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Short Communication

Suspected spontaneous hyperadrenocorticism in a young experimental beagle dog

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Abstract: A 6-month-old female beagle dog, assigned to the low-dose group in a toxicity study, was evaluated for compound toxicity, and spontaneous hyperadrenocorticism was suspected. The animal had an externally apparent distended abdomen on clinical examination upon arrival. Pre-dose clinical pathology showed slightly higher erythroid parameters and stress leukogram on hematology; plasma biochemistry showed higher total protein, gamma-glutamyl transferase, total cholesterol, and triglyceride levels than the reference data. On necropsy, a prominent increase in adipose tissues of the subcutis and abdomen and increased weight of the adrenal gland and liver were observed. Histopathology revealed diffuse hyperplasia of adrenocortical cells in the zona fasciculata and reticularis, cortical atrophy of the thymus, and abundant glycogen accumulation in the hepatocytes. These findings were incidental and not test-substance-related. Electron microscopy of the adrenocortical cells in the zona fasciculata revealed decreased typical translucent lipid droplets, increased electron-dense lipid droplets, and abundant smooth endoplasmic reticulum and lysosomes. Additionally, increased numbers of various sizes and forms of mitochondria with tubular, vesicular, or lamellar cristae compared to that of normal animals were observed. These ultrastructural characteristics of the adrenocortical cells suggested hyperfunction. The pre-dose plasma cortisol levels were slightly higher than those of other females assigned to the toxicity study, while plasma adrenocorticotropic hormone levels were within the normal range. These findings indicate that hyperadrenocorticism is a possible cause of the systemic changes in this case. (DOI: 10.1293/tox.2020-0072; J Toxicol Pathol 2021; 34: 261–267)

Key words: adrenocortical hyperfunction, adrenocorticotropic hormone, cortisol, Cushing's syndrome, electron microscopy, endocrine diseases

Hyperadrenocorticism (HAC), also known as Cushing's syndrome, is an endocrine disease commonly observed in middle- or old-aged dogs[1, 2]. Persistent cortisol excess from the adrenal cortex leads to combined gluconeogenic, lipolytic, lipogenic, protein catabolic, and anti-inflammatory effects on many organs[3, 4]. HAC is suspected when dogs show clinical symptoms like polyuria, polydipsia, polyphagia, central obesity, hepatomegaly, panting, muscle atrophy, progressive bilateral alopecia, and systemic hypertension[5]. Abdominal distention, often described as the "potbellied" or pendulous abdomen, is also a classic symptom and the cumulative result of increased abdominal organ weight and decreased abdominal muscle strength due to the catabolic effects of cortisol[6]. Herein, we report a case of suspected spontaneous HAC in a young experimental beagle dog assigned to a toxicity study with hematology, plasma biochemistry, histopathology, and electron microscopy.

Six-month-old female beagle dogs were purchased from Beijing Marshall Biotechnology Co., Ltd. (Beijing, China) for a screening toxicity study. The animals were assigned to the control, low-, middle-, and high-dose groups (n=1), and the animal in the low-dose group was the present case. This animal was kept in an animal room for 15 days of quarantine, 22 days of acclimation, and 14 days of administration, and was housed individually in a stainless-steel cage. The study protocol was approved by the Institutional Animal Care and Use Committee of Shionogi Pharmaceutical Research Center. Blood samples were collected from the cephalic vein during the quarantine and acclimation periods and once during the administration period (data not shown for the administration period). The animal's room was maintained at an appropriate temperature and relative humidity with a 12-h light/dark cycle (8 a.m. to 8 p.m.). A 2-week toxicity study with clinical examination, urinalysis, hematological, plasma biochemical, and pathological examinations were routinely conducted. In addition, plasma cortisol and adrenocorticotropic hormone (ACTH) levels were evaluated using the chemiluminescent enzyme immunoassay as additional examinations because hormonal abnormalities were suspected in the present case. At the completion of the

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administration period, the animal was necropsied following anesthesia with pentobarbital sodium and euthanasia by exsanguination. Organ weight measurement was conducted, including for the adrenal glands, liver, and pituitary gland. The collected organs were fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin stain for microscopic examination. Liver sections were also stained with periodic acid-Schiff staining. Formalin-fixed adrenal glands were refixed in 3% glutaraldehyde, followed by 2% osmium tetroxide, and embedded in an epoxy resin. After staining with platinum blue, followed by lead citrate, ultrathin sections were observed using a JEM 1400 plus electron microscope (JEOL, Tokyo, Japan).

The animal had a distended abdomen upon arrival. In addition, no abnormalities were detected on clinical examination or urinalysis throughout the quarantine, acclimation, and administration periods (data not shown). The body weight in the present case was slightly higher than that of other females assigned to the toxicity study during the quarantine and acclimation periods. Pre-dosing hematological examination revealed abnormalities in erythroid parameters, such as mildly higher red blood cell counts, hemoglobin level, and hematocrit than the reference data (in-house background data of Marshall beagle dogs collected from toxicity studies, Table 1). Abnormalities in the leukocyte parameters, such as mildly higher neutrophil and monocyte counts and lower lymphocyte and eosinophil counts (stress leukogram), were also observed. Plasma biochemical examination revealed higher total protein, gamma-glutamyl transferase, total cholesterol, and triglyceride levels than the reference data (Table 2). These abnormalities in hematological and plasma biochemical parameters were consistent throughout the quarantine and acclimation periods. Since these changes were considered to be of little significance with regard to toxicological evaluation, this animal was assigned to a low-dose group in the toxicity study. No test-substance-related observation was noted in this animal during the administration period, and the animal was subjected to necropsy after completion of the administration period. At necropsy, prominently increased subcutaneous and visceral fat, hepatomegaly, and a small thymus were observed. During organ weight measurement, an increase in the weights of the adrenal gland and liver and a decrease in the weight of the thymus were noted (Table 3). Histopathological examination revealed a thickened cortex of the bilateral adrenal glands. Diffuse hyperplasia with decreased lipid droplets and eosinophilic cytoplasm of the adrenocortical cells from the zona fasciculata to the zona reticularis were observed (Fig. 1A and B). In the liver, disarrangement of the hepatic cords and a clear and vacuolated cytoplasm with a variation in the size of centrilobular hepatocytes was observed (Fig. 1C and D). Abundant glycogen accumulation characterized by periodic acid-Schiff reaction was observed in the cytoplasm around vacuoles (Fig. 1D, inset). Furthermore, cortical atrophy of the thymus was observed. These findings were not test-substance-related since no similar findings were observed in the higher dose groups. No peculiar observations were noted in the pituitary gland.

Electron microscopy of the adrenocortical cells in the zona fasciculata revealed a decrease in typical translucent lipid droplets and an increase in electron-dense lipid droplets and swollen mitochondria in the cytoplasm (Fig. 2A and B). Some electron-dense lipid droplets had a halo or showed a mottled pattern (Fig. 2C and D). In addition, abundant smooth endoplasmic reticulum, lysosomes, and increased mitochondria of various sizes and forms with tubular, vesicular, or lamellar cristae compared to those in normal animals were observed in the cytoplasm (Fig. 2C and D).

The pre-dose plasma cortisol levels in this animal were slightly higher than those of other females assigned to the toxicity study, while the plasma ACTH levels were near the lower limit of the normal range (Table 4). These findings indicate that HAC is a possible cause of the systemic changes observed in this case.

Diffuse hyperplasia of the adrenal cortex accompanied by increased adrenal weights, as observed in our case, indicate alterations in adrenal function. Fine structural characteristics of the adrenal cortex in Cushing's syndrome include well-developed smooth endoplasmic reticulum, variations in the size of the mitochondria, and various morphologies of cristae in humans, which are consistent with this case[7]. The conversion of cholesterol to pregnenolone, which is an early step in steroid hormone biosynthesis, occurs in the mitochondria, and oxidative reactions by cytochrome P450 enzymes occur in both the smooth endoplasmic reticulum and mitochondria[8]. The ultrastructural characteristics of the adrenocortical cells observed in this case suggested hyperfunction with respect to morphology. The density of the lipid droplets observed in electron micrographs varies from electron lucent to markedly electron dense, and this is considered to be correlated with the saturated and unsaturated fatty acid content and the degree of unsaturation of fatty acids present[9]. Since the steroid hormone is derived from cholesterol ester in the adrenocortical cells and used as cholesterol after hydrolysis, it is possible that the morphological characteristics of lipid droplets in the present case represent an increased consumption of cholesterol and cholesterol esters because of hyperactivated steroid biosynthesis in adrenocortical cells[10]. However, the cause of the morphological variations of the electron-dense lipid droplets observed in this case was not completely clear.

Three major pathogeneses result in HAC. The most common mechanism is a functional ACTH-secreting adenoma of the pituitary gland that causes bilateral adrenal cortical hypertrophy and hyperplasia, which is described as pituitary-dependent HAC (PDH)[3]. The second mechanism is adrenal-dependent Cushing's syndrome, in which elevated concentrations of cortisol are attributable to adrenal gland tumors, hyperplastic adrenal glands, or adrenal glands with nodular adrenal hyperplasia[2]. Iatrogenic HAC induced by long-term administration of corticosteroids has also been reported in dogs[3], but is not applicable in the present case. The bilateral diffuse hyperplasia of the adrenal glands observed in the present case was indicative of hypersecretion

Table 1. Hematology Data for the Present Case

Parameter		*Blood collection day			**Reference data		
		Day −20	Day −12	Day −7	mean	−2SD	+2SD
RBC	$\times 10^6$/ μL	7.17	7.55	7.93	6.52	5.59	7.44
HGB	g/dL	16.0	17.2	17.9	14.9	12.8	17.1
HCT	%	48.9	51.7	54.6	44.4	39.0	49.8
WBC	$\times 10^3$/ μL	9.32	8.43	10.55	8.39	5.59	11.18
Neut	$\times 10^3$/ μL	5.80	5.33	6.84	4.35	1.99	6.71
Lymph	$\times 10^3$/ μL	2.44	2.22	2.54	3.18	2.11	4.26
Mono	$\times 10^3$/ μL	0.79	0.46	0.83	0.52	0.25	0.79
Eos	$\times 10^3$/ μL	0.11	0.25	0.10	0.23	0.06	0.39
Neut	%	62.3	63.2	64.9	51.4	39.4	63.4
Lymph	%	26.2	26.3	24.1	38.3	27.2	49.4
Mono	%	8.5	5.5	7.9	6.2	3.3	9.1
Eos	%	1.2	2.9	0.9	2.8	0.7	4.9

RBC: red blood cell; HBG: hemoglobin; HCT: hematocrit; WBC: white blood cell; Neut: neutrophil; Lymph: lymphocytes; Mono: monocytes; eosinophils: eosinophils; SD: standard deviation. *: The first day of administration was designated as Day 1. Days prior to administration are expressed with a minus sign before numerals. **: In-house background data collected from toxicity studies (Female Marshall beagle dog from China assigned to a control group in toxicity studies, 6 months of age, n=24)

Table 2. Plasma Biochemistry Data for the Present Case

Parameter		*Blood collection day			**Reference data		
		Day −20	Day −12	Day −7	mean	−2SD	+2SD
T.Pro	g/dL	5.79	6.06	6.38	5.48	4.93	6.03
Alb	g/dL	3.63	3.76	3.93	3.25	3.06	3.43
GGT	U/L	7.52	7.81	9.04	4.18	3.10	5.26
TG	mg/dL	70	32	79	30	6	53
T.Cho	mg/dL	213	211	208	126	93	159

T.Pro: total protein; Alb: albumin; GGT: gamma-glutamyl transferase; TG: triglyceride, T.Cho: total cholesterol; SD: standard deviation. *: The first day of administration was designated as Day 1. Days prior to administration are expressed with a minus sign before numerals. **: In-house background data collected from toxicity studies (Female Marshall beagle dog from China assigned to a control group in toxicity studies, 6 months of age, n=11).

Table 3. Absolute and Relative Organ Weights in the Present Case

Organ	Organ weight	*Reference data		
		Mean	−2SD	+2SD
Adrenal gland (left)	862 mg	484	335	632
	12 mg /100 g BW	7.6	5.4	9.7
Adrenal gland (right)	791 mg	466	314	617
	11 mg /100 g BW	7.3	4.6	10
Liver	317 g	205	160	250
	4.6 g /100 g BW	3.2	2.3	4.2
Pituitary gland	36 mg	49	33	64
	0.52 mg /100 g BW	0.77	0.49	1.0
Thymus	0.707 g	5.64	0.709	10.6
	0.010 g /100 g BW	0.089	0.014	0.16

BW: body weight; SD: standard deviation. *: In-house background data collected from toxicity studies (Female Marshall beagle dog from China assigned to a control group in toxicity studies, 6 months of age, n=10).

of ACTH; however, it was found that the plasma ACTH levels were within the normal range and no observation was detected in the pituitary gland. The precise pathogenesis of adrenocortical hyperplasia remained uncertain in this case.

In the liver, hepatocytes were swollen due to the accumulation of glycogen granules in the cytoplasm because

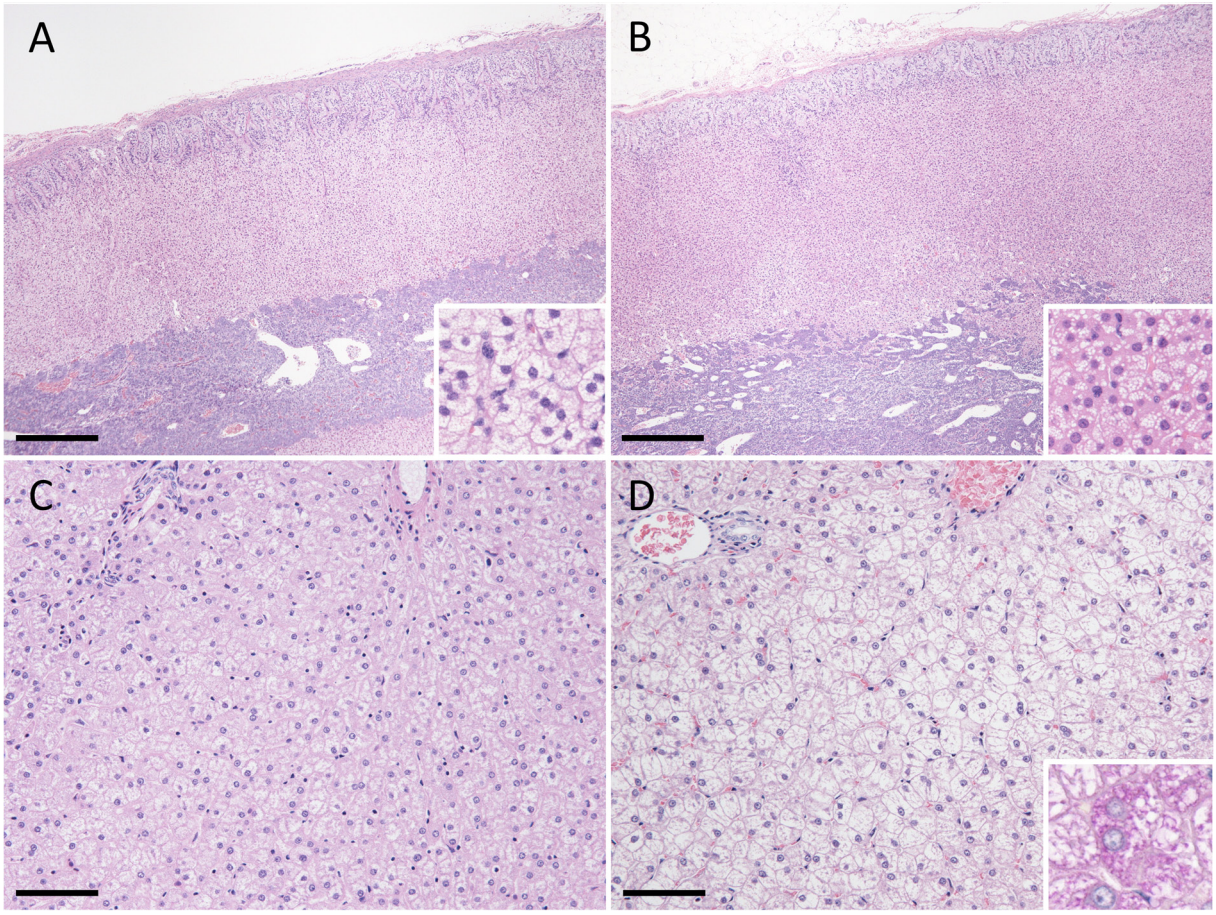

Fig. 1. Photomicrographs showing the adrenal gland and liver in a normal dog and in this case (Hematoxylin & eosin staining). (A) Adrenal gland of a normal dog assigned to the control group in this study. Scale bar=500 µm. (B) Adrenal gland in this case. Thickened cortex owing to diffuse hyperplasia with decreased lipid and eosinophilic cytoplasm of the adrenocortical cells from the zona fasciculata to zona reticularis. Scale bar=500 µm. (C) Liver of a normal dog assigned to the control group in this study. Scale bar=200 µm. (D) Clear and vacuolated cytoplasm with a variation in size of centrilobular hepatocytes and disarrangement of hepatic cords in the liver in this case. Abundant glycogen accumulation corresponded to clear cytoplasm characterized by periodic acid-Schiff staining-positive reactivity (Inset). Scale bar=200 µm.

of the suspected effect of glycogenic metabolism alterations caused by HAC[3]. In addition, because of the long-term treatment with glucocorticoids, thymic involution occurs as a biological response[11, 12]. Therefore, atrophy of the thymic cortex in our case was considered to be caused by HAC.

Common clinical signs and physical and laboratory examination findings related to canine HAC are shown in Table 5. Common clinico-pathological abnormalities in dogs with HAC include neutrophilic leukocytosis, lymphopenia, eosinopenia, thrombocytosis, and mild erythrocytosis in the complete blood count and increased alkaline phosphatase, alanine aminotransferase, total cholesterol, triglyceride, and glucose levels in the serum[13]. Alterations in the number of leukocytes with increased serum cortisol concentration characterized by neutrophilia, monocytosis, lymphopenia, and eosinopenia are termed “stress leukogram” and are popularly recognized in many species, including dogs[14]. Mild erythrocytosis may occasionally be noted in dogs with HAC, owing to ventilatory problems, or in females because of androgenic stimulation of the bone marrow[5]. Dyslipidemia, such as an increase in serum total cholesterol and triglyceride levels, is common in dogs with HAC[13, 15]. The pathogenetic mechanisms of dyslipidemia are multifactorial, including the direct and indirect action of cortisol on lipolysis, free fatty acid production and turnover, very-low-density lipoprotein synthesis, and fatty accumulation in the liver[15]. Major clinical findings in HAC, such as polyuria, polydipsia, and alopecia, and increased blood parameters, such as platelets, alkaline phosphatase, alanine amino transferase, and glucose levels, were not observed in our case, probably because the animal was at an early onset stage. In addition, increased gamma-glutamyl transferase and total protein levels were observed in plasma biochemistry. As most of the reported HAC dogs are companion animals, it is possible that the symptoms of HAC manifest differently in experimental beagle dogs because the rearing conditions are considerably different.

Stress often occurs during toxicity studies in many

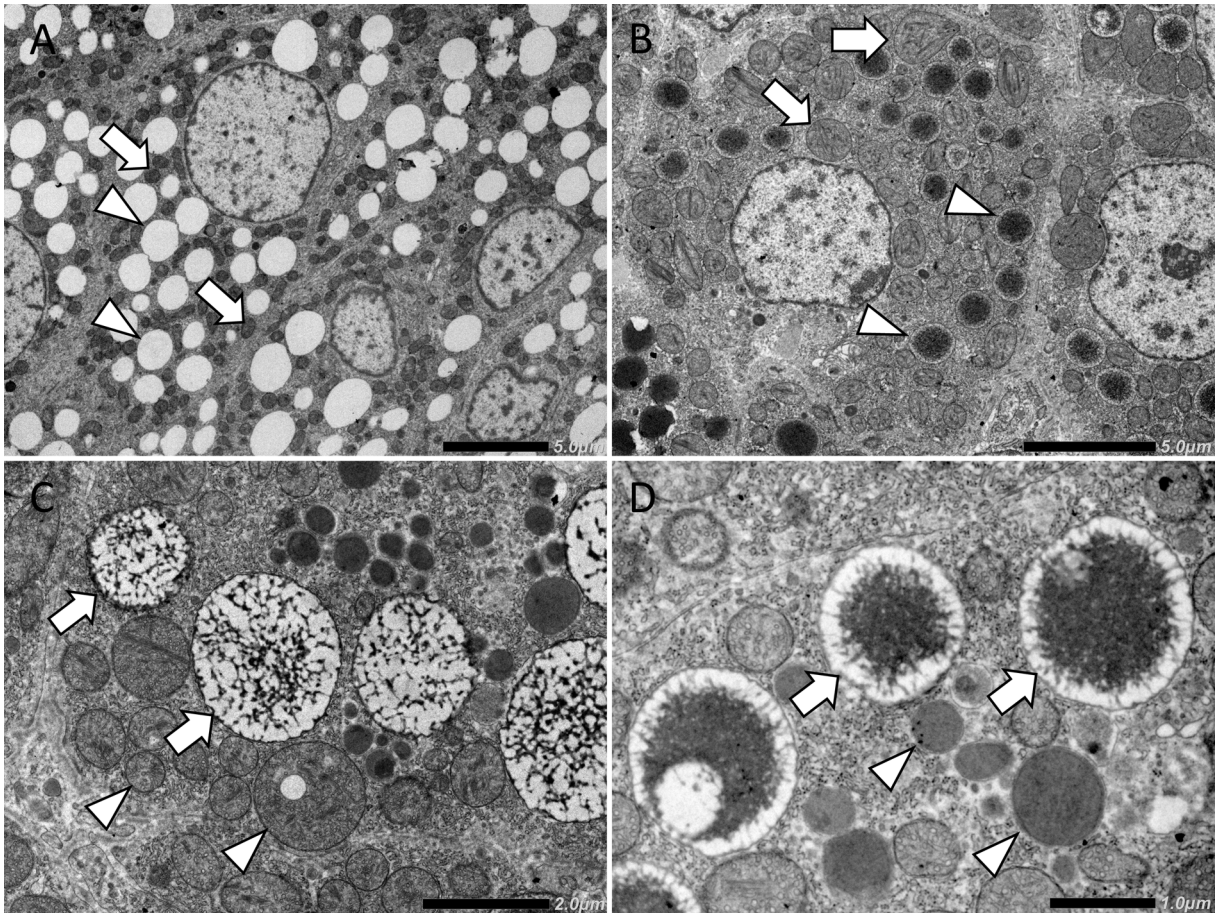

Fig. 2. Ultrastructure of the adrenocortical cells in the zona fasciculata in a normal dog assigned to the control group in this study (A) and in this case (B–D). (A) Uniform mitochondria in size (arrows) and multiple translucent lipid droplets scattered in the cytoplasm (arrow heads). (B) Decreased translucent lipid droplets, swollen mitochondria (arrows), and electron-dense lipid droplets with a halo (arrow heads) in the cytoplasm. (C) Increased electron-dense lipid droplets with a mottled pattern (arrows), mitochondria of various sizes showing tubular, vesicular, or lamellar cristae (arrow heads), and abundant smooth endoplasmic reticulum in the cytoplasm. (D) Increased electron-dense lipid droplets with a lucent halo (arrows) and lysosomes (arrowheads) in the cytoplasm.

Table 4. Plasma Cortisol and ACTH Concentrations in Female Beagle Dogs Assigned to the Toxicity Study

Animals (n=1)	*Blood collection day		Mean	Reference data
	Day −12	Day −7		
Cortisol (μg/dL)				
Control	2.3	3.8	3.1	0.5–6.0[1]
Low-dose group**	3.5	4.7	4.1	
Middle-dose group	1.4	1.1	1.3	
High-dose group	2.1	1.6	1.9	
ACTH (pg/mL)				
Low-dose group**	6.3	7.4	6.9	5–36***

ACTH: adrenocorticotropic hormone. *: The first day of administration was designated as Day 1. Days prior to administration are expressed with a minus sign before numerals. **: An animal in the low-dose group is indicative of the present case. ***: Reference data of the test facility.

species, and the manifestations may include decreased total body weight or body weight gain; altered organ weights, such as decreased weights of the thymus and spleen and increased weight of the adrenal gland; and altered circulating leukocyte counts, such as increased neutrophils with decreased lymphocytes and eosinophils[16]. Since these are caused by the activation of the hypothalamic-pituitary-adrenal axis to increase serum glucocorticoid concentrations,

Table 5. Comparison of Tables According to Common Clinical Signs, Physical, and Laboratory Examination Findings of Spontaneous Canine HAC in the 2012 ACVIM Consensus Statement (Small Animal)[13]

1. Clinical manifestations of canine HAC. Categorization of frequency was based on the identification at the time of initial presentation.		
Common	Less common	Uncommon
Polydipsia	Lethargy	Thromboembolism
Polyuria	Hyperpigmentation	Ligament rupture
Polyphagia	Comedones	Facial nerve palsy
Panting	Thin skin	Pseudomyotonia
Abdominal distention*	Poor hair regrowth	Testicular atrophy
Endocrine alopecia	Urine leakage	Persistent anestrus
Hepatomegaly*	Insulin-resistant diabetes mellitus	
Muscle weakness		
Systemic hypertension		
2. Common laboratory abnormalities in dogs with HAC.		
Complete blood count	Serum biochemistry panel	Urinalysis
Neutrophilic leukocytosis*	Increased alkaline phosphatase	Specific gravity ≤ 1.018–1.020
Lymphopenia*	Increased alanine aminotransferase	Proteinuria
Eosinopenia*	Hypercholesterolemia*	Indicators of urinary tract infection
Thrombocytosis	Hypertriglyceridemia*	
Mild erythrocytosis*	Hyperglycemia	

HAC: hyperadrenocorticism. *: Observations consistent with the present case.

the pathophysiology of stress-related effects is considerably similar to HAC. However, the present case was assigned to a low-dose group of a short-period (2-week) toxicity study, no other animals in the control, and higher-dose groups presented with stress-related observations, and no specific stress inducers existed in the rearing conditions. In addition, observations in the present case were consistent throughout the quarantine, acclimation, and administration periods, which may exclude stress-derived effects induced by environmental changes, including transportation. Furthermore, abdominal distention, increases in subcutaneous and visceral fat, and hepatomegaly, as observed in the present case, are generally not seen in stress-influenced dogs. Therefore, the observations in the present case were not considered to be chronic stress-derived effects.

This case showed slightly higher plasma cortisol levels throughout the acclimation and quarantine periods than the other females assigned to the toxicity study, although the values were within the range of reference data for normal dogs. Pulsatile ACTH secretion results in variable cortisol concentrations, which may at times be within the reference range, even in animals with HAC[13]. It has been reported that hourly measured serum cortisol concentrations in PDH dogs were 4.3 ± 1.5 (mean, SD), while in normal control dogs, they were 1.4 ± 0.6 (mean, SD)[17]. Although the serum cortisol values of PDH dogs were within the normal range in these dogs, 42% of these dogs showed exaggerated cortisol levels (>22 μg/dL) after ACTH stimulation[17]. Adrenal function testing procedures, such as low-dose dexamethasone suppression or an ACTH stimulation test, are commonly required for the diagnosis of HAC[1]. Unfortunately, none of these tests were performed in the toxicity study.

HAC is commonly observed in middle- or old-aged dogs; however, juvenile onset has also been reported in dogs[18]. This dog showed skin disorders, abdominal enlargement, hematological abnormalities like marked elevation of alkaline phosphatase, alanine aminotransferase and cholesterol, hepatomegaly, and no adrenal gland enlargement. In the present case, abdominal enlargement, hematological abnormalities such as mild erythrocytosis, stress leukogram, elevation of total protein, gamma-glutamyl transferase, total cholesterol, triglyceride, hepatomegaly, and adrenal gland enlargement were observed, while no skin disorders were observed. Therefore, initial or early symptoms of HAC in young dogs may be seen in hematological studies and in organs such as the liver. No previous reports describing spontaneous HAC in experimental beagle dogs are available.

In conclusion, distended abdomen and morphological characteristics of the adrenocortical cells, suggesting hyperfunction with slightly higher plasma cortisol levels were observed in the present case. Various observations, such as increased adipose tissues of the subcutis and abdomen, a small thymus, an increase in weight of the adrenal gland and the liver, and hematological abnormalities suggesting a hypercortisolemic condition, were indicative of HAC. In case the characteristic appearance of a distended abdomen and consistent hematological abnormalities are observed in the experimental beagle dogs before dosing in toxicity studies, spontaneous lesions may be observed in the adrenal gland and liver, which are the major target organs in toxicity studies[19, 20]. Since animals with such abnormal findings can make the toxicological evaluation complicated and difficult, they should be excluded from the toxicity study.

Disclosure of Potential Conflicts of Interest: The authors declare no conflicts of interest.

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