

## EXPERIMENTAL STUDY

# Hemopressin increases penicillin-induced epileptiform activity in rats

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**ABSTRACT**

**OBJECTIVE:** Hemopressin (Hp) is the first peptide ligand described for the CB1 cannabinoid receptor. Therefore, we aimed to investigate the effect of hemopressin on penicillin-induced epileptiform activity by using electrophysiological recording (ECoG) technique.

**METHODS:** Male Wistar rats were anesthetized with urethane (1.25 g/kg), and epileptiform activity was induced by intracortical injection of penicillin (500 IU). Animals were randomly divided into eight groups. Subsequently, the rats were administered with saline or hemopressin as follows: saline control group (Group I: 2 µl/i.c.v./saline), hemopressin groups (Group II: 0.025 µg/i.c.v.; Group III: 0.075 µg/i.c.v.; Group IV: 0.15 µg/i.c.v.; Group V: 0.3 µg/i.c.v.; Group VI: 0.6 µg/i.c.v.; Group VII: 1.2 µg/i.c.v.; Group VIII: 2.4 µg/i.c.v.). The various doses of hemopressin were injected intracerebroventricularly (i.c.v) 30 minutes after penicillin (2.5µl) injection. After hemopressin injection, ECoGs were recorded for three hours.

**RESULTS:** Hp at doses of 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 µg/kg significantly increased the frequency of epileptiform ECoG activity compared to penicillin-injected group without changing the amplitude. The 0.6 µg hemopressin was the most effective dose to increase the epileptiform activity ( $p < 0.05$ ) while the dose of 0.025 µg hemopressin was non-effective ( $p > 0.05$ ).

**CONCLUSIONS:** The results of this study provided electrophysiological evidence for hemopressin to be modulating penicillin-induced epileptiform activity by acting as CB<sub>1</sub> receptor antagonist. Further studies are required to elucidate the involved mechanism underlying this effect (Fig. 3, Ref. 40). Text in PDF [www.elis.sk](http://www.elis.sk).

**KEY WORDS:** cannabinoid, CB<sub>1</sub>, epilepsy, hemopressin, penicillin, receptor.

**Introduction**

Epilepsy is one of serious chronic neurologic disorders characterized by a lasting predisposition to have epileptic seizures potentially leading to neurological, cognitive, physiological and social problems (1). Studies conducted so far have shown that systems, receptors and peptides involved in epilepsy could be used to protect brain cells during and after seizures or to minimize the resulting damage (2, 3, 4). Recent studies revealed that the cannabinoid system has considerable roles in controlling and preventing epileptic seizures (5, 6, 7).

Cannabinoids exert their behavioral and neuronal effects through CB<sub>1</sub> receptor (8, 9, 10, 11). It has been shown that endocannabinoids such as arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) have a neuroprotective effect on the hippocampus via activating the CB<sub>1</sub> receptor (12, 13). Endocannabinoids are synthesized from membrane lipids when needed

as a response to a prolonged neuronal depolarization and elevated intracellular calcium levels. It is known that a retrograde inhibition of neurotransmitter release and control of neuronal stimulation are conditioned via this mechanism (9, 10, 11, 14). It has been reported that these events take place during seizure (15). Endocannabinoids prevent seizures through a CB<sub>1</sub> receptor-dependent mechanism (16, 17). CB<sub>1</sub> receptor antagonists have been shown to lower the seizure threshold, thereby facilitating epileptogenesis (17). Our group previously showed that the cannabinoid CB<sub>1</sub> receptor agonist, arachidonyl-2'-chloroethylamide hydrate (ACEA), is a anticonvulsant, and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1Hpyrazole-3carboxamide (AM-251) has a proconvulsant effect (18, 19). In addition, down-regulation of CB<sub>1</sub> receptor and associated molecules have been shown in hippocampus regions which were removed surgically due to temporal lobe epilepsy (20). Thus, CB<sub>1</sub> receptors seem to play a significant role in pathophysiology of convulsive seizures.

Hemopressin is the first peptide ligand identified for CB<sub>1</sub> cannabinoid receptor (21). Hemopressin (PVNFKFLSH) was previously isolated from rat brain homogenates and was shown to cause hypotension in anesthetized rats (22). Later, Heiman et al (21) discovered hemopressin, a peptide ligand selectively binding CB<sub>1</sub> cannabinoid receptors, using conformation state-sensitive antibodies to screen the endogenous peptide panel of rat brain and

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adipose tissue. By using nuclear magnetic resonance spectroscopy and circular dichroism method, Scrima et al (23) verified that hemopressin is indeed the CB1 receptor ligand. Dodd et al (24) studied the effect of hemopressin on dietary behaviors and found no pharmacological effects of it in rats lacking CB1 receptors. Hemopressin is selectively bound to cannabinoid receptors and does not have any effect on receptor family related to other G-proteins, including CB2 receptor (21, 24).

Although it has been well established that hemopressin is a CB1 receptor ligand, the question whether it is an agonist or antagonist of cannabinoid CB1 receptor is still disputed. Heilmann et al (21) showed that hemopressin decreased the increase in antibody recognition induced by CB1 agonist, which was the same as the decrease by CB1 receptor antagonist SR141716 (rimonabant). A study in which neuroprotective effects of cannabinoid agonists and antagonists were investigated in rat cerebral ischemia/reperfusion models found that hemopressin slightly suppressed the neuroprotective effect exerted by cannabinoid receptor agonist ACEA (25). Some *in vivo/in vitro* hemopressin studies showed that cannabinoid receptors acted as an antagonist and passed the blood-brain barrier, while suppressing the appetite and leading to antinociception (24, 26, 27). Some other studies revealed that hemopressin acted as CB1 receptor agonist. Li et al (28) showed that the effect of hemopressin on gastrointestinal motility was the same as the effect of cannabinoid receptors. Similarly, Pan et al (29) found that hemopressin had an antinociceptive effect in a rate-dependent manner, similar to that of CB1 receptor agonists.

Hemopressin has recently been shown to be closely associated with cannabinoid CB1 receptor system. Cannabinoid CB1 receptor system has been clearly indicated to play a role in epilepsy, but the effect of hemopressin on epilepsy has not been investigated so far. Elucidation of the effect of hemopressin on epileptic activity could probably increase the significance of cannabinoid system in epilepsy. In addition, this will allow a detailed understanding of hemopressin CB1 receptor agonism and antagonism. Thus, the aim of the present study was to investigate the effect of hemopressin on penicillin-induced epileptiform activity in rat. Penicillin epilepsy model is known as a simple partial epilepsy model in the clinic.

## Methods

### Animals

Fifty-six adult male Wistar Albino rats ( $210 \pm 10$  g) were used for the experiments. Experimental designs and procedures were approved by Ondokuz Mayıs University Animal Research Committee in accordance with the European Commission regulations (2014-HAYDEK-16). Rats were obtained from Ondokuz Mayıs University Medical and Surgical Research Center (Samsun, Turkey). The rats were housed in cages with 3–4 rats per cage and were kept under standardized conditions (at  $20 \pm 3$  °C temperature; 12-hour light - 12-hour dark cycle, lights on at 06:00 a.m.; 55–60 % relative humidity). Standard food pellets and water were available *ad libitum*.

### Experimental groups

After seven days of adaptation to laboratory conditions, the animals were randomly divided into eight groups of seven rats per group. Each rat was used only once. All experimental procedures were performed between 10.00 a.m. and 3.00 p.m. Experimental groups were as follows:

Experiment 1: dose-response relationship of hemopressin (Hp)

- (1) 500 units penicillin (2.5 µl, i.c.)
- (2) 500 IU penicillin (2.5 µl, i.c.) + Hp (0.025 µg, i.c.v.)
- (3) 500 IU penicillin (2.5 µl, i.c.) + Hp (0.075 µg, i.c.v.)
- (4) 500 IU penicillin (2.5 µl, i.c.) + Hp (0.15 µg, i.c.v.)
- (5) 500 IU penicillin (2.5 µl, i.c.) + Hp (0.3 µg, i.c.v.)
- (6) 500 IU penicillin (2.5 µl, i.c.) + Hp (0.6 µg, i.c.v.)
- (7) 500 IU penicillin (2.5 µl, i.c.) + Hp (1.2 µg, i.c.v.)
- (8) 500 IU penicillin (2.5 µl, i.c.) + Hp (2.4 µg, i.c.v.)

### Surgical procedure

The rats were starved for one day before the operation. Prior to surgery, animals were given anesthesia with an intraperitoneal injection of urethane (1.25 g/kg, i.p). Then the rats were placed in a stereotaxic apparatus. The periosteum of the skull was removed by scraping, and small bleeding foci were cauterized by electrocautery to prevent bleeding. In all experiments, bregma was used as a reference point, and implantation co-ordinates were taken from the atlas according to Paxinos and Watson (30). Then, four holes of 0.2 mm diameter were opened with the help of a micro-drill. Tripolar electrodes were placed over the left somatomotor cortex (electrode coordinates: first electrode placed 3mm lateral to sagittal suture and 4 mm rostral to bregma; second electrode was placed 3 mm lateral to sagittal suture and 4 mm caudal to bregma). The common reference electrode was fixed on the pinna. During this procedure, bleeding which may occur in the bone tissue was inhibited by bone wax (W810, ETHICON). The body heat of the rats was kept constant with electric blankets (Harvard 7087). Rectal temperature was maintained between 36.0 and 37.5 °C using a feedback-controlled heating system.

### Induction of epileptiform activity

Penicillin G potassium salt selectively antagonizes the  $GA_{A}$ -receptor and mediates inhibitory postsynaptic potentials in the central nervous system. The induction of epileptiform activity was carried out as described previously by Ayyıldız et al (31); Esen and Aygun (3). Intracortical (i.c.) injection of penicillin was applied to the cerebral cortex (2 mm lateral and 2 mm rostral to bregma) through a Hamilton microinjector at a depth of 3.2 mm from the skull bone system. The epileptic focus was produced by 500 units of penicillin G potassium injection.

### Drugs administration

Hemopressin, urethane and Penicillin G potassium salt were purchased from Sigma Chemical Co., St. Louis, USA. All drugs dissolved in in physiological saline.

Hemopressin (PVNFKFLSH; Sigma Chemical Co., St. Louis, MO, U.S.A.) were used in the experiments. Hemopressin and Penicillin G potassium salt was dissolved in physiological saline

and administered intracortically in a volume of 2.5  $\mu$ l. Intracerebroventricular injections were administered into the left lateral ventricle of each rat through a stereotaxic apparatus with coordinates of 1.5 mm lateral and 1.1 mm rostral to bregma. Intracerebroventricular (i.c.v.) injection of the drug was applied to this hole through a Hamilton microinjector at a depth of 4.2 mm from the skull bone system (33). In the first set of experiments, hemopressin, was administered 30 min after penicillin (i.c.) application at doses of 0.025, 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4  $\mu$ g (i.c.v.).

### ECoG recordings

The electrocorticography (ECoG) activity was continuously monitored on a 16-channel recorder (PowerLab, 16/35, AD Instruments, Castle Hill, Australia). All recordings were made under anesthesia and stored on a computer. The frequency and amplitude of epileptiform ECoG activity was analyzed off-line.

### Statistical analyses

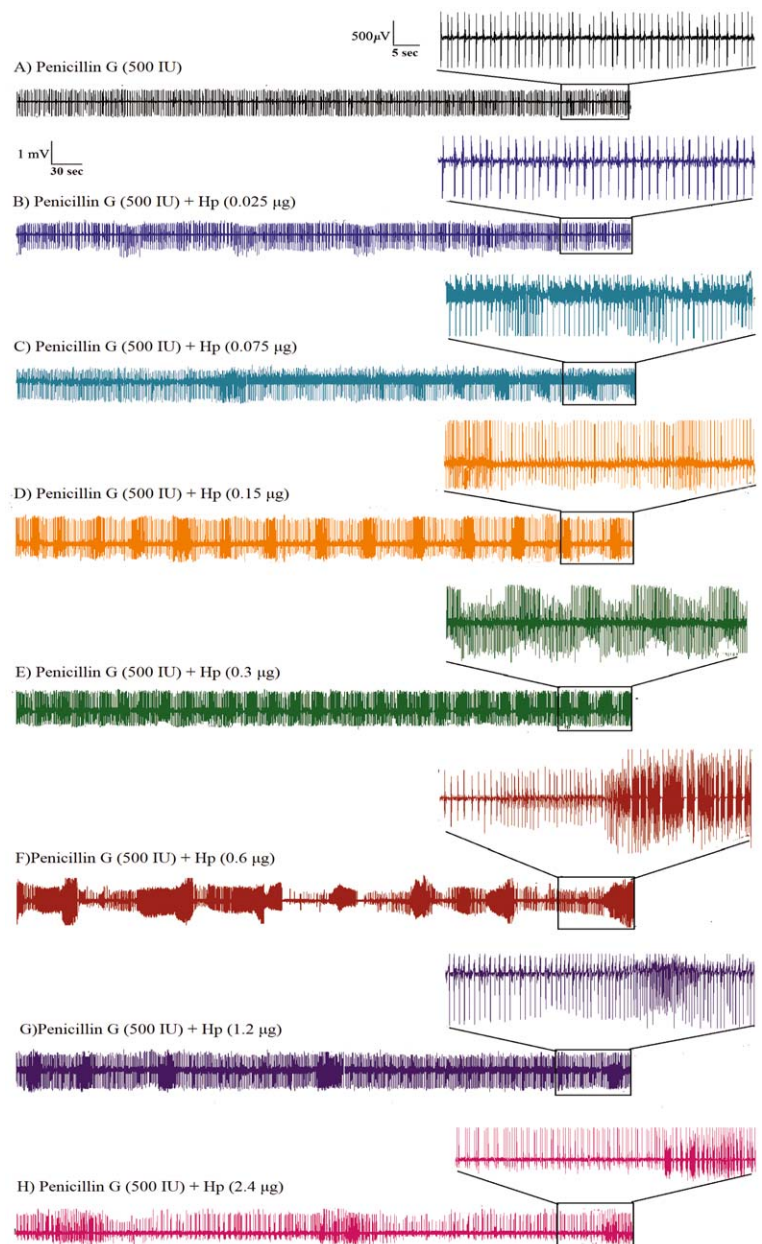
The results for each group are expressed as means  $\pm$  standard error of the mean (SEM). Statistical comparisons were made using SigmaStat software (SPSS Science, Chicago, IL, U.S.A.). The data analysis was performed using one-way analysis of variance (ANOVA) and Tukey tests for comparisons. Differences were considered statistically significant at  $p < 0.05$ .

### Results

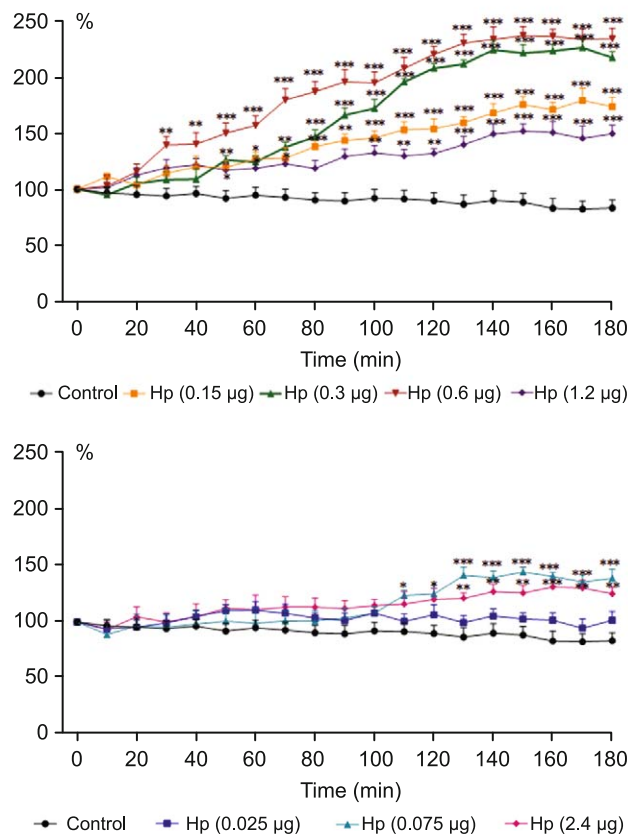
Intracortical injection of penicillin (500 IU) induced an epileptiform ECoG activity characterized by bilateral spikes and spike-wave complexes (Fig. 1A). This ECoG activity began within 3–5 min after penicillin injection and lasted for 5 h. It reached a constant level of frequency and amplitude in 30 min. The mean spike frequency and amplitude were  $37.54 \pm 3.21$  spike/min and  $932.97 \pm 30.24$   $\mu$ V within 90 min after physiological saline injection (i.c.v.), respectively. Hemopressin (Hp) was administered 30 min after penicillin injection.

Hemopressin, at a dose of 0.025  $\mu$ g, did not affect the mean frequency and amplitude of penicillin-induced epileptiform activity (Figs 1B, 2 and 3). Hemopressin at doses of 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4  $\mu$ g significantly enhanced the mean frequency of epileptiform activity without changing the amplitude (Figs 2 and 3). However, the dose of 0.6  $\mu$ g was the most effective in increasing the mean frequency.

The mean frequency of epileptiform ECoG activity in groups administered with hemopressin at



**Fig. 1.** The intracortical injection of penicillin (500 IU) induced the epileptiform activity on ECoG (A). The intracerebroventricular (i.c.v.) administration of Hp at the low doses of 0.025  $\mu$ g/kg, i.p., 30 min after penicillin injection did not significantly change the frequency and amplitude of epileptiform ECoG activity (B). The administration of 0.075  $\mu$ g Hp caused a marked increase in the frequency of penicillin-induced epileptiform activity within 110 min after Hp injection without changing the amplitude (C). The administration of 0.15  $\mu$ g Hp caused a marked increase in the frequency of penicillin-induced epileptiform activity within 50 min after Hp injection without changing the amplitude (D). The administration of 0.3  $\mu$ g Hp caused a marked increase in the frequency of penicillin-induced epileptiform activity within 50 min after Hp injection without changing the amplitude (E). The administration of 1.2  $\mu$ g Hp caused a marked increase in the frequency of penicillin-induced epileptiform activity within 100 min after Hp injection without changing the amplitude (F). The administration of 2.4  $\mu$ g Hp caused a marked increase in the frequency of penicillin-induced epileptiform activity within 130 min after Hp injection without changing the amplitude (G). Hp (0.075, 0.15, 0.3, 0.6, 1.2 and 2.4  $\mu$ g) also caused the development of status epilepticus-like activity.

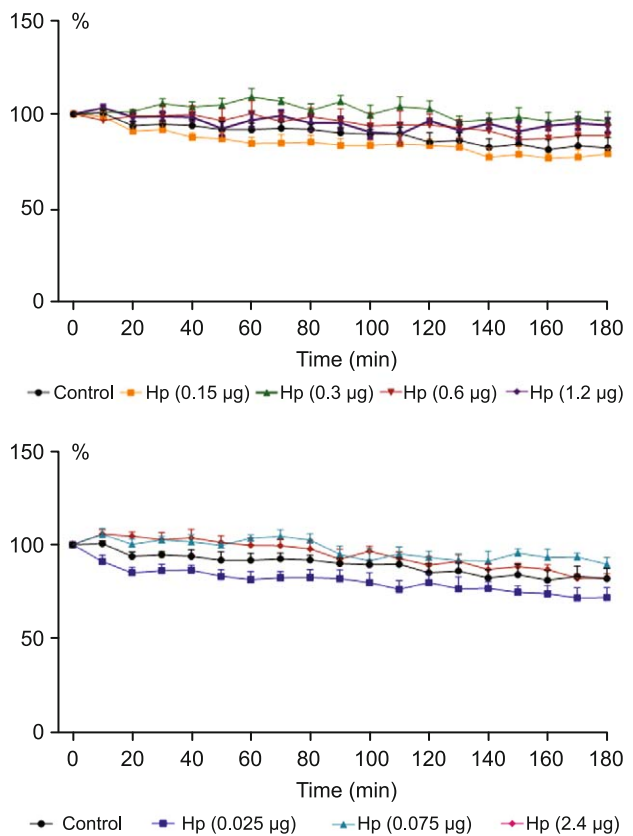


**Fig. 2.** The effects of intracerebroventricular administration of Hp on mean spike frequency of penicillin-induced epileptiform activity. Hp at a rate of 0.025 µg/kg did not influence mean frequency or amplitude of epileptiform activity. Hp rates of 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 µg/kg i.c.v. increased the mean spike frequency of epileptiform activity within 110, 50, 50, 30, 100 and 130 min after Hp injection, respectively, compared to penicillin-injected groups (control groups); \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. The percent frequency of epileptiform ECoG activity value (defined as mean of spike frequency after substance is administered / mean of spike frequency before substance is administered \* 100) depends on both frequencies of epileptiform ECoG activity before and after the substance is administered.

doses of 0.025, 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 µg were 42.00 ± 3.07, 55.26 ± 2.66; 51.30 ± 1.65, 51.61 ± 1.72, 58.65 ± 3.86, 52.85 ± 3.45, and 50.88 ± 2.08 spike/min, respectively, within 90 min after Hp (0.025 µg); 110 min after Hp (0.075 µg); 50 min after Hp (0.15 µg); 50 min after Hp (0.3 µg); 30 min after Hp (0.6 µg); 100 min after Hp (1.2 µg) and 130 min after Hp (2.4 µg) injection, respectively. The amplitude of ECoG activity in the latter groups was 820.90 ± 45.77, 998.94 ± 37.99, 880.50 ± 32.16, 1096.96 ± 35.39, 1063.94 ± 65.73, 943.45 ± 36.28, and 979.55 ± 38.69 µV respectively (Figs 2 and 3).

**Discussion**

In the present study, the effect of hemopressin was investigated in penicillin-induced experimental epilepsy model. Hemopressin caused an increment in the mean frequency of epi-



**Fig. 3.** The effects of Hp on mean spike amplitude of penicillin-induced epileptiform activity. Hp at rates of 0.025, 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 µg/kg, i.c.v. did not change amplitude of epileptiform activity. The percent amplitude of epileptiform ECoG activity value (defined as mean of spike amplitude after substance is administered / mean of spike amplitude before substance is administered\*100) depends on both frequencies of epileptiform ECoG activity before and after the substance administration.

leptiform activity in various doses of hemopressin injection. The dose of hemopressin 0.6 µg was most effective in increasing the epileptiform activity. The small dose of hemopressin (0.025 µg) was not effective.

Heimann et al (21) used 50 µg/kg (i.p.) and 500 µg/kg (i.p.) rates of hemopressin and observed antinociceptive effect by both rates. They found that the antinociceptive effect was highest at 500 µg/kg rate. In another study, 500 nmol/kg (i.p.) application of hemopressin decreased food intake in rats (24). Fogaça et al (34) showed that 0.05 mg/kg (i.p) and 0.5 mg/kg (oral) administration of hemopressin resulted in anxiogenic-like effects in Wistar rats. In a study dealing with neuroprotective effects of cannabinoid agonist and antagonists using cerebral ischemia/reperfusion rat model, administration of 1 mg/kg i.p. of hemopressin partly suppressed the neuroprotective effect induced by cannabinoid receptor agonist ACEA (25). In general, ICV applications are one in one thousand of IP applications. In the present study, the most effective dose of hemopressin was 0.6 µg (ICV) while that of 0.025 µg was non-effective.

Heiman et al (21) screened endogenous peptide panel of rat brain tissue using conformation state-sensitive antibodies and found that only hemopressin could modulate antibody binding considerably and thus could be a potential ligand for CB1 receptors. More recently, Scrima et al (23) studied structural features of hemopressin using circular dichroism and nuclear magnetic resonance spectroscopy. Based on findings about the coefficients of energy value, binding and clamping, hemopressin was shown to interact with CB1 receptor. On the other hand, another study using CB1 knock-out rats failed to show the effects of hemopressin on food uptake (24). All studies with hemopressin showed that it selectively binds to CB1 receptor. However, the question of whether it is an agonist or antagonist is still controversial. While in some cases, hemopressin appears to be a CB1 receptor antagonist because of its effect on appetite and some main pathways, in other cases, it acts as a CB1 receptor agonist for gastrointestinal system.

There are many studies in literature reporting that CB1 receptors affect epileptiform activity (6, 7, 11, 18, 35). Most studies showed that cannabinoid CB1 receptor agonists have anticonvulsant effect. Wallace et al. found that an elimination of endogenous cannabinoid tonus increased the seizure susceptibility (17). In other studies, the release of glutamate through CB1 receptor in glutamatergic axon terminals was reduced as a result of anticonvulsant effect (36, 37). In pilocarpine epilepsy model established in rats,  $\Delta^9$ -THC (10 mg/kg) completely eliminated epileptic discharges of CB1 receptor agonist WIN55,212 (5 mg/kg), while CB1 receptor antagonist SR141716A significantly increased both length and frequency of seizures (38). If hemopressin had acted as a CB1 receptor agonist, it would have exerted an anticonvulsant effect. However, hemopressin had a proconvulsant effect in that study. Therefore, it could be stated that hemopressin functions as a CB1 receptor antagonist.

In the present study, hemopressin showed a proconvulsant effect albeit sometimes late in all rates except for 0.025  $\mu$ g. Hemopressin application at the rate of 0.6  $\mu$ g significantly increased the spike activity after 30 minutes. In addition, discharges similar to *status epilepticus* were observed during seizures. Our findings are in accordance with the reports in literature where CB<sub>1</sub> receptor antagonists had proconvulsant effects through CB<sub>1</sub> receptors in experimental epilepsy models. Thus, it could be stated that hemopressin acts as a CB<sub>1</sub> receptor antagonist. In addition, it was also suggested that hemopressin could be a negative allosteric modulator (26, 39, 40). However, considering the fact that hemopressin alone could increase seizure, it could be stated that it is a CB<sub>1</sub> receptor antagonist rather than a negative allosteric modulator of CB<sub>1</sub> receptors.

## Conclusions

Based on the results from the present study, it could be stated that hemopressin could be an endogenous CB<sub>1</sub> receptor antagonist. However, this conclusion needs to be verified through immunohistochemical, PCR and western blot studies along with electrophysiological findings. The presence of antagonization for the endogenous cannabinoid system in brain could correct mood disorders involving endocannabinoid system.

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