The effect of 1 year of trilostane treatment on peripheral lymphocyte subsets in dogs with pituitary-dependent hyperadrenocorticism

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ABSTRACT. This study investigated the changes in lymphocyte subsets during the trilostane medication of Pituitary-dependent hyperadrenocorticism (PDH) dogs. The cortisol level and lymphocyte subsets of eight dogs with PDH were monitored 0, 1, 3, 6, 9 and 12 months after the initiation of trilostane treatment. White blood cells (WBC), lymphocytes, $CD3^+$ (T lymphocyte), $CD4^+$ (helper T lymphocyte), $CD8^+$ (cytotoxic T lymphocyte) and $CD21^+$ (B lymphocyte) cells were measured. Although the post-ACTH stimulation test cortisol level was significantly lower during trilostane treatment, changes in the $CD3^+$, $CD4^+$, $CD8^+$ and $CD21^+$ counts were not observed. Meanwhile, significant decrease was observed in WBC counts during trilostane treatment. These indicate that long-term trilostane treatment has little effect on the lymphocyte subsets in PDH dogs.
KEY WORDS: ACTH stimulation test, cortisol, immune suppression, white blood cells

Pituitary-dependent hyperadrenocorticism (PDH) is a common endocrine disease in dogs that presents with symptoms, such as polyuria-polydipsia (PU/PD), polyphagia, panting, pendulous abdomens and dermatological problems. These symptoms are caused by an excess production of glucocorticoids [7, 18]. In addition, an increased production of glucocorticoids suppresses the immune system, resulting in infectious diseases, such as pyoderma and urinary tract infections being frequently observed in dogs with PDH [7, 9].

Various studies have demonstrated the use of lymphocyte subset counts to investigate the immune state of dogs [2, 11]. Furthermore, the increased cortisol concentrations and consequent disease conditions characterized by PDH affect the lymphocyte subsets in dogs with this condition [16]. However, no studies have investigated whether a change in lymphocyte subset counts occurs during trilostane treatment of dogs with PDH. Trilostane is an adrenal steroid biosynthesis inhibitor that targets 3β-hydroxysteroid dehydrogenase and is considered an efficacious and safe medication for the treatment of dogs with PDH [19]. The purpose of this study was thus to determine whether 1 year of trilostane treatment has any effect on the lymphocyte subsets of dogs suffering from PDH.

Eight dogs with untreated PDH that visited the Nippon Veterinary and Life Science University animal clinic were used in this study (Table 1). PDH diagnosis was based on the clinical condition of the dogs, the results of an ACTH stimulation test and the ultrasonographic appearance of the adrenal glands. Dogs were included in the study, if at least three clinical signs of hyperadrenocorticism were detected. A serum cortisol concentration of above 20 μg/dl in a sample obtained 1 hr after an ACTH stimulation test was considered abnormal. Furthermore, PDH diagnosis was confirmed based on a bilateral symmetrical appearance or bilateral enlargement of the adrenal glands (minor axis over 6−7 mm) on ultrasonographic examination (Vivid 7, GE Yokokawa Medical System, Tokyo, Japan). Informed consent was obtained from all the dog's owners after the purpose, nature, and potential risks and benefits of the study had been reviewed. This work was approved by the Nippon Veterinary and Life Science University Animal Research Committee.

The ACTH stimulation test was carried out by collecting blood samples for the determination of the serum cortisol concentration 1 hr after (post-ACTH) the intravenous injection of 0.25 mg of ACTH (Cortrosyn, Daiichi Sankyo, Tokyo, Japan). Serum cortisol concentrations were measured using the chemiluminescence method (IMMULYZE 1000, Mitsubishi Chemical Medience Corporation, Tokyo, Japan). All dogs were administered trilostane tablets (Desopan, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) once or twice a day with food. The initial dosages were determined based on body weight (1.84 ± 0.94 (mean ± SD) mg/kg/day; range: 5.48–15.0 mg/dog/day) and were further adjusted throughout the treatment period according to the dog's clinical condition −such as an improvement in PU/PD−and the post-ACTH serum cortisol concentration. Trilostane was administered by the pet owner before visiting hospital, and ACTH stimulation test was performed 2 to 6 hr after administration of trilostane.

Blood was obtained from the cephalic vein of each dog after 0, 1, 3, 6, 9 and 12 months of trilostane treatment. Sample collection and processing were performed using similar methods to those described by Mori *et al*. [16]. Blood samples for lymphocyte subsets analysis were collected be-

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Table 1. The physical profiles of eight dogs diagnosed with pituitary-dependent hyperadrenocorticism treated with trilostane in this study.

No.	Breeds	Age (years)	Sex	Body weight (kg)	Cortisol concentration after 1 hour ACTH stimulation test (μg/dl)		Concurrent disease before treatment	Concurrent disease after one year
					Initial day	Treated		
1	Mix	11	spayed	18.5	66.7	5.76		hepatocarcinoma
2	Mix	6	spayed	16.8	31.5	12.4		
3	Miniature Dachshund	9	male	6.35	82.8	0.94		hypothyroidism
4	Papillon	9	male	5.4	92	2.22		
5	Miniature Dachshund	9	male	5.7	28.5	6.65		
6	Miniature Dachshund	11	male	6	48.5	1.05	periodontal disease	periodontal disease
7	Miniature Schnauzer	13	castrated	10.1	31.5	4.21		
8	Pug	12	female	7.4	53.3	7.81	dermatosis	dermatosis

fore the injection of ACTH. Samples were immediately analyzed within 3 hr from each sampling for lymphocyte subset distribution using flow cytometry (BD FACSCalibur, Becton Dickinson, Tokyo, Japan) to calculate the relative percentages of CD3$^+$ (T lymphocytes), CD4$^+$ (T$_H$ lymphocytes), CD8$^+$ (T$_C$ lymphocytes) and CD21$^+$ (B lymphocytes). Percentages of CD3$^+$, CD4$^+$, CD8$^+$ and CD21$^+$ were determined according to differences in the surface markers of lymphocytes for 5,000 events. Absolute values for lymphocyte subsets were calculated using white blood cell (WBC) counts (Automated hematology analyzer pocH-100i, Sysmex Corp., Kobe, Japan) in combination with the flow cytometer results. The WBC, CD3$^+$ cell, CD4$^+$ cell, CD8$^+$ cell and CD21$^+$ cell counts, the CD4$^+$/CD8$^+$ ratio and the cortisol concentration 1 hr after an ACTH stimulation test (post-ACTH cortisol level) were determined for each blood sample.

Data are presented as mean ± standard deviation (SD). Statistical significance was determined using one-way repeated measures ANOVA and Dunnett's post hoc test using GraphPad Prism 5 analysis software (GraphPad Software, Inc., San Diego, CA, U.S.A.). Differences were considered significant at values of $P<0.05$.

Significant changes in the post-ACTH cortisol level and WBC count were observed ($P<0.05$), with lower cortisol levels being recorded at 1, 3, 6, 9 and 12 months relative to the pretreatment level (Fig. 1). Moreover, the WBC count was lower ($P<0.05$,) at 3, 6, 9 and 12 months than at 0 months (Fig. 2a). The lymphocyte (Fig. 2b), CD3$^+$ T lymphocyte (Fig. 2c), CD21$^+$ B lymphocyte (Fig. 2d), CD4$^+$ T$_H$ lymphocyte (Fig. 2e) and CD8$^+$ T$_C$ lymphocyte (Fig. 2f) counts, and the CD4$^+$/CD8$^+$ ratio (Fig. 2g) did not change significantly over the treatment period.

The reduction in the cortisol level of PDH dogs after the initiation of trilostane treatment and the maintenance of the mean cortisol level below 9.1 μg/dl for the 3 to 12 months of treatment suggest that the trilostane treatment was successful [6, 17].

Long-term cortisol administration contributes to the development of both neutrophilia and lymphopenia [15]. In addition, endogenous glucocorticoids accelerate neutrophil maturation in the bone marrow and transference into the peripheral compartment, resulting in neutrocytosis [5, 22]. In the current study, WBC counts prior to treatment (13,750
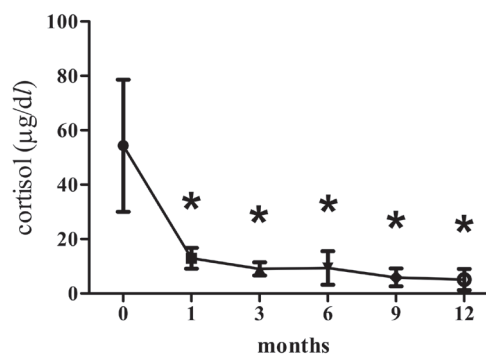

Fig. 1. The change in the post-ACTH stimulation cortisol concentration of dogs with pituitary-dependent hyperadrenocorticism during 12 months of trilostane treatment. Values are expressed as mean ± SD. Asterisks indicate significant differences ($P<0.05$) as compared with the 0-month value.

± 4,696 cells/μl) were high relative to the reference range for normal dogs (6,000–17,000 cells/μl) [8]. Thus, the increased WBC counts prior to the initiation of treatment may have been caused by neutrophilia in response to high cortisol exposure. The decreased WBC counts from 0 to 3, 6, 9 and 12 months (13,750 ± 4,696, 8,938 ± 3,092, 9,463 ± 3,010, 8,350 ± 2,789 and 9,750 ± 7,559 cells/μl, respectively) may thus have been due to the decreased cortisol concentration during the trilostane treatment period.

Lymphocyte counts prior to treatment were low (594 ± 188 cells/μl) relative to the reference range for normal dogs [8] and did not change during the trilostane treatment period (Fig. 2). Cortisol modulates the regulation of leukocyte recruitment [5], especially hematological changes including an immediate increase in the numbers of mature neutrophils and monocytes and a reduction in the numbers of eosinophils and lymphocytes [12]. Therefore, a rise in cortisol levels in the absence of active inflammation, such as in Cushing's syndrome, can cause immune suppression associated with lymphopenia [14]. This is in agreement with the previous finding that PDH dogs have lower lymphocyte counts than healthy dogs [16]. Long-term glucocorticoid exposure may therefore have caused irreversible dysregulation of the thymus, which is a main organ responsible for CD3$^+$ T lymphocyte production and CD4$^+$ T$_H$ lymphocyte selection and maturation.
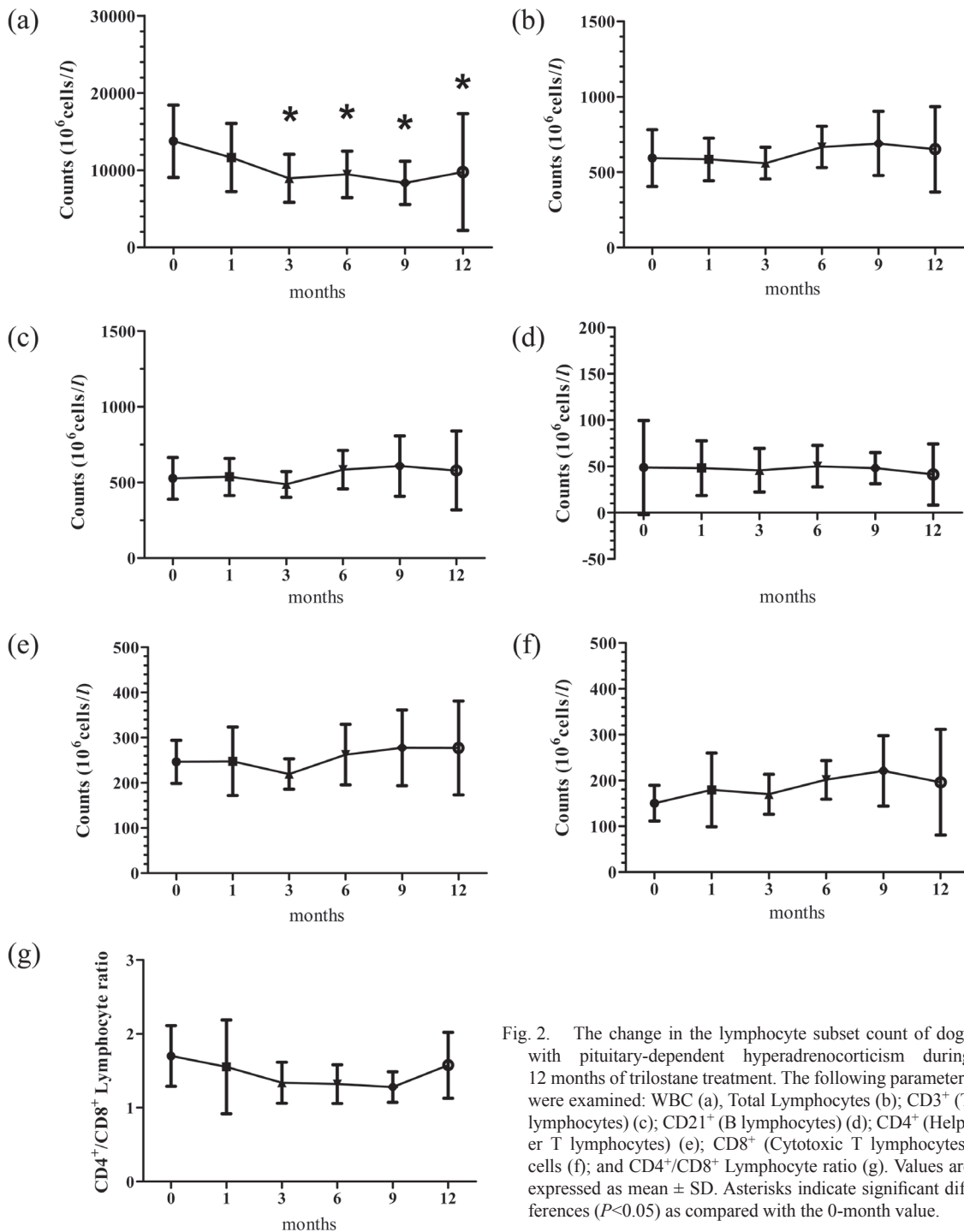
Fig. 2. The change in the lymphocyte subset count of dogs with pituitary-dependent hyperadrenocorticism during 12 months of trilostane treatment. The following parameters were examined: WBC (a), Total Lymphocytes (b); $CD3^+$ (T lymphocytes) (c); $CD21^+$ (B lymphocytes) (d); $CD4^+$ (Helper T lymphocytes) (e); $CD8^+$ (Cytotoxic T lymphocytes) cells (f); and $CD4^+/CD8^+$ Lymphocyte ratio (g). Values are expressed as mean ± SD. Asterisks indicate significant differences ($P<0.05$) as compared with the 0-month value.

Similar to the lymphocyte count, there was no significant change in the $CD3^+$ T lymphocyte, $CD4^+$ T_H lymphocyte and $CD21^+$ B lymphocyte subset counts. However, $CD8^+$ T_C lymphocyte cells showed a tendency to increase. T_H lymphocytes are divided into two subsets based on their cytokine production profiles. T_H1 cells mainly secrete interferon-gamma (IFN-γ) and interleukin (IL)-2 which promote the develop-ment of macrophages, $CD8^+$ T_C lymphocyte cells and $CD21^+$ B lymphocyte cells. Meanwhile, T_H2 cells generally produce IL-4, 5 and 10 which have been implicated in the stimulation of $CD21^+$ B lymphocyte cells to produce the antibody IgE [3, 10]. The administration of glucocorticoids or the occurrence of Cushing's syndrome suppresses T lymphocyte counts, with $CD4^+$ counts being more depleted than $CD8^+$

counts [13, 20]. In human adult atopic dermatitis patients, hydrocortisone butyrate ointment reduced numbers of CD3$^+$ and CD4$^+$, but not CD8$^+$ in atopic dermatosis lesions. Furthermore, hydrocortisone butyrate ointment also induced lower expression of IL-4 and IL-5, but not IFN-γ [4]. These changes were considered indicative of a shift toward T$_H$2 dominance. Furthermore, changes in lymphocyte subset counts might be related to lymphocyte cytokine expression [10], with cortisol potentially altering cytokine production from a T$_H$1 to a T$_H$2 pattern [1, 21]. Unfortunately, lymphocyte cytokine production was not evaluated in the current study, and the relationship between the lymphocyte subsets and lymphocyte cytokine production should thus be further studied. CD21$^+$ B lymphocyte cell counts in PDH dogs were almost unchanged during trilostane medication. Reduced CD21$^+$ B lymphocyte cell counts may be as a result of decreased signaling activity by cytokines secreted from CD4$^+$ cells [3].

In conclusion, long-term trilostane treatment of PDH dogs resulted in almost no variation in lymphocyte counts and lymphocyte subset counts. Previous studies comparing the lymphocyte subsets in PDH dogs with or without concurrent disease with healthy control dogs found that increased cortisol concentrations and concurrent disease changed the lymphocyte subsets in PDH dogs [16]. However, the current study suggests that untreated PDH dogs with or without concurrent diseases were immune suppressed, and long term successful trilostane treatment had little effect on their immune state, especially regarding the lymphocyte subsets. Future studies assessing lymphocyte function should thus be done in order to provide a better understanding of these results.

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