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Heart Rate Variability and Electroencephalogram as Predictors of the Therapeutic Outcome of Vagus Nerve Stimulation in Dogs with Epilepsy: A Preliminary Study

Junya HIRASHIMA, Miki TAKAGI, Miyoko SAITO*

Laboratory of Small Animal Surgery (Neurology), School of Veterinary Medicine, Azabu University,
1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

Abstract: This preliminary study aimed to investigate the potential of heart rate variability (HRV) and electroencephalogram (EEG) as predictors of a therapeutic outcome of vagus nerve stimulation (VNS) therapy in dogs with epilepsy. We performed serial HRV and EEG analyses befor and until 6 months after VNS initiation and assessed temporal changes in HRV and EEG in four dogs that underwent VNS therapy. Two of the four dogs had reductions in seizure frequency and were determined to be responders, and the other two were non-responders. No differences in temporal changes in HRV and EEG were observed between responders and non-responders. However, HRV before VNS initiation showed that responders had higher parasympathetic activity than non-responders. EEG before VNS initiation revealed that both responders had their irritative zone in the temporal lobe. HRV and EEG assessments before VNS therapy probably helps predict its therapeutic outcome in dogs.
Keywords: vagus nerve stimulation, heart rate variability, electroencephalogram, dog, epilepsy

Introduction

Epilepsy is a chronic neurological disorder that causes epileptic seizures. The prevalence of this disease in dogs has been reported to be 0.6–0.75%[1, 2]. Similar to humans, medication failed to control seizures in about 20–30% of the dogs with epilepsy, despite using several antiseizure drugs (ASDs) appropriately[3]. In both humans and dogs, this condition is called drug-resistant epilepsy (DRE). For humans with DRE, additional non-pharmacological therapy is required, and different types of treatment modalities are currently available[3]. As DRE is a reason for euthanasia in dogs because it decreases dogs' quality of life and increases the financial burden of medical treatment[4, 5], non-pharmacological treatment is also urgently needed in dogs with DRE.

Vagus nerve stimulation (VNS) therapy is considered an adjunctive effective treatment for humans with DRE[3, 6]. This therapy suppresses seizures with intermittent stimulation to the left cervical vagus nerve using surgically implanted devices. The effect of VNS therapy is known to increase over time; thus, around a 2-year treatment period is required to assess whether a patient responds to VNS therapy or not[7]. After 2 years of observation, little or no effect of VNS therapy was observed among one-third of human patients who received VNS therapy[8]. In veterinary medicine, one short-term clinical assessment and one long-term case report investigated the effects of conventional implantable VNS therapy in dogs with DRE[9, 10]. Moreover, a short-term assessment of non-invasive transcutaneous VNS, a new method of VNS therapy in dogs with DRE, was reported[11]. These studies reported the potential efficacy of VNS therapy in dogs; however, VNS therapy had not reduced the seizure frequency in several dogs in these studies[9, 11]. The evaluation period of the two short-term assessments was about 3 months. Thus, it might be too early to conclude that VNS therapy had only a little effect on those dogs, because human

*Corresponding author: Miyoko Saito (msaito@azabu-u.ac.jp)

patients need a 2-year observation period for assessment. However, observing dogs for 2 years is too long as they have a much shorter lifespan than humans. If a dog has a low likelihood of responding to VNS therapy, it is preferable to try other treatment options. Therefore, predictors that indicate whether a dog can benefit from VNS therapy or not will be valuable.

Heart rate variability (HRV) is considered a non-invasive indicator of the balance of the autonomic nervous system[12]. This indicator has been used widely in dogs to assess their stress level or emotional state[13, 14]. As VNS stimulates the vagus nerve in humans and the vagosympathetic trunk in dogs, VNS would affect HRV because the vagus nerve is a major component of the autonomic nervous system. An electroencephalogram (EEG) is a record of the electrical activity of the brain. Furthermore, VNS has been known to influence brain activity as an experimental study reported that the stimulation of the vagus nerve produced EEG desynchronization in cats under anesthesia[15]. Thus, HRV and EEG have been targeted as biomarkers of VNS in humans[16–26].

In the literature, many studies have investigated whether HRV or EEG could be used to predict the therapeutic outcomes of VNS therapy in humans; however, the results are still controversial[16–26]. Two studies of HRV showed that patients with higher high-frequency (HF) power tend to respond to VNS therapy[24, 25]; however, another study reported that HF power was lower in responders than in non-responders[26]. One study showed focal or multifocal epileptic activities in EEG related to the effects of VNS[21]. Nevertheless, another study reported conflicting results[22]. There is no such study in veterinary medicine. Thus, this preliminary study aimed to investigate whether serial HRV or EEG analyses could predict the therapeutic outcomes of VNS therapy in dogs with epilepsy early (before or within 6 months after VNS initiation). We hypothesized that some sort of change in HRV or EEG could be seen over time if dogs respond to VNS therapy, so that the therapeutic outcome could be predicted. In other words, if dogs did not respond to VNS therapy, there would be no changes in HRV or EEG. We also hypothesized that there could be a difference in HRV or EEG before VNS initiation between responders and non-responders and that either parameter could be a predictor.

Materials and Methods

Study design

The study period was 3 years, from June 2018 to June 2021. Candidates for VNS therapy were recruited until January 2021 so that follow-up was possible for at least 6 months. The therapeutic outcomes of VNS therapy were assessed prospectively and serial HRV and EEG analyses were performed in the dogs before and until 6 months after VNS initiation.

Dogs

The inclusion criteria for candidates of VNS therapy included (1) dogs with DRE, with a seizure frequency of at least two seizures every 3 months despite using more than three ASDs appropriately; (2) dogs diagnosed with idiopathic epilepsy (IE) or structural epilepsy (SE) caused by non-progressive brain diseases; and (3) dogs in which VNS therapy was considered an appropriate additional therapy based on seizure semiology, a magentic resonance imaging (MRI) scan of the brain, and results of ictal or interictal EEG recording[27]. The diagnosis of IE was made according to the tier III level of the International Veterinary Epilepsy Task Force (IVETF) consensus proposal[28]. The diagnosis of SE was made based on an MRI scan and/or cerebrospinal fluid analysis. In dogs that met the inclusion criteria, the VNS devices were implanted surgically in the manner described previously[9, 10]. Breeds with the neck length of less than 15 cm were excluded because their body size was too small to implant the VNS devices. Two laboratory Beagles with epilepsy that had more than two seizures every 3 months despite using one or two ASDs were also included. In these two laboratory dogs, the plasma concentration of the ASD was within the therapeutic range. The study protocol was approved by the Ethics Committee of Azabu University, Japan (approval number: 180724-1). Written informed consent was obtained from the owners for the participation of their dogs in this study.

Assessment of therapeutic outcome

In accordance with the IVETF proposal, the observation period was decided to be at least 6 months after VNS initiation, to determine whether a dog responded to VNS therapy or not[29]. After determining a dog as a candidate, the owner of the dog was instructed to record the number of seizures in a diary until VNS implantation surgery. The period before VNS implantation surgery was considered as the baseline period. If the owner already had a diary with detailed records of the seizure frequency, the duration in the diary was also included in the baseline period. The baseline period of the two laboratory dogs was based on the medical records in our laboratory. The owners continued recording the number of seizures after VNS initiation. The number of seizures after VNS initiation in the patient dogs and the laboratory dogs was counted in the owner's diary and by daily checking of the 24-hour video recorded by investigators, respectively. The seizure frequency (per month) during the baseline period and the observation period was compared. The observation period was defined as the period from VNS initiation until the end of the study period. Dogs that had a "relevant reduction" in seizure frequency: ≥ 20% seizure reduction,[30] were classified as responders, and dogs that had an increase or no reduction in seizure frequency were classified as non-responders. During the observation period, the dogs' ASD regimen was unchanged.

Procedure of electrocardiogram recording

To assess HRV, the electrocardiogram (ECG) was recorded in a counseling room at Azabu University Veterinary Teaching Hospital (AUVTH) for the patient dogs and in an authors' laboratory room for the laboratory dogs. If allowed, the HRV was evaluated two times before VNS implantation surgery and the average of these results was considered as the baseline. The HRV was also assessed 1, 2, 3, 4, 5, and 6 months after VNS initiation. The analysis period of 6 months was adopted because 6 months is the shortest recommended period to assess the treatment outcomes of a therapy for epilepsy in veterinary medicine[29]. The ECG recording was conducted at the same time and in the same place for each dog. All ECG recordings were carried out in dogs without sedation or anesthesia. Wireless biological measuring equipment (Polymate Mini AP108, Miyuki Giken Co., Ltd., Japan) was used for the ECG recording. To reduce the contact impedance between the skin and electrodes, the skin was scrubbed with alcohol on cotton and a skin-prepared gel (Skinpure, Nihon Kohden Co., Ltd., Japan). Four electrodes connected to the sensor were placed on the chest of the dogs with the electrode paste (Ten20 Conductive Paste, Weaver and Company, USA), and lead I ECG was recorded. A self-adherent bandage was applied around the chest and the dogs wore a jacket to prevent the dislocation of the electrodes. The sensor was attached to the dogs' back via the jacket.

To keep the consistency of the state of rest in the dogs during each ECG recording, all ECG recordings were started when the dogs were determined to be in the resting state. The resting state was defined as the state that the dog lays down with the head down and has a repiratory rate below 30 breathes per minute[31] in this study. If the dog did not become in the resting state in an hour, the experiment was stopped.

A 5-minute ECG recording was performed. The resting state of the dogs was maintained during the 5-minute ECG recording. The heart rate was also maintained by keeping the dogs in the resting state. If the dogs moved (e. g., standing up or elevating their head) and the heart rate increased during the 5-minute ECG recording, the recording was stopped. The ECG recording was restarted after the dogs become in the resting state again.

HRV analyses

From the 5-minute ECG data recorded, HRV analysis was performed using a bioelectric signal analysis program (BIMUTAS II, KISSEI COMTEC Co., Ltd., Japan). Time-domain and frequency-domain HRV parameters were assessed. Time-domain HRV parameters contained the standard deviation of all R-R intervals for a 5-minute period (SD), the root mean square of successive R-R intervals for a 5-minute period (RMSSD), and the percentage of R-R intervals that differs more than 50 milliseconds for a 5-minute period (pNN50). SD represents the sympathetic activity, and RMSSD and pNN50 indicate the parasympathetic activity[12]. The frequency-domain HRV parameters

included a low-frequency (LF) band (0.04–0.15 Hz), a HF band (0.15–0.4 Hz), and the LF/HF ratio. LF shows the sympathetic activity influenced by the parasympathetic activity. HF represents only the parasympathetic activity. The LF/HF ratio reflects the balance of the autonomic nervous system or the sympathetic activity[12]. The heart rate per minute was also assessed.

Procedure of EEG recording

Before VNS implantation surgery, interictal EEG recording was performed under medetomidine sedation. If allowed, the EEG was evaluated two times before the surgery and the average of these results was considered as the baseline. The EEG was also assessed 1, 3, and 6 months after VNS initiation. The analyses period of 6 months was adopted for the same reason as described in the procedure of ECG recording. To sedate the dogs, 10 μg/kg medetomidine was administered intravenously. Immediately after, an additional 10 μg/kg medetomidine was administered intramuscularly. The sedation level was maintained for a drowsy or light-sleep stage. Based on the sedation level, an additional medetomidine 10 μg/kg was administered intravenously or intramuscularly. These stages were determined by the dogs' appearance and finding vertex sharp waves, sleep spindles, or K complexes in the EEG trace[32, 33]. The additional medetomidine administration was stopped when the total dosage reached 70 μg/kg. The EEG was recorded using a digital electroencephalograph (Neurofax EEG-9100, NIHON KOHDEN Co., Ltd., Japan), with sensitivity = 50 μV/mm, time constant = 0.3 seconds, and high cut filter = 120 Hz. A needle electrode (stainless steel EEG needle, 22.5 mm long, 0.22 mm diameter, 1.5 m cable, NE-224s, NIHON KOHDEN Co., Ltd., Japan) was used as the recording electrode. The electrodes were placed using a slight modification of the method of Hasegawa and Utsugi[27, 32]. Each electrode was placed subcutaneously at both sides symmetrically in the frontal (LF and RF), parietal (LP and RP), occipital (LO and RO), temporal (LT and RT), and three midline regions (Fz, Cz, and Oz). A reference electrode was placed on the left and right ears. A ground electrode was placed on the dorsal aspect of the neck above the atlas. Electrodes to record eye movement were placed above the left and right eyelids. The ECG on the digital electroencephalograph was recorded with electrodes placed on both axillae. Bilateral ear reference electrode derivation or average derivation and bipolar derivation were performed for the EEG recording. The EEG recording was performed for 30 minutes. To reverse the sedation after the EEG recording, atipamezole was administered intramuscularly.

EEG analyses

Visual and quantitative EEG analyses were performed. For visual EEG analysis, the number of epileptiform discharges (EDs) in each 30-minute EEG recording was counted visually by the investigators. EDs are defined as transient waves that stand out against background activity, such as spikes, sharp waves, polyspikes, spike–wave complexes, and rhythmic spikes[32, 34, 35]. A series of polyspikes or rhythmic spikes was counted as one ED. EDs with an amplitude of more than 50 μV were counted because low-amplitude EDs are considered borderline findings[32]. The number of EDs per minute was calculated and considered as the ED frequency. The irritative zone, which is the cortex area where EDs are generated[27], was also assessed as visual EEG analysis.

Quantitative EEG analysis was performed using an EEG mapping program (ATAMAP II, KISSEI COMTEC Co., Ltd., Japan). Sixty replications of 2-second epochs without artifacts and EDs were extracted randomly from the entire EEG. Thus, 2 minutes of recording was analyzed. Fast Fourier transform was used for each referential channel, and the relative power (%) of the spectral bands, delta (0.5–4.0 Hz), theta (4.0–8.0 Hz), alpha (8.0–13 Hz), and beta (13–30 Hz), was expressed. The spectral bands were calculated for every lead and averaged.

Outcome measures

Whether the dogs were a responder or not was assessed at the end of the study period. Additionally, the temporal changes in HRV parameters (time-domain and frequency-domain parameters) and EEG parameters (ED frequency and the relative power of each spectral band) from pre-VNS surgery to 6 months after VNS initiation were evaluated. Furthermore, the differences in the results of HRV and EEG

Table 1. Information about the dogs and the antiseizure drugs (ASD) administered in each dog

Dog	Breed	Age (months)	Sex	Body weight (kg)	Seizure type	ASDs
A	Shetland sheepdog	64	Male	13.75	FS-GTCS	ZNS, KBr, LEV, GBP
B	Weimaraner	100	Neutered male	32.60	FS-GTCS	ZNS, KBr, LEV
C	Beagle	60	Female	8.30	FS-GTCS	PB, KBr
D	Beagle	121	Neutered female	9.20	GTCS	PB

Dogs A–C had focal epileptic seizures evolving into generalized tonic–clonic seizures (FS-GTCS) and dog D had generalized tonic–clonic seizures (GTCS). ZNS: zonisamide; KBr: potassium bromide; LEV: levetiracetam; PB: phenobarbital; and GBP: gabapentin.

Table 2. Therapeutic outcomes of VNS therapy in each dog

Dog	Baseline period (months)	Seizure frequency (per month) during the baseline period	Observation period (months)	Seizure frequency (per month) during the observation period	Reduction rate (%)	Determination
A	6	62.00	20	6.30	89.8	Responder
B	9	1.67	8	1.00	40.0	Responder
C	11	1.45	23	1.74	−20.0	Non-responder
D	4	5.00	8	8.25	−65.0	Non-responder

parameters before VNS initiation between responders and non-responders were assessed, and the irritative zone of each dog was evaluated.

Results

Dogs

Two dogs (A and B) referred to the neurology service at AUVTH because of DRE met the criteria for candidates of VNS therapy. Dog A and B were diagnosed with IE. During the study period, four dogs received VNS therapy (two patient dogs and two laboratory dogs). The median age of the dogs at enrollment was 82 months (range: 60–121 months). There was one each of a neutered male, male, neutered female, and female. Their median body weight was 11.48 kg (range: 8.3–32.6 kg). Two of the four (dogs C and D) were laboratory dogs with epilepsy: dog C with IE and dog D with SE. The diagnosis of IE in dog C was made according to the tier III level of the International Veterinary Epilepsy Task Force (IVETF) consensus proposal[28]. Dog D had porencephaly of the left cerebral hemisphere, diagnosed by an MRI scan. Dogs A, B, and C had focal epileptic seizures evolving into generalized tonic–clonic seizures. Dog D had generalized tonic–clonic seizures. The details, including the ASD regimen in each dog, are described in Table 1.

Therapeutic outcomes

The median observation period at the end of the study period was 14 months (range: 8–23 months). Dogs A and B had ≥ 20% reduction in seizure frequency, and dogs C and D had an increase in seizure frequency after VNS initiation (Table 2). Thus, dogs A and B were determined to be responders, and dogs C and D were determined to be non-responders. The stimulation parameters of VNS at the end of the study period in each dog are shown in Table 3.

HRV analyses

Dog A had only a single ECG recording before VNS. HRV analyses 4 and 5 months after VNS initiation were not performed for dog C because the dog had received surgery for gastrointestinal foreign body obstruction 4 months after VNS initiation. HRV analyses 5 months after VNS initiation was also excluded due to suspected influence of the surgery to HRV. HRV could not be assessed in dog D 5 months after VNS initiation because the dog did not become in the resting state in an hour. The temporal changes in time-domain and frequency-domain parameters in all dogs are presented in

Table 3. Stimulation parameters at the end of the study period in each dog

Dog	Output current (mA)	Pulse width (μs)	Frequency (Hz)	Stimulation ON-time (sec)	Stimulation OFF-time (min)
A	0.50	250	20	30	1.1
B	0.75	250	20	30	1.8
C	1.00	250	20	30	3.0
D	0.50	250	20	30	1.1

Fig. 1. The results of the LF/HF ratio showed a tendency to decrease after VNS initiation in all dogs. No other parameters had a tendency of increasing or decreasing over time. Furthermore, there were no notable differences in the temporal changes in each parameter between responders and non-responders.

The baseline of each HRV parameter in the responder and non-responder groups is shown in Fig. 2. Responders had a lower LF/HF ratio and a higher RMSSD than non-responders.

EEG analyses

Only dog A had a single EEG recording before VNS initiation. As dog D had porencephaly and human patients with porencephaly have been known to have low-amplitude EEG[35], EDs with amplitude <50 μV were also counted in this dog. Both the right and left temporal lobes were the irritative zones of dogs A and B. The left parietal lobe and right frontal lobe were the irritative zones of dogs C and D, respectively. The temporal changes in ED frequency and the relative power of each spectral band in all dogs are presented in Fig. 3. No parameters had a tendency of increasing or decreasing over time. There were no notable differences in the temporal changes in each parameter between responders and non-responders.

The baseline ED frequency and the relative power in each band in the responder and non-responder groups are shown in Fig. 4. No differences were observed between responders and non-responders.

Discussion

This preliminary study investigated whether HRV or EEG could predict the therapeutic outcomes of VNS therapy in dogs with epilepsy before or within 6 months after VNS initiation. The temporal changes in HRV and EEG parameters between responders and non-responders of VNS therapy were compared. Moreover, the results of HRV and EEG parameters before VNS implantation surgery between responders and non-responders were compared. No differences were observed in the temporal changes in HRV and EEG parameters between responders and non-responders. Therefore, we did not find a parameter that could predict the future outcome by the temporal change. However, we found two parameters in HRV (LF/HF ratio and RMSSD) that had differences in the preoperative results between responders and non-responders. Additionally, the irritative zone on the EEG was different between responders and non-responders.

The LF/HF ratio tended to decrease after VNS initiation in all dogs over time (Fig. 1). We hypothesized that there would be a parameter that presents a notable difference between responders and non-responders; however, no parameter showed a notable difference between groups. The low LF/HF ratio means an increase in parasympathetic activity[37]. A study in humans that investigated HRV before and 6 and 12 months after VNS initiation showed a decrease in the LF/HF ratio in all patients, regardless of the outcomes of VNS therapy[37]. These results are consistent with our study results. As this change in the LF/HF ratio was found in all dogs regardless of their treatment outcome, this change was presumably due to chronic stimulation of the left vagosympathetic trunk.

In the literature, a study evaluated the influence of VNS on HRV in dogs[38]. This report showed that VNS did not affect HRV. However, our study found a decrease in the LF/HF ratio. This inconsistency may be due to a difference in the stimulation period of VNS. The previous report activated VNS for only 55 minutes. Thus, we considered that chronic

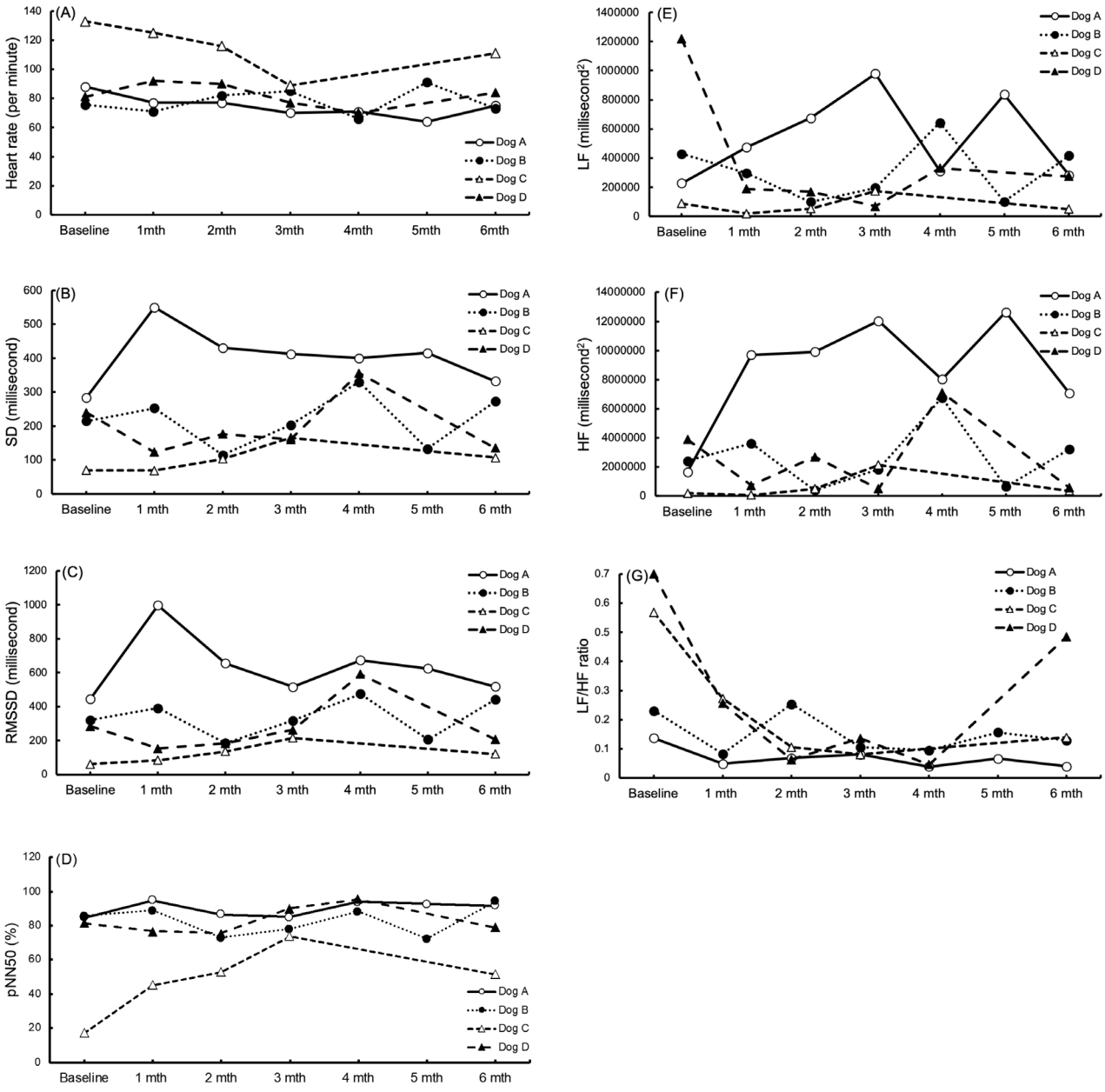

Fig. 1 Temporal changes in the results of HRV analysis.
The temporal changes in the heart rate (A), SD (B), RMSSD (C), pNN50 (D), LF (E), HF (F), and LF/HF ratio (G). In all dogs, the LF/HF ratio showed a tendency to decrease from the baseline (G). No parameters showed a difference between responders (dogs A and B) and non-responders (dogs C and D).

VNS might affect HRV analysis, especially the LF/HF ratio, in dogs.

In this study, the responder group included one Shetland Sheepdog and one Weimaraner, and the non-responder group included two Beagles. In a previous study, the influence of HRV analysis on dog breeds was investigated, and only brachycephalic dogs showed a higher vagal tone than the other breeds[39]. Another study reported that body weight, age, and sex did not affect HRV analysis[40]. Thus, HRV can be assessed regardless of the physical characteristics of dogs, except for brachycephalic dogs. The dogs in this study were administered ASDs. It was found that phenobarbital had no influence on HRV analysis in dogs[41]. We could not deny interference with HRV by the other ASDs as there are

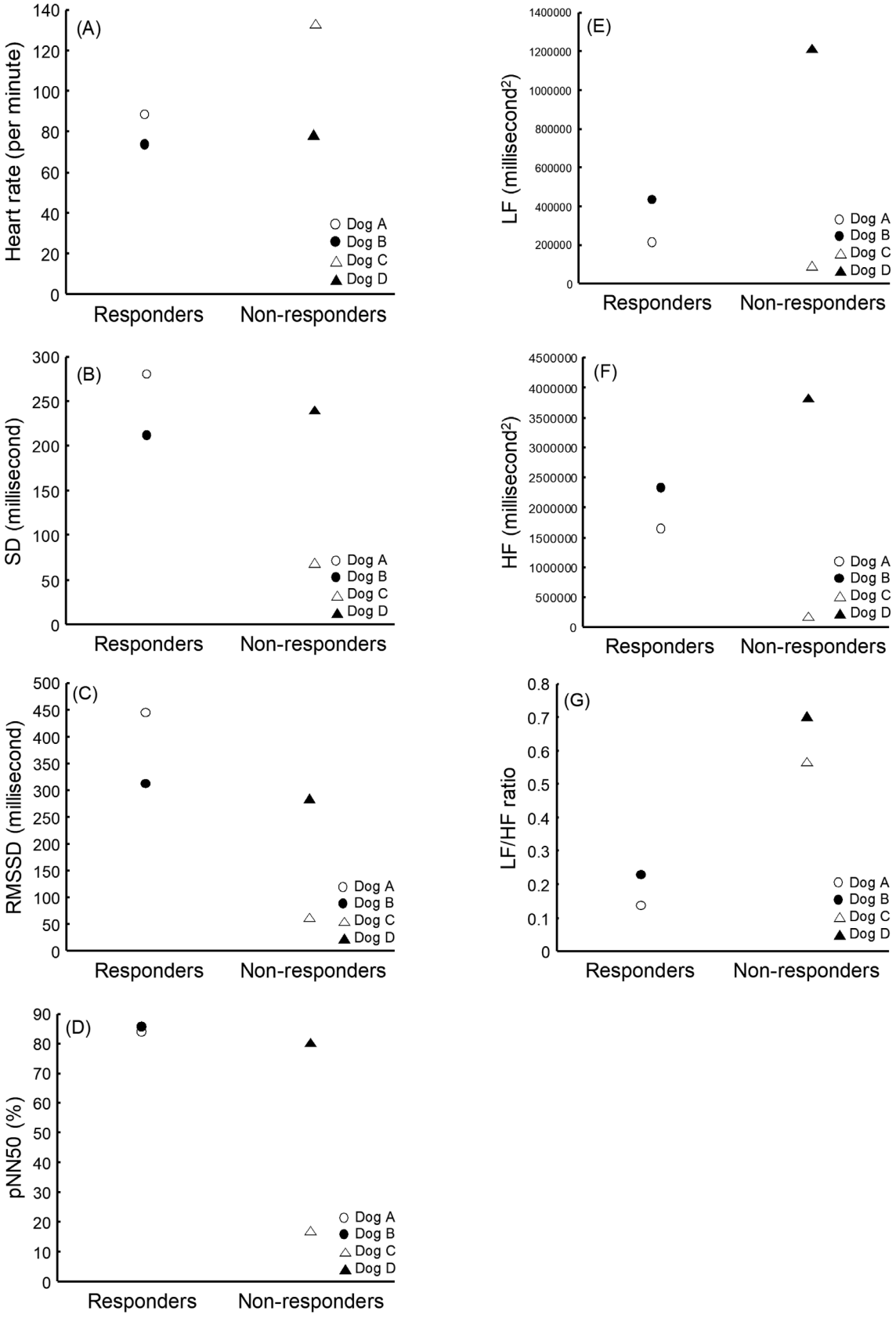

Fig. 2 Comparison of the baseline HRV analysis between responders and non-responders.
The baseline heart rate (A), SD (B), RMSSD (C), pNN50 (D), LF (E), HF (F), and LF/HF ratio (G). Responders showed a higher RMSSD (C) and a lower LF/HF ratio (G) than non-responders.

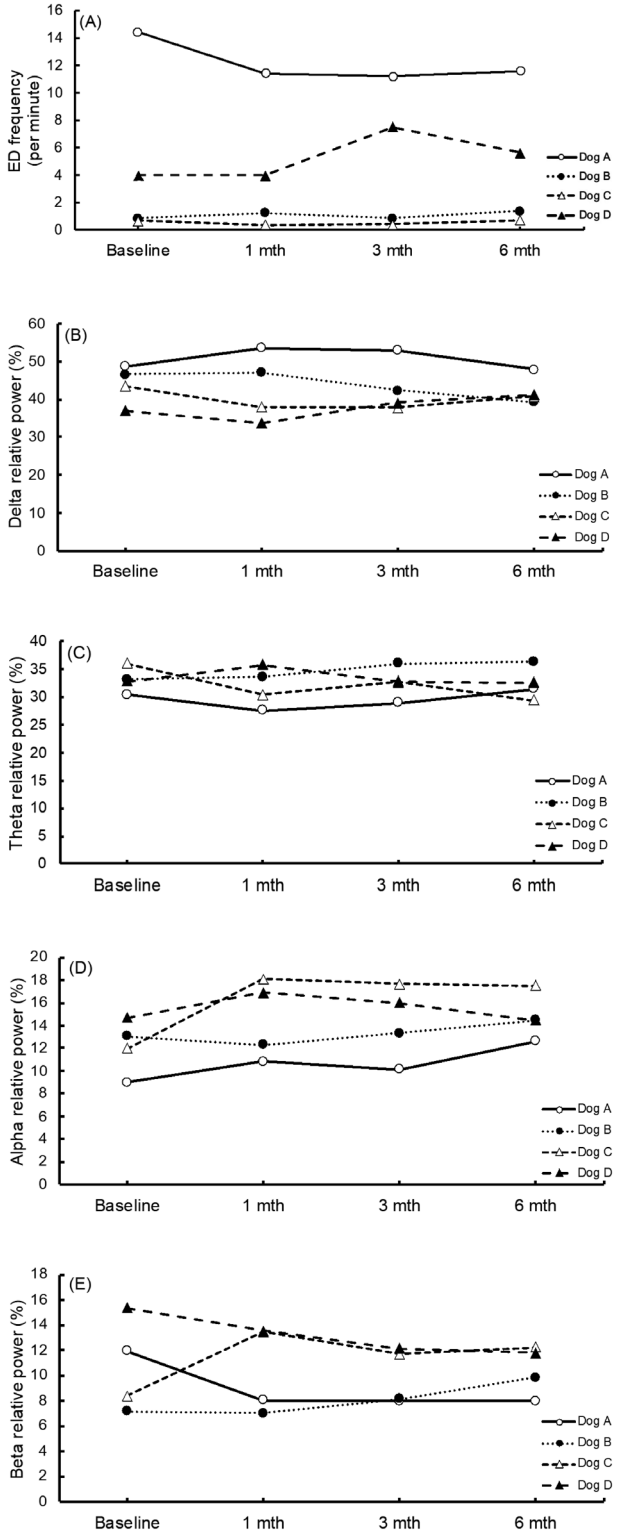

Fig. 3 Temporal changes in the results of EEG analysis. The temporal changes in the ED frequency (A), delta relative power (B), theta relative power (C), alpha relative power (D), and beta relative power (E). No parameters showed a difference between responders (dogs A and B) and non-responders (dogs C and D).

no such reports of the other ASDs in veterinary medicine. However, we did not change the ASD regimen of the dogs during the study period. Therefore, in this study, we determined that VNS therapy can be the cause for the change in HRV over time.

When comparing HRV before VNS initiation between responders and non-responders, we found that responders had a lower LF/HF ratio and a higher RMSSD than non-responders. These results are consistent with a previous study in humans[24, 25]. The LF/HF ratio represents the balance of the autonomic nervous system: a high LF/HF ratio shows an increase in sympathetic activity and a low LF/HF ratio reflects an increase in parasympathetic activity. A high RMSSD represents an increase in parasympathetic activity. Therefore, dogs with predominant parasympathetic activity presumably respond well to VNS therapy, so low LF/HF ratio and high RMSSD might be preoperative predictors of the therapeutic outcomes of VNS therapy. We need to note that responders were administered zonisamide, potassium bromide, or levetiracetam, which were not used in non-responder dogs. Thus, there may be a possibility that these ASDs decreased the LF/HF ratio and increased the RMSSD. However, finding high parasympathetic activity in preoperative HRV has the potential to be a predictor. As our preliminary study found that higher parasympathetic activity in preoperative HRV is a candidate for a positive predictor of VNS outcome, larger studies should be conducted to evaluate this finding.

Although the mechanism is still elusive, one study indicated that dogs with epilepsy have higher parasympathetic activity than dogs without epilepsy[41]. Our findings suggested that epileptic dogs with higher parasympathetic activity than other epileptic dogs respond to VNS therapy. Based on these results, we assumed that epileptic dogs show an increase in parasympathetic activity because of a resistant reaction to epilepsy rather than a result of epilepsy. Furthermore, there may be pathophysiology in which seizures are suppressed by increased parasympathetic activity. Indeed, inhaling lavender essential oil showed an increase in parasympathetic activity[42] and an anticonvulsant effect[43] in humans. Some epileptic dogs with such pathophysiology probably tend to increase their parasympathetic tone on

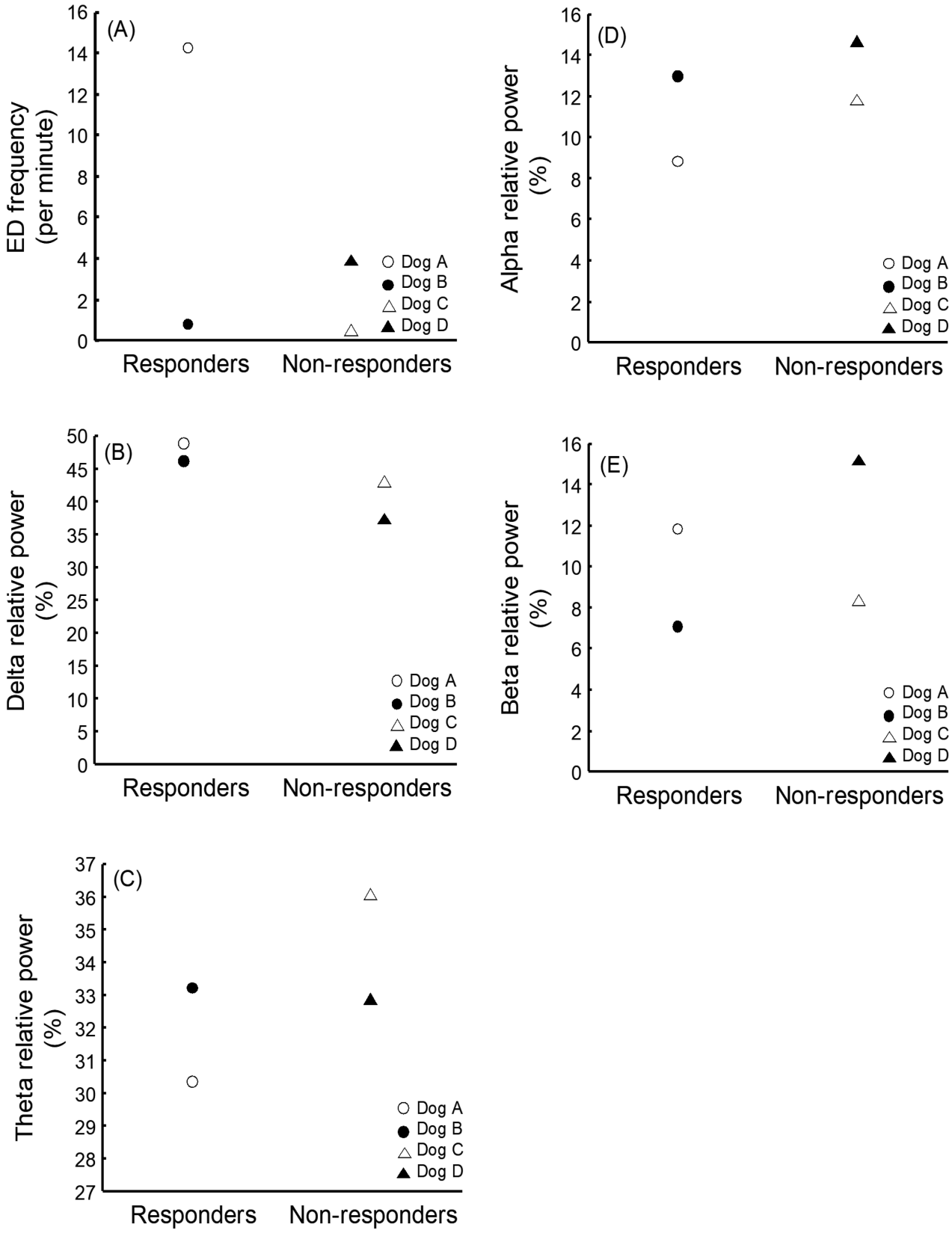

Fig. 4 Comparison of the baseline EEG analysis between responders and non-responders.
The baseline ED frequency (A), delta relative power (B), theta relative power (C), alpha relative power (D), and beta relative power (E). There were no notable findings between the results of responders and non-responders.

their own. Hence, we hypothesized that epileptic dogs with higher parasympathetic activity than other epileptic dogs respond to VNS therapy because VNS reinforced their resistant reaction to epilepsy.

As there is no standard protocol for ECG recording to assess HRV analysis in veterinary medicine, there are slight differences in recording methods among studies in dogs[14, 39–41]. Twenty-four-hour long-term ECG and 5-minute recordings were both recommended in humans[12]. We decided to record the ECG for 5 minutes because the ECG recording was performed during the reevaluation of the effect of VNS therapy. Additionally, HRV analysis could be affected by several factors, particularly circadian rhythm and heart rate[12]. Thus, we started recording the ECG when the dogs relaxed to maintain their heart rate, and all ECG recordings were performed in the same time frame to exclude the influence of circadian rhythm.

In visual EEG analysis, we could not find a difference in the temporal change in ED frequency between responders and non-responders. Further, there were no notable differences in the results of ED frequency before VNS initiation between responders and non-responders. Several studies in humans reported that patients whose seizures decreased by VNS achieved a reduction in the number of EDs[44–46]. In our study, one responder dog had a slight decrease in ED frequency after VNS initiation; however, the other responder had no reduction. Thus, the temporal change in ED frequency might not be used as a predictor of the treatment outcome of VNS therapy in dogs. The reason why one dog had no reduction in ED frequency despite a seizure frequency decrease is unclear.

In this study, although there were no notable findings in ED frequency, we found that responders had their irritative zone in the temporal lobe, whereas the irritative zone of non-responders was in another cortex area. The focality and localization of EDs as a preoperative biomarker have been well investigated in humans[17, 23]. Although it is still controversial, studies have indicated that patients with multifocal or bilateral EDs and patients who had EDs in the temporal lobe responded well to VNS therapy[17, 23]. Thus, we considered that the irritative zone might be a predictor of the therapeutic effect of VNS therapy in dogs.

No considerable differences were observed in the temporal change in quantitative EEG analysis results between responders and non-responders. The results of quantitative EEG analysis before VNS initiation between responders and non-responders also showed no differences. Several studies reported that chronic VNS altered the background activity of EEG, such as increasing the power spectrum of gamma frequencies and desynchronization of EEG activity, in humans[47, 48]. An experimental study in cats showed the desynchronization of EEG activity with VNS[15]. However, it is difficult to compare our results with those in cats because the immobilization method was different. Contrary to the results in humans and cats, VNS did not affect the background activity of EEG in our study. To keep the sedation level in the dogs at a drowsy or light-sleep stage, medetomidine was administered several times. Thus, we considered that medetomidine might have masked the influence of VNS on the background activity of EEG.

There were considerable difference in the obseravation period between each dog; the longest duration was 23 months and the shortest was 8 months. We did not align the observation period to the shortest period. This study compared the results of HRV and EEG between responders and non-reponders. Thus, the determination of responders or non-reponders was the most important point. Since the therapeutic effect of VNS therapy is known to increase over time in humans, we considered that a long obseravation period provides higher confidence in the determination of whether the dogs to be a responder or not. Therefore, the long observation period was preferable for this study. Nevertheless, the observation period in two out of four dogs were 8 months due to the end of the study period. However, 8 months is an enough period to assess a short-term outcome of a therapeutic intervention against canine epilepsy[29].

Adjustment of the stimulation intensity is considered an important factor in VNS therapy. Six months after VNS initiation, two dogs had an output current of 0.50 mA, and the output current in other two dogs was 0.75 and 2.25 mA. The output current in one dog was 2.25 mA and considerably higher than the other dogs. We increased the output current of this dog as high as humans' setting for the aim of our another study (manuscript in preparation), and the output

current was decreased to 1.0 mA at 18 months after VNS initiation. This dog had a higher output current than the other dogs; however, the temporal changes in HRV and EEG parameters had no notable differences between this dog and the other dogs. Thus, the stimulus intensity had no influence on HRV and EEG in our dogs.

There are several limitations to this study. The observation period of one non-responder dog was 8 months. As the effect of VNS therapy increases over time, this dog might have become a responder later. However, the dog is unlikely to become a responder because the dog showed an increase in its seizure frequency during the observation period. VNS turns on according to the programmed stimulation cycle (e.g., 5 minutes OFF and 30 seconds ON), and the VNS ON condition has been known to affect the result of quantitative EEG analysis[26]. Thus, it might be preferable to analyze only the period of the VNS OFF condition to evaluate the chronic influence of VNS on EEG, and the same might be true in HRV. However, we could not completely exclude the period of the VNS ON condition from the assessment because it was impossible to know the timing of the VNS ON condition in most of the dogs. In this preliminary study, non-responders included only a single breed. Although the previoud study indicated that breeds did not affetc HRV, our results in non-responders (e.g., non-responders had higher LF/HF ratio than responders) should assess with more breeds. Another limitation is that there was no control group: in this study, non-epileptic dogs underwent VNS therapy. However, VNS implantation surgery is invasive, and performing the surgery in non-epileptic dogs may be an ethical problem. Furthermore, the devices are expensive. Thus, we decided not to have a control group. As this was a preliminary study, the sample size was small. We would like to apply our findings in a clinical setting and evaluate the preoperative HRV and irritative zone on the EEG as predictors of VNS outcomes in dogs with DRE.

Conclusions

This preliminary study was the first to investigate the predictors of treatment outcomes of VNS therapy in dogs with epilepsy. The preoperative assessment of HRV, especially the LF/HF ratio and RMSSD, and the irritative zone on the EEG showed their potential as predictors. Thus, larger studies in dogs with DRE are warranted to evaluate the findings.

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