INTRODUCTION

Epilepsy is the most common and serious neurological disorder that affects millions of people worldwide (Moshé et al. 2015). This neurological disorder characterized by the repeated occurrence of bursts of electrical activity (seizures) in specific brain areas such as limbic system and cerebral cortex (Avazin and Franceschetti 2003). Various antiepileptic drugs (AEDs) are widely used as long-term adjunctive therapy or as monotherapy in epilepsy (Johannessen and Landmark 2010). However, approximately one-third of epileptic patients do not respond adequately to existing AEDs (Franco et al. 2013). In addition, many available AEDs cause toxicity (Lösch and Leppik 2002). Therefore, more effective and safer new therapeutics are needed.

Vitamin B\textsubscript{12} is necessary for the development and initial myelination of the central nervous system as well as for the maintenance of its normal function (Stabler 2013). Promoting neurite growth, neuroregeneration and antinoceptive properties of vitamin B\textsubscript{12} were studied in animal models (Okada et al. 2010, Hosseinzadeh et al. 2012, Erfanparast et al. 2014, Romano et al. 2014, Tamaddonfard et al. 2014). Neurological problems resulting from vitamin B\textsubscript{12} deficiency have wide spectrum, from asymptomatic to life-threatening pancytopenia or myelopathy (Stabler 2013). In addition, vitamin B\textsubscript{12} deficiency has been shown to cause epileptic seizures (Sklar et al. 1986, Kumar 2004, Erol et al. 2007). Although there are increasing evidences indicating neuroprotective effect for vitamin B\textsubscript{12} (Romano et al. 2014, Tamaddonfard et al. 2014), the antiepileptic effect of this vitamin has not been documented well.

The present study was designed to investigate the effect of focal administration of vitamin B\textsubscript{12} on penicil-
Vitamin B12 and epileptiform activity

Fig. 1. Electroencephalographic (EEG) recording samples obtained from right sensory-motor cortex. (a) Shows the baseline activity recorded at 10th min before penicillin. (b) Shows the first spike wave (arrowhead) induced by penicillin. (A) Shows the epileptiform activity induced by microinjection of penicillin after normal saline. (B, C and D) Show the effects of vitamin B₁₂ at doses of 50, 100 and 200 ng/site, respectively, on penicillin-induced epileptiform activity. (E and F) Show the effects of diazepam at doses of 50 and 200 ng/site on epileptiform activity induced by penicillin. (G) Shows the effect of flumazenil at a dose of 50 ng/site on penicillin-induced epileptiform activity. (H and I) Show the effects of combined treatments with ineffective and effective doses of vitamin B₁₂ and diazepam, respectively, on the epileptiform activity induced by penicillin. (J and K) Show the effects of flumazenil on antiepileptiform activities induced by vitamin B₁₂ and diazepam, respectively. EEG was recorded with two speeds (0.05 and 0.25 cm/s) under calibration of 100 μV/1 mm (i.e., 500 μV/5 mm).
lin-induced seizures. In addition, the contribution of GABA<sub>A</sub> benzodiazepine receptors was assessed using a GABA-benzodiazepine receptor agonist, diazepam (Middendrop et al. 2014), and a GABA-benzodiazepine receptor antagonist, flumazenil (May et al. 2013), with and without vitamin B<sub>12</sub>. It is well known that diazepam has sedative, hypnotic, anxiolytic, muscle relaxant, and anticonvulsant effects (Middendrop et al. 2014).

**METHODS**

**Animals**

Healthy adult male Wistar rats, weighing 280–320 g were used in this study. Rats were maintained in polyethylene cages with food and water available ad libitum in a laboratory with controlled ambient temperature (22±0.5°C) and under a 12 h light–dark cycle (lights on at 07:00 AM). Six rats were used in each experiment. Experiments were performed between 10:00 AM and 13:00 PM Laboratory Animal Care and Use Center of the Faculty of Veterinary Medicine of Urmia University approved the experimental protocol.

**Drugs**

Drugs used in the present study included urethane, vitamin B<sub>12</sub>, diazepam, flumazenil and penicillin G potassium. The drugs were purchased from Sigma-Aldrich Co., St Louis, MO, USA. They were dissolved in normal saline (a sterile solution of 0.9% of NaCl). A drop of Tween 80 was added to diazepam plus normal saline solution.

**Experimental groups**

Rats were divided into 11 groups with six rats in each group as follows: group 1 received intracortical microinjection of normal saline. In groups 2, 3 and 4, intracortical microinjection of vitamin B<sub>12</sub> at doses of 50, 100 and 200 ng/site was performed, respectively. Groups 5, 6 and 7 treated with intracortical microinjection of diazepam at doses of 50 and 200 ng/site and flumazenil at a dose of 50 ng/site, respectively. Group 8 received a mixture of flumazenil (50 ng/site) plus diazepam (200 ng/site). Group 9 and 10 treated with intracortical microinjection of vitamin B<sub>12</sub> (50 ng/site) plus diazepam (50 ng/site) and vitamin B<sub>12</sub> (100 ng/site) plus diazepam (200 ng/site), respectively. Group 11 received intracortical microinjection of a mixture of flumazenil (50 ng/site) plus vitamin B<sub>12</sub> (200 ng/site). Intracortical microinjection of penicillin was performed five min after above-mentioned chemical compounds. The microinjection volumes of the chemical agents and penicillin were 1 and 1.5 µl/site, respectively.

**Surgical procedure and induction of epileptiform activity**

Induction of epileptiform activity was previously described in detail (Kozan et al. 2008, Tamaddonfard et al. 2012a). The animals were anesthetized with intraperitoneal injection of urethane (1.2 g/kg), and were placed in a stereotaxic apparatus (Stoelting, Wood Lane, IL, USA). Body temperature was maintained between 36 and 37°C using a controlled heating pad system. Thereafter, the scalp was incised, and the skull was leveled off around the bregma.
The epileptic focus was produced by intracortical microinjection of penicillin. For this purpose, a hole with 0.8 mm in diameter was made in the right parietal bone overlying the right sensory-motor cortex (1 mm posterior to the bregma and 3 mm lateral to the midline). Penicillin G potassium (300 IU, 1.5 μl) was microinjected 1 mm beneath the surface of the skull using a 1 μl Hamilton’s syringe in a period of 90 s.

**Electroencephalographic (EEG) recordings**

For EEG recordings, two 5-mm length pin electrodes (0.5 mm in diameter) were implanted in right frontal and parietal bones according to the following coordinates; first electrode, 1 mm anterior to the bregma and 2 mm lateral to the midline (frontal electrode); second electrode, 5 mm posterior to the bregma and 2 mm lateral to the midline (parietal electrode). The common reference electrode was fixed on the left pinna. The electrodes were connected to a 4-channel physiograph (Physiograph 4-channels, MK-III-P, NARCO Bio-systems, USA) via a universal coupler (Universal coupler, type 7189, NARCO Bio-systems, USA) for EEG activity recordings. The EEG recordings were performed at 15 min before (baseline activity) and at 10, 30 and 60 min after intracortical microinjection of penicillin. In each of the above-mentioned times, EEG activity was recorded for a period of 1 min with two speeds (0.05 and 0.25 cm/s). The number and amplitude of spike waves were manually calculated from the recorded EEGs.

**Statistical analyses**

Statistical comparisons were performed using the GraphPad Prism (5) software (GraphPad Software, San Diego, CA, USA). Data obtained from the number and amplitude of spike waves were analyzed by two-way analysis of variance (ANOVA). Bonferroni post-test was applied for showing significant differences among groups. All the values are expressed as the mean±SEM. Statistical significance was set at \( P<0.05 \).

**RESULTS**

Figure 1 shows the EEG recordings obtained from 30th min after intracortical microinjection of chemical agents followed by penicillin. Baseline activities of each animal were recorded before administration of substances and it has been confirmed that none of the animals had spontaneous epilepsy (Fig. 1). The intracortical microinjection of penicillin (300 IU) induced an epileptiform activity characterized by spike waves with high number and amplitude (Fig. 1). Epileptiform activity began 3–5 min after penicillin microinjection and continued with constant levels of number and amplitude of spike waves to the end of experiment.

Figure 2 (A and B) shows the effects of focal administration of vitamin B12 on the number and amplitude of spike waves induced by penicillin. In the normal saline treated group (control group), the number of spikes/min were 29.7±2.9, 26.1±3 and 23±1.4 spike/min at 10th, 30th and 60th min after penicillin microinjection, respectively. In addition, the spike amplitudes were 1483±176, 1426±140 and 1154±145 μV, respectively. While vitamin B12 at a dose of 50 ng/site produced no significant effect, at doses of 100 and 200 ng/site it significantly...
attenuated the number ($F_{3,60}=56.68$, $P<0.05$, Fig. 2A) and amplitude ($F_{3,60}=22.38$, $P<0.05$, Fig. 2B) of spike waves induced by penicillin. No significant differences were observed between the antiepileptiform effects of 100 and 200 ng/site of vitamin B$_{12}$.

Figure 3 (A and B) shows the effects of microinjection of diazepam and flumazenil on the number and amplitude of spike waves induced by penicillin. Focal administration of diazepam at a dose of 50 ng/site did not alter penicillin-induced epileptiform activity, whereas at a dose of 200 ng/site, it significantly decreased both the number ($F_{4,75}=18.84$, $P<0.05$, Fig. 3A) and amplitude ($F_{4,75}=17.16$, $P<0.05$, Fig. 3B) of spike waves induced by penicillin. In addition, flumazenil alone at a dose of 50 ng/site did not change the number (Fig. 3A) and amplitude (Fig. 3B) of spike waves induced by penicillin. When flumazenil (50 ng/site) co-administered with diazepam (200 ng/site), the number ($F_{4,75}=18.84$, $P<0.05$, Fig. 3A) and amplitude ($F_{4,75}=17.16$, $P<0.05$, Fig. 3B) of spikes significantly increased in comparison with diazepam (200 ng/site) alone.

Co-administration of ineffective doses of vitamin B$_{12}$ (50 ng/site) and diazepam (50 ng/site) significantly decreased the number ($F_{3,60}=18.04$, $P<0.05$, Fig. 4A) and amplitude ($F_{3,60}=11.28$, $P<0.05$, Fig. 4B) of spike waves induced by penicillin as compared with normal saline. In addition, co-administration of effective doses of vitamin B$_{12}$ (100 ng/site) and diazepam (200 ng/site) significantly decreased the number ($F_{3,60}=34.92$, $P<0.05$, Fig. 5A) and amplitude ($F_{3,60}=44.54$, $P<0.05$, Fig. 5B) of spike waves when compared with alone used of them.

Figure 6 gives data about the effect of flumazenil (50 ng/site) on the antiepileptic effects of vitamin B$_{12}$ at a dose of 200 ng/site. Co-administration of flumazenil (50 ng/site) with vitamin B$_{12}$ (200 ng/site) significantly increased the number ($F_{3,60}=34.85$, $P<0.05$, Fig. 6A) and amplitude ($F_{3,60}=15.26$, $P<0.05$, Fig. 6B) of spike waves when compared with vitamin B$_{12}$ at a dose of 200 ng/site used alone.

**DISCUSSION**

In the present study, intracortical microinjection of penicillin (300 IU, 1.5 μl) produced an epileptiform activity characterized by spike waves with high frequency and amplitude. Penicillin has been widely used to produce seizures in laboratory animals, particularly in rats. Our results are approximately consistent with other findings in which the doses of intracortical microinjected penicillin were 200–500 IU (Kozan et al. 2008, Yildirim et al. 2011, Tamaddonfard et al. 2012a,b, Arslan et al. 2014, 2013). Penicillin exerts its epileptiform activity in cortical tissues by inhibiting gamma-aminobutyric acid (GABA) receptor, owing to its structural resemblance to a specific GABA$_A$ receptor antagonist, bicuculline, and thus leads to rhythmic epileptiform discharges (Fisher 1989, Arik et al. 2014).

In the present study, focal administration of vitamin B$_{12}$ significantly decreased the number and amplitude of epileptiform spikes. Vitamins have been considered important patterns in controlling certain types of seizures or even preventing adverse effects of AEDs (Ayyıldız et al. 2006, Ranganathan and Ramaratnam 2009, Sawicka-Glazer and Czuczwar 2014). There are several studies indicating an association between vitamin B$_{12}$ deficiency and EEG abnormalities in epilepsy (Lundgren and Blennow 1999, Biancheri et al. 2002, İncecik et al. 2010). For example, Biancheri and coworkers (2002) reported epilepsy in nine patients

![Fig. 4. The effect of focal co-microinjection of ineffective doses of vitamin B$_{12}$ and diazepam on the number (A) and amplitude (B) of spike waves induced by intracortical microinjection of penicillin in rats. Vitamin B$_{12}$ and diazepam were co-microinjected 5 min before injection of penicillin. Values are expressed as the mean±SEM (n=6); *P<0.05 compared with normal saline.]
with vitamin B₁₂ deficiency. On the other hand, there are increasing evidences indicate neuroprotective properties for vitamin B₁₂ in peripheral and central nervous systems. The reason behind the idea of researching the neuroprotective efficacy of vitamin B₁₂ is that its deficiency can cause an impairment of brain and nerve tissue function (Miodownik and Lerner 2010). In the previous studies, scholars evaluated the neuroprotective actions of vitamin B₁₂ in rats with sciatic and corneal nerves crush injury models (Romano et al. 2014, Tamaddonfard et al. 2014). In addition, studies have shown that vitamin B₁₂ is able to protect cortical neurons and retinal cell cultures against glutamate cytotoxicity (Akaike et al. 1993, Okada et al. 2010). On the other hand, neuroprotection is increasingly considered as a promising therapy for preventing and treating epilepsy (Acharya et al. 2008). These findings open a possibility of exploring the potential of vitamin B₁₂ in the treatment epilepsy. However, the potential use of vitamin B₁₂ in the treatment of epilepsy has not suggested yet.

In the present study, focal administration of diazepam significantly decreased the number and amplitude of epileptiform activity. These effects were inhibited by co-administration of flumazenil with diazepam. Benzodiazepines, including diazepam, are important anticonvulsants used in the treatment of epilepsy, which act through γ₂-subunit of GABAₐ receptor to enhance GABA-mediated inhibition (Riss et al. 2008, Joshi et al. 2013, Middendrop et al. 2014). Intracerebroventricular injection of diazepam reduced the number and amplitude of the spike waves induced by intracortical microinjection of penicillin in rats (Tamaddonfard et al. 2012a,b). Activation of GABAₐ receptor opens channels largely permeable to chloride ions and an impairment of GABAergic signaling is involved in various neurological disorders including epilepsy (Ben-Ari et al. 2012, Arik et al. 2014).

Several mechanisms are recognized for current antiepileptic drugs: modulation of voltage-gated ion channel (phenytoin); enhancement of GABA-mediated inhibitory neurotransmission (benzodiazepines); and...
attenuation of excitatory amino acids neurotransmission (felbamate) (Lasoń et al. 2011). Many available AEDs cause toxicity (Löschner and Leppik 2002). Combinations of two or more drugs reduce the intensity and incidence of unwanted effects. In the present study, the number and amplitude of spike waves were also reduced when ineffective and effective doses of vitamin B$_{12}$ and diazepam were focally co-administered. This result indicates that a potentiation effect may exist between vitamin B$_{12}$ and diazepam in producing antiepileptic effect. On the other hand, the inhibitory effect of flumazenil on vitamin B$_{12}$ antiepileptiform activity observed in our study can clarify the involvement of GABA-benzodiazepine receptor complex in the antiepileptiform activity of vitamin B$_{12}$. In this context, it has been reported that vitamin B$_{12}$ increased the GABA contents of suprachiasmatic nucleus and cerebral cortex in rats (Ikeda et al. 1997).

**CONCLUSION**

The results of the present study showed antiepileptiform effects for vitamin B$_{12}$ and diazepam after intracortical microinjection of them. Combined treatments with vitamin B$_{12}$ and diazepam produced better antiepileptiform effects. GABA$_{A}$- benzodiazepine receptor complex system might be involved in antiepileptiform activity of vitamin B$_{12}$.

**REFERENCES**


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