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Gas Chromatography-Mass Spectrometry-Based Metabolic Profiling of Cerebrospinal Fluid from Epileptic Dogs

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ABSTRACT. Epilepsy is a common neurological disorder with seizures, but diagnostic approaches in veterinary clinics remain limited. Cerebrospinal fluid (CSF) is a body fluid used for diagnosis in veterinary medicine. In this study, we explored canine epilepsy diagnostic biomarkers using gas chromatography-mass spectrometry (GC-MS)-based metabolic profiling of CSF and multivariate data analysis. Profiles for subjects with idiopathic epilepsy differed significantly from those of healthy controls and subjects with symptomatic epilepsy. Among 60 identified metabolites, the levels of 20 differed significantly among the three groups. Glutamic acid was significantly increased in idiopathic epilepsy, and some metabolites including ascorbic acid were changed in both forms of epilepsy. These findings show that metabolic profiles of CSF differ between idiopathic and symptomatic epilepsy and that metabolites including glutamic acid and ascorbic acid in CSF may be useful for diagnosis of canine epilepsy.
KEY WORDS: epilepsy, GC-MS, metabolic profiling.

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Epilepsy is a common chronic neurological disorder in dogs with an estimated prevalence of 0.5% to 5.7% [16]. The terminology of epilepsy is complex and often inconsistent in the literature [14]. There are over 40 epileptic syndromes and related conditions in humans, and epileptic syndromes are classified by such phenotypic criteria as age of onset, type of electroencephalographic abnormalities, seizure characteristics and type of stimulus that induces seizures [6]. In veterinary neurology, epilepsy is categorized as idiopathic, symptomatic, probable symptomatic or reactive seizure. Classification of canine seizures is difficult, because veterinarians usually have to determine the seizure type from the owner's observations [3]. Therefore, epilepsy is not usually differentiated into syndromes in dogs.

Differential diagnosis of idiopathic and symptomatic epilepsy in canine cases is important, because of differences in prognosis and treatment. Diagnosis of idiopathic epilepsy (IE) is performed based on exclusion, age of onset, evidence of forebrain dysfunction in an interictal neurological examination or the presence of a macroscopically identifiable intracranial lesion; however, prolonged seizures or status epilepticus may cause transient neurological defects and changes on MRI [18]. Symptomatic epilepsy (SE) is easier to diagnose correctly, because of the increased availability of MRI in veterinary practice. Most dogs with recurrent seizures with no identifiable underlying cause are classified as having IE when there is no interictal abnormality. This may be due to the difficulty of seizure description or the lack of a functional diagnostic method, such as electroencephalography (EEG), which is not routinely used in veterinary practice.

Metabolomics is a fast and reproducible method that is particularly useful in toxicology and pathognomy and directly reflects biological events [4] through evaluation of changes in levels of endogenous metabolites in biological samples due to physiological stimuli or genetic changes. Metabolomics can also be used for global determination of metabolites or patterns of biomarkers that change as a result of a drug toxicity or in relation to a particular disease [9, 12]. The utility of the method is further increased by the use of peripheral fluids, such as urine and plasma, for metabolomic analysis. Current metabolomic technologies are typically based on gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR). These techniques provide direct information on metabolism and on reciprocal relationships between metabolic networks and underlying mechanisms. In particular, GC and CE offer rapid analysis and efficient resolution, and MS has excellent selectivity and sensitivity. Thus, various clinical and toxicological applications of GC-MS and CE-MS, together with sample collection and pretreatment methods, have been developed for detection and identification of endogenous low-molecular-weight biomarkers [11, 17].

Here, we examined GC-MS-based metabolic profiling of low-molecular-weight metabolites in CSF from cases of ca-

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nine epilepsy. The results provide new insights into changes in metabolites based on multivariate analyses and conventional diagnoses made in a veterinary clinic. These findings may contribute to development of diagnostic and therapeutic agents for canine epilepsy.

MATERIAL AND METHODS

Subjects and data collection: The subjects were dogs examined at Kakogawa Animal Hospital, Kakogawa, Hyogo, Japan between March 2005 and April 2010. Concurrent MRI and CSF analysis were evaluated retrospectively using case records. Cases were excluded, if permission of the owner, interictal, hematological and biochemical data from physical and neurologic examinations, CSF findings or MRI data were unavailable.

Routine CSF analysis included the total nucleated cell count (TNCC) and measurement of microprotein concentration using a Micro Total Protein Kit (Wako Pure Chemicals, Osaka, Japan). Protein concentration was considered to be elevated if>25 mg/d*l*, and pleocytosis was judged to be present if TNCC in the CSF was>5 /*μl*. If CSF analysis was abnormal, titers for infectious causes of encephalitis were evaluated, including canine distemper virus, *Ehrlichia canis*, *Neospora canis*, *Toxoplasma gondii*, *Aspergillus sp*. and *Cryptococcus sp*.

MRI was performed with 0.2 T system (Signa, GE Medical Imaging Systems, Tokyo, Japan), and MRI findings were evaluated independently of CSF analysis. Routine imaging included T2, fluid attenuation inversion recovery and T1-weighted sequences performed before and after administration of intravenous gadolinium (Gadoteridol, Eizai, Tokyo, Japan). Images were obtained in at least 3 orthogonal planes relative to the region of interest.

Clinical diagnosis of IE was defined retrospectively by exclusion of cases that with a positive CSF titer or fungal or bacterial culture or showed morphological abnormality on MRI or based on the results of clinical and neurologic examinations. Cases that responded to anticonvulsant therapy (oral phenobarbital or bromide) and required this therapy after a presumptive diagnosis were included in the IE group. SE due to intracranial diseases was diagnosed when abnormalities were found in brain MRI, CSF tap results or clinical course after presentation. Particular emphasis was placed on the MRI findings. Normal CSF was sampled from a control group of 18 healthy domestic dogs with the owner's consent.

CSF preparation: CSF preparation for extraction and derivatization of low-molecular weight metabolites was performed as described by Kuhara *et al* [11]. Collected CSF was immediately centrifuged at 5,000 g for 10 min at 4°C, and the supernatant was transferred to a clean tube and stored at −80°C until use. To extract low-molecular-weight metabolites, 25 *μl* of CSF was mixed with 900 *μl* of 70% ethanol containing 10 *n*g of 2-isopropyl malic acid and centrifuged at 15,000 g for 10 min at 4°C. The solution was then evaporated to dryness. Metabolites in the dried residue were converted to trimethylsilyl derivatives with 100 *μl* of *N,O*-bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (10:1) (Thermo, Tokyo, Japan) and analyzed by GC-MS.

GC-MS analysis and data processing: GC-MS analysis was carried out using a GC-MS-QP2010plus system (Shimadzu, Kyoto, Japan) with Inert Cap 5 MS/NP (0.25 mm I.D. × 30 m, 0.25 *μ*m; GL Science, Tokyo, Japan), as described in Kuhara *et al* [11]. Chromatogram acquisition, detection of mass spectral peaks and waveform processing were performed using Shimadzu GC-MS solution software (ver. 2.53). Identification of low-molecular-weight metabolites was carried out using the library of the National Institute of Standards and Technology. Semiquantitative assessment was performed using the peak intensity of 2-isopropylmalic acid as an internal standard.

Multiple classification analysis and statics: A dataset for multiple classification analysis was compiled from the metabolic profiling data. A three-dimensional matrix was constructed based on sample names (observations), metabolite name (variable indices) and normalized peak intensities (variables). Partial least square discriminant analysis (PLS-DA) was performed in MetaboAnalyst 2.0 [20]. Statistical significance was analyzed by Steel-Dwass test. In all analyses, $P<0.05$ was taken to indicate statistical significance.

RESULTS

Subject characteristics and CSF analysis: A total of 16 cases met the inclusion criteria for canine IE. There were subtle neurological deficits in some cases, including delayed proprioceptive positioning on one side, in interictal neurological examinations (Table 1). The mean age at CSF collection was 5.5 ± 4.5 years (median 4.3, range 0.5 to 14.2), and that at first onset of seizure was 3.9 ± 2.6 years (median 3.4, range 0.5 to 8.2). SE was diagnosed in 19 cases, including 3 with meningoencephalitis, 7 with meningoencephalomyelitis of undetermined etiology (MUE) and 9 with brain tumors. The mean age at CSF collection was 8.9 ± 4.0 years (median 8.5, range 2.9 to 15.9), and that at first onset of seizure was 8.8 ± 4.1 years (median 8.2, range 2.6 to 15.8). Background data did not differ significantly among the disease categories in the SE group. The mean age of the healthy controls was 4.8 ± 4.7 years (median 2.3, range 0.5 to 15.2). Age at CSF collection in the IE group was slightly lower than that in the SE group (P=0.026) and not significantly different from that in the control group. Age at CSF collection in the SE group was significantly higher than that in the control group (P=0.006). All CSF was collected more than three days after the last seizure. The mean protein concentrations in the CSF were 22.0 mg/d*l* (median 18.4, range 8.1 to 57.1 mg/d*l*) in controls, 20.4 mg/d*l* (median 16.6, range 3.6 to 46.8 mg/d*l*) in the IE group and 54.9 mg/d*l* (median 36.3, range 2.3 to 202.9 mg/d*l*) in the SE group with no significant differences among the groups.

Evaluation of differences in CSF profiles using multiple classification analysis: A total of 60 metabolites identified in the CSF were analyzed by PLS-DA. A PLS-DA score plot showed clear separation among the groups (Fig. 1A). The metabolites largely responsible for this separation included ascorbic acid, glyceric acid, threonic acid, glutaric acid,

Table 1. Characteristics of subjects

Breed	Sex	Age at CSF collection	Age at first seizure onset	Neurologic status at rest	Diagnosis	CSF protein (mg/dl)
Beagle	S	0.8	0.8	N	IE	15.7
Pomeranian	C	4.3	3.9	N	IE	46.8
Chihuahua	F	3.0	3.0	N	IE	33.0
Bernese Mountain	C	4.3	3.4	N	IE	9.9
Chihuahua	M	4.9	2.0	N	IE	26.4
Yorkshire Terrier	C	1.1	3.4	N	IE	8.3
Toy Poodle	M	2.0	0.5	N	IE	13.2
Beagle	C	1.6	1.4	N	IE	15.2
Miniature Dachshund	M	5.6	3.5	N	IE	17.1
Shetland Sheepdog	C	14.2	7.2	N	IE	3.6
Chihuahua	M	0.5	0.5	N	IE	27.2
Mong	S	13.5	7.3	N	IE	39.5
Welsh Corgi	M	8.2	6.4	N	IE	16.8
Welsh Corgi	C	7.2	7.5	N	IE	20.3
Miniature Schnauzer	C	13.6	8.2	A	IE	16.4
Yorkshire Terrier	S	3.5	2.8	N	IE	16.4
Chihuahua	M	2.9	2.6	N	SE (MUE)	2.3
Pug	M	3.2	3.2	A	SE (MUE)	17.7
Labrador Retriever	M	8.5	8.2	A	SE (MUE)	43.0
Shih-tzu	M	3.5	3.5	A	SE (MUE)	8.2
Mong	F	6.7	5.8	A	SE (MUE)	194.7
Mong	M	13.9	13.9	A	SE (MUE)	5.8
Mong	C	15.0	14.9	A	SE (MUE)	2.4
Golden Retriever	F	8.1	7.3	N	SE (brain tumor)	10.0
Labrador Retriever	F	7.8	7.8	A	SE (brain tumor)	80.7
Welsh Corgi	M	8.7	8.6	A	SE (brain tumor)	89.0
Golden Retriever	M	10.6	10.6	N	SE (brain tumor)	2.6
Shiba	M	11.1	11.0	N	SE (brain tumor)	83.7
Welsh Corgi	C	11.8	11.8	A	SE (brain tumor)	56.2
Shiba	M	12.5	12.5	A	SE (brain tumor)	30.5
Mong	M	15.9	15.8	A	SE (brain tumor)	36.3
Pug	M	3.5	3.5	A	SE (brain tumor)	64.5
Beagle	F	6.1	6.1	N	SE (meningoencephalitis)	29.7
French Bulldog	F	6.3	6.3	A	SE (meningoencephalitis)	202.9
Pug	M	13.3	13.2	A	SE (meningoencephalitis)	82.9
Maltese	C	1.1	-	N	Control	40.3
Mong	S	1.4	-	N	Control	12.1
Shiba	M	0.6	-	N	Control	32.0
Toy Poodle	M	10.3	-	N	Control	27.8
Miniature Dachshund	C	8.3	-	N	Control	9.2
West Highland White Terrier	S	7.3	-	N	Control	57.1
Papillon	M	0.8	-	N	Control	21.2
Shih-tzu	C	15.2	-	N	Control	8.6
Mong	C	12.0	-	N	Control	18.2
Corgi	F	10.4	-	N	Control	23.2
Italian Greyhound	M	7.8	-	N	Control	20.1
Welsh Corgi	M	2.3	-	N	Control	17.7
Maltese	F	0.6	-	N	Control	8.9
Welsh Corgi	M	0.5	-	N	Control	46.3
Toy Poodle	M	0.5	-	N	Control	8.1
Golden Retriever	M	4.9	-	N	Control	13.3
Shiba	M	2.3	-	N	Control	13.1
Pomeranian	F	0.6	-	N	Control	18.6

F, female; M, male; C, casted; S, spayed; A, abnormal; N, normal.

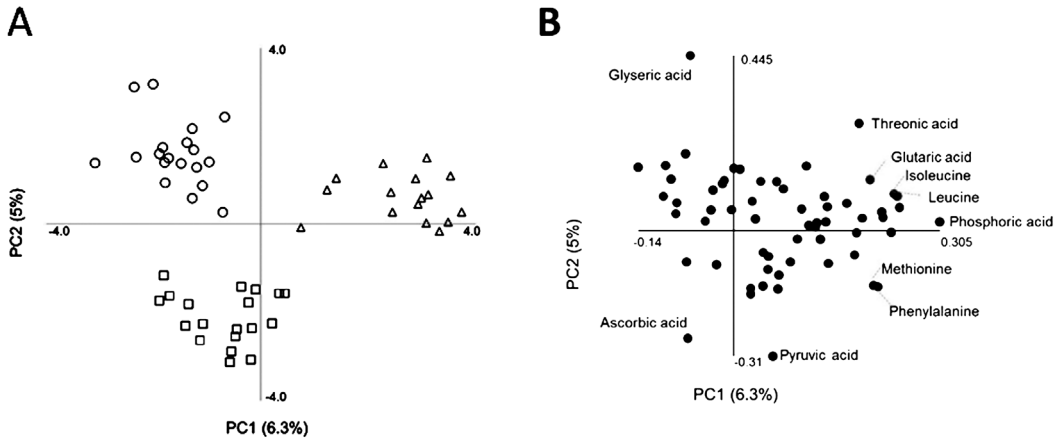

Fig. 1. Classification of canine CSF metabolites by PLS-DA. A. GC-MS data for the control (squares), IE (triangles) and SE (circles) groups were analyzed using PLS-DA, and the results are represented as a score plot. B. Loading plot of canine CSF metabolites.

isoleucine, leucine, phosphoric acid, methionine, phenylalanine and pyruvic acid (Fig. 1B). The levels of 20 metabolites differed significantly among the groups, and 16 metabolites showed significant changes in IE cases compared to controls (Fig. 2). The levels of glutamic acid, isoleucine, phosphoric acid, malonate, stearic acid, glutaric acid, glutamine, acetic acid, oxanilic acid, glyceric acid, threonic acid and succinic acid increased, and those of ascorbic acid, pyruvic acid and 3-hydroxy-2-butanone decreased in IE. The levels of 13 metabolites differed significantly between IE and SE, including higher levels of glutamic acid, isoleucine, methionine and serine in IE. The levels of 9 metabolites were significantly different in SE cases compared to controls with glutamic acid, methionine, phenylalanine, ascorbic acid, malic acid, acetone and pyruvic acid all decreasing in SE. Only glutamic acid showed significant differences among all three groups.

DISCUSSION

Epileptic seizure is a common neurological disease, but is difficult to diagnose by observation. In most patients, seizures start in focal brain regions following a variety of brain insults. The present study was performed in a population of epileptic dogs encountered commonly in veterinary practice. Classification of canine epilepsy as IE or SE is currently performed by exclusion of identifiable underlying causes for epileptic seizures, and there is a need to establish a method for rapid diagnosis of these types of epilepsy.

Recent developments in metabolomics permit mining of metabolic information in disease states and during treatment [15]. The approach is founded on the assumption that a disease signature exists within a given biological sample, but requires no prior knowledge of sample content. Suitable body fluids for metabolomics include urine, plasma, serum, saliva and tissue homogenates, all of which carry the signature of biochemical and metabolic responses to a myriad of genetic and environmental influences [15]. Metabolomics also holds promise for early diagnosis of disease and for identification of metabolic pathways as targets for disease amelioration [13]. Serum metabolomics of CNS disorders has been vindicated by identification of blood biomarkers of Parkinson's disease [2]. Serum is the basic sample for metabolomics, being readily collectable by minimally invasive techniques, but serum metabolic profiles of responders and non-responders to anti-epilepsy drugs may not be clearly distinguishable [1]. This suggests that serum may not always be optimal for evaluation of heterogeneous disorders, such as epilepsy, because of significant blood-brain barrier interference with metabolite exchange between blood and CSF. Thus, collection of CSF may be better for diagnosis of epilepsy. For this reason, we used GC-MS-based canine CSF metabolic profiling to detect potential biomarkers applicable for diagnosis of epilepsy.

In canine CSF, 16 of 60 identified metabolites had significantly different levels in IE cases compared to controls. PLS-DA separated IE from SE and controls, and metabolites with higher loading values in this analysis were identified (Fig. 1). The levels of 20 metabolites, including amino acids, fatty acids and organic acids that could be potential diagnostic biomarkers for epilepsy, showed significant differences among the groups (Fig. 2).

It has been proposed that the balance of excitatory and inhibitory neurotransmitters is a key regulator of seizure [5]. Glutamic acid and γ-aminobutyric acid (GABA) are major excitatory and inhibitory compounds in brain, respectively. In this study, the glutamic acid content in IE increased significantly compared to those in SE and in controls. Levels of related compounds (glutamine, which is synthesized from glutamic acid and serine) in IE were also high compared to those in controls, whereas GABA levels in IE and SE did not differ from that in controls (data not shown). The glutamic acid level in CSF is regulated by glutamate transporters located on the surface of epithelial cells of veins, glia and neuronal cells and depends on the concentration in serum [7]. It

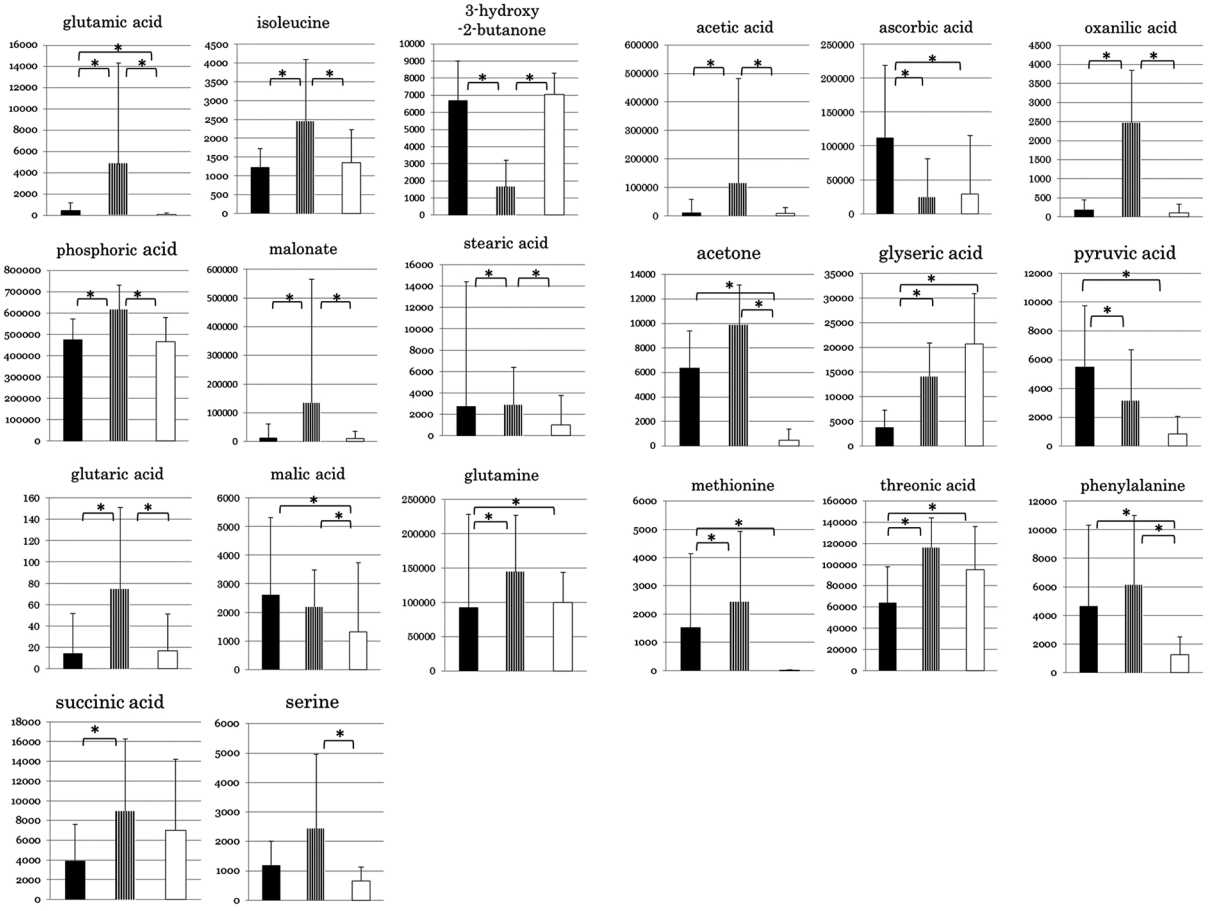

Fig. 2. Levels of metabolites in CSF in the control (black), IE (stripes) and SE (white) groups. * $P<0.05$ between two groups. Levels are expressed in arbitrary units (see Materials and Methods).

was also reported that some metabolites in brain are often excluded into extracellular fluid [18] and that the glutamic acid level in CSF has been related to the severity of neurological disorder in dog and cat models [8], although the level in CSF has also been found to decrease in canine epilepsy [5]. These findings indicate that a disorder of glutamic acid regulation and metabolism might be related to the pathogenesis of canine IE and that further evaluation of glutamic acid is required as a potential diagnostic biomarker for IE.

It was shown that levels of ascorbic acid, pyruvic acid, glyceric acid, threonic acid and glutamine were significantly changed in epilepsy (Fig. 2), indicating these can be a biomarker of epileptic symptom.

Reactive oxygen species (ROS) formation increases during seizures and removal of ROS depends on the antioxidant system. The protective effect of antioxidants may partly be due to antioxidant nutrients, such as ascorbic acid and carotenoids [19]. Ascorbic acid is a powerful water-soluble antioxidant that has a protective role against oxidative damage in tissues, including in central nervous system. Ascorbic acid contents in CSF of IE and SE cases were significantly lower than that in controls, but there was no significant difference between IE and SE. Threonic acid, a metabolite of ascorbic acid, was also elevated in both epilepsy groups. These findings indicate that ROS-related oxidative stress might be present in the brain in IE and SE and that ascorbic acid may be a potent biomarker for epilepsy. Further investigation is needed to evaluate these findings in chronic intracranial disease, but it is possible that ascorbic acid and its metabolite in CSF could be a marker of epileptic symptoms.

The decrease of pyruvic acid and increase of glyceric acid in epilepsy were significant in SE (Fig. 2). High glycolytic activity is common in tumors known as the Warburg effect, and serum levels of metabolites related to it are also known to be changed in tumors [10]. Thus, SE cases with tumors might have contributed to the differences in the metabolites among the groups in this study. Thus, in cases with epileptic symptoms, investigation of metabolic changes in CSF in those with brain tumors is needed to distinguish these cases from those with inflammatory disease.

In conclusion, GC-MS-based canine CSF metabolic profiles in IE differed from those in SE and in healthy controls. Glutamic acid may be a potent biomarker for IE, and ascorbic acid and threonic acid may be useful for general

detection of epilepsy. These findings will help to establish rapid diagnostic methods for canine epilepsy.

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REFERENCES

1. Al Zweiri, M., Sills, G. J., Leach, J. P., Bodie, M. J., Robertson, C., Watson, D. G. and Parkinson, J. A. 2010. Response to drug treatment in newly diagnosed epilepsy: A pilot study of ^{1}H NMR- and MS-based metabonomic analysis. *Epilepsy Res.* **88**: 189–195. [Medline] [CrossRef]
2. Bogdanov, M., Matson, W. R., Qang, L., Matson, T., Saunders-Pullman, R., Bressman, S. S. and Flint Beal, M. 2008. Metabolomic profiling to develop blood biomarkers for Parkinson's disease using ^{1}H NMR-Base metabonomics. *Nat. Med.* **8**: 1439–1444.
3. Chandler, K. 2006. Canine epilepsy: what can we learn from human seizure disorders? *Vet. J.* **172**: 207–217. [Medline] [CrossRef]
4. De Vos, R. C., Moco, S., Lommen, A., Keurentijes, J. J., Bion, R. and Hall, R. D. 2007. Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* **2**: 778–791. [Medline] [CrossRef]
5. Ellenberger, C., Mevissen, M., Doherr, M., Scholtysik, G. and Jaggy, A. 2004. Inhibitory and excitatory neurotransmitters in the cerebrospinal fluid of epileptic dogs. *Am. J. Vet. Res.* **65**: 1108–1113. [Medline] [CrossRef]
6. Engel, J. Jr. 1996. Introduction to temporal lobe epilepsy. *Epilepsy Res.* **26**: 141–150. [Medline] [CrossRef]
7. Gottlieb, M., Wang, Y. and Teichberg, V. 2003. Blood-mediated scavenging of cerebrospinal fluid glutamate. *J. Neurochem.* **87**: 119–126. [Medline] [CrossRef]
8. Hasegawa, D., Masuki, N., Fujita, M., Ono, K. and Orima, H. 2004. Kinetics of glutamate and γ-aminobutyric acid in cerebrospinal fluid in a canine model of complex partial status epilepticus induced by kainic acid. *J. Vet. Med. Sci.* **66**: 1555–1559. [Medline] [CrossRef]
9. Hasegawa, M., Ide, M., Takenaka, S., Yamate, J. and Tsuyama, S. 2007. Urinary metabolic fingerprinting for thioacetamide-induced rat acute hepatic injury using Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS) with reference to detection of potential biomarkers for hepatotoxicity. *Toxicol. Pathol.* **35**: 570–575. [Medline]
10. Kim, J. W. and Dang, C. V. 2006. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res.* **66**: 8927–8930. [Medline] [CrossRef]
11. Kuhara, T. 2001. Diagnosis of inborn errors of metabolism using filter paper urine, urease treatment, isotope dilution and gas chromatography-mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* **758**: 3–25. [Medline] [CrossRef]
12. Kuhara, T. 2005. Gas chromatographic-mass spectrometric urinary metabolome analysis to study mutations of inborn errors of metabolism. *Anal. Chim. Acta* **19**: 47–59.
13. Lindon, J. C., Holmes, E. and Nicholson, J. K. 2006. Metabonomics techniques and applications to pharmaceutical research & development. *Pharm. Res.* **23**: 1075–1088. [Medline] [CrossRef]
14. Monteiro, R., Adamis, V., Keys, D. and Platt, S. R. 2012. Canine idiopathic epilepsy: prevalence, risk factors and outcome associated with cluster seizures and status epilepticus. *J. Small Anim. Prac.* **53**: 526–530. [CrossRef]
15. Nicholson, J. K., Connelly, J., Lindon, J. C. and Homes, E. 2002. Metabolomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discov.* **1**: 153–161. [Medline] [CrossRef]
16. Patterson, E. E., Armstrong, P. J., Obrien, D. P., Roberts, M. C., Johnson, G. S. and Mickelson, J. R. 2005. Clinical description and mode of inheritance of idiopathic epilepsy I English springer spaniels. *J. Am. Vet. Med. Assoc.* **226**: 54–58. [Medline] [CrossRef]
17. Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M. and Nishioka, T. 2003. Quantitative metabolomic analysis using capillary electrophoresis mass spectrometry. *J. Proteome Res.* **2**: 488–494. [Medline] [CrossRef]
18. Wang, Y., Wang, Y., Li, M., Xu, P., Gu, T., Ma, T. and Gu, S. 2013. ^{1}H NMR-based metabolomics exploring biomarker in rat cerebrospinal fluid after cerebral ischemia/reperfusion. *Mol. Biosyst.* **9**: 431–439. [Medline] [CrossRef]
19. Xavier, S. M. I., Barbosa, C. O. and Barros, D. O. 2007. Vitamin C antioxidant in hippocampus of adult Wistar rats after seizures and status epilepticus induced by pilocarpine. *Neurosci. Lett.* **420**: 76–79. [Medline] [CrossRef]
20. Xia, J., Mandal, R., Sinelnikov, I.V., Broadhust, D. and Wishart, D. S. 2012. Metaboanalyst 2.0—a comprehensive server for metabolomics data analysis. 1–7.