (26). This heteroexchange might protect neurons by facilitating glutamate uptake in glial cell membranes. On the other hand, ascorbic acid was shown to have antioxidant properties in various experiments. It was reported to reduce lipid peroxidation induced by fenthion in rat tissues (27) and also to significantly prevent 3-nitrotyrosine, a marker of oxidant peroxy-nitrite formation in guinea pigs (28). Oliveira et al. (29) found that a high dose of ascorbic acid (300 mg/kg) protected rat brain against pentylentetrazol-induced seizures. They

also reported that at intermediate doses, it potentiated the duration of convulsive episodes. Yamamoto et al. (30) also showed that ascorbate pretreatment (200 mg/kg) 60 min before the induction of epileptic activity prevented or delayed the occurrence of epileptic discharges in the ferrous chloride model of experimental epilepsy. In penicillin-induced epilepsy, ascorbic acid decreased the frequency of the epileptiform activity at doses of 50–400 mg/kg (i.p.); moreover, the occurrence of epileptiform activity was delayed in the ascorbic acid-pretreated group (100 mg/kg, i.p.) (8).

Additionally, the combination of anticonvulsant drugs and vitamins may provide enhanced protection against several diseases (31–34). Pretreatment with ascorbic acid just prior to valproic acid (VPA) exposure prevents VPA-induced down regulation of the *Hoxa2* gene expression in concert with enhanced glutathione status, indicating a protective effect on embryos (34). Vitamin C and vitamin E were shown to enhance the catalepsy induced by nitric oxide synthase inhibitors in mice (33) and also decrease the oxidative stress produced by cell phores in rats (35). Chronic administration of ascorbic acid combined with phenytoin augments the resistance to urethane-induced loss of righting reflex in experimental animals (32). Moreover, the combination of memantine with vitamin D may enhance the protection against Alzheimer disease (31).

In agreement with previous studies, the results of the present study indicate that coadministration of memantine with pyridoxine, alpha-tocopherol, and ascorbic

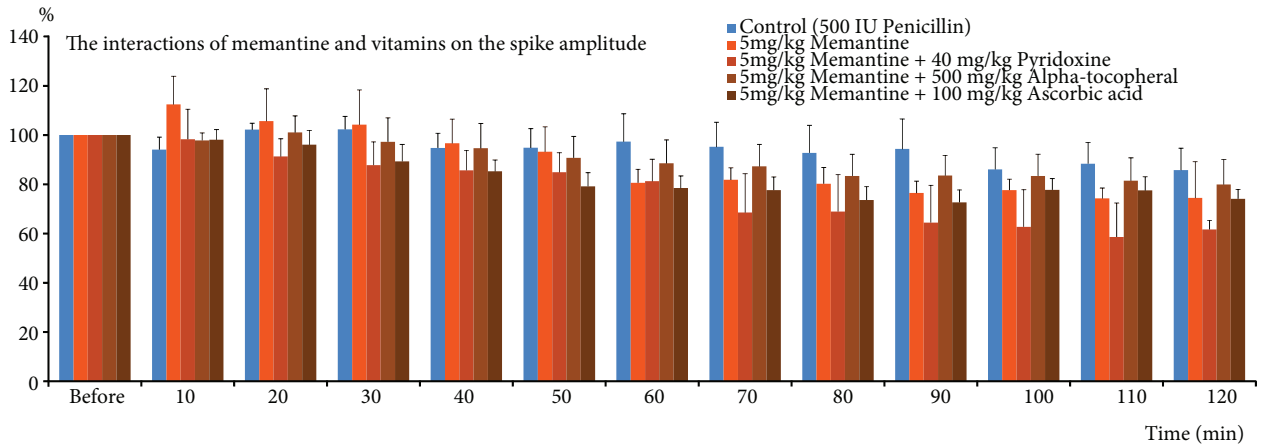


Figure 3. The effects of memantine (5 mg/kg, i.p.), memantine + ascorbic acid (100 mg/kg, i.p.), memantine + alpha-tocopherol (500 mg/kg, i.p.), and memantine + pyridoxine (40 mg/kg, i.p.) on the mean spike amplitude of penicillin-induced epileptiform activity in rats. There were no significant differences in the mean spike amplitude of any experimental groups. The percentage amplitude of epileptiform ECoG activity value depends on both the amplitude of epileptiform ECoG activity before and after the substance administered and is defined as: amplitude value (%) = (the mean of spike amplitude after substance administered / the mean of spike amplitude before substance administered) × 100.

acid significantly decreased the penicillin-induced epileptiform activity in the 30, 40, and 40 min after the memantine injection, respectively. Since the antiepileptic effects of pyridoxine, alpha-tocopherol, ascorbic acid, and memantine on penicillin-induced epilepsy appeared at 40, 60, 30, and 50 min after injection, respectively (3,8–10), it can be concluded that all memantine + vitamin combinations showed earlier antiepileptic effects compared to memantine applied alone. Furthermore, in our previous study, coadministration of memantine with alpha-tocopherol and pyridoxine, but not with ascorbic acid, showed earlier antiepileptic effects compared to the vitamins applied alone (3). The antiepileptic effect of memantine + ascorbic acid appeared earlier than it did when memantine was applied alone, but later than when ascorbic acid was applied alone. Unfortunately, we did not have the chance to compare these results with others,

since no data are available on the effects of the combined treatment of alpha-tocopherol, ascorbic acid, and pyridoxine with the NMDA blocker memantine on other experimental models of epilepsy. These results give rise to the thought that an interaction might exist between the antiepileptogenic effects of vitamins and NMDA system on penicillin-induced epilepsy.

The present study demonstrates that coadministration of memantine with effective doses of pyridoxine, ascorbic acid, and alpha-tocopherol can cause earlier antiepileptic effects on penicillin-induced epilepsy in rats compared with memantine alone. However, it is important to note that the results of the present study do not explain the pathways these drug combinations use to suppress epileptiform activity. Therefore, the eventual relationship between these vitamins and the NMDA system must be clarified with further electrophysiological investigations.

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