Trilostane-induced inhibition of cortisol secretion results in reduced negative feedback at the hypothalamic–pituitary axis

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Abstract

Cushing’s disease caused by pituitary corticotroph adenoma in dogs is usually treated by medical treatment, and the efficacy of this treatment has been reported. However, controversy remains as to whether reduced negative feedback through the inhibition of cortisol secretion, similar to Nelson’s syndrome, may appear as an adverse effect. The purpose of this study was to investigate the effect of reduced negative feedback through the inhibition of cortisol secretion by daily trilostane administration on the pituitary–adrenal axis in clinically normal dogs. Dogs were administered 5 mg/kg trilostane twice a day every day for 8 weeks (n = 8) or 16 weeks (n = 3). After the initiation of trilostane administration, plasma adrenocorticotropic hormone (ACTH) concentrations were increased remarkably. As assessed by magnetic resonance imaging (MRI) during administration, the pituitary became enlarged. After trilostane administration, the cytoplasmic areas of the pituitary corticotrophs were increased and the ratio of pituitary corticotrophs to all cells in the anterior lobe was greater in the trilostane-treated dogs than that in untreated animals. In addition, histological examinations revealed bilateral adrenal cortical hyperplasia. Using real-time PCR quantification, the expression of proopiomelanocortin (POMC) mRNA in the pituitary and ACTH receptor (ACTH-R) mRNA in the adrenal gland was greater in the dogs treated with trilostane than in untreated dogs. These results indicate that reduced negative feedback induced hyperfunction of the pituitary corticotrophs and pituitary enlargement in healthy dogs. These changes suggest that the inhibition of cortisol secretion by trilostane may increase the risk for accelerating the growth of corticotroph adenomas in dogs with Cushing’s disease.

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Keywords: Corticotroph; Cushing’s disease; Dog; Nelson’s syndrome; Trilostane

1. Introduction

Pituitary-dependent hyperadrenocorticism (PDH) accounts for approximately 80–85% of hyperadreno-
corticism, a major endocrine disorder in dogs, and the majority of these cases, which represent Cushing’s disease, had adenomas of the pituitary corticotroph [1,2]. In veterinary clinical medicine, the prevalence of advanced imaging modalities for diagnosis, such as computer tomography (CT) and magnetic resonance imaging (MRI), has enabled a detailed visualization of the pituitary, and, in combination with endocrinological tests, has enabled the accurate diagnosis of Cushing’s disease.
In regard to the treatment of PDH dogs, although surgical treatment by transsphenoidal hypophysectomy has been reported to be useful [3–5], most dogs with PDH are treated clinically to inhibit cortisol excess [6].

Recently, trilostane has been used increasingly for the treatment of PDH dogs, and its efficacy has been reported [7–12]. Trilostane, a competitive inhibitor of 3β-hydroxysteroid dehydrogenase, inhibits the synthesis of several steroids in the adrenal cortex including cortisol [13,14]. Mitotane, which has been used for a long time, inhibits the secretion of cortisol by cell necrosis due to cytotoxicity on the zona fasciculata (ZF) and zona reticularis (ZR) in the adrenal cortex [15–17]. However, trilostane inhibits cortisol secretion by competitively inhibiting enzymes and adverse effects are less frequent than those that appear with mitotane treatment.

Although there are previous reports demonstrating that the clinical symptoms of PDH dogs were improved by treatment with trilostane [7–9,12], neither mitotane or trilostane administration is aimed at a radical cure of the disease arising from adenomas of the pituitary. Moreover, reduced negative feedback due to the inhibition of cortisol secretion by mitotane or trilostane may increase the risk for accelerating growth of the corticotroph adenoma in dogs with Cushing’s disease. However, no studies have investigated the effect of cortisol inhibition by trilostane on the pituitary corticotroph that is the cause the disease. In this study, in order to investigate the effect of inhibition of cortisol secretion from the adrenal cortex by trilostane on the pituitary corticotroph, we examined the effect of daily administration of trilostane on the hypothalamic–pituitary–adrenal (HPA) axis in normal Beagle dogs.

2. Materials and methods

2.1. Dogs

Twenty-one healthy Beagles were used. The group characteristics were as follows: 9 males and 12 females; 1–6 years old (mean: 2.1 years old); and 7.0–15.0 kg body weight (mean: 9.8 kg). Dogs were randomly assigned to a control group (n = 10, 4 males and 6 females, mean age was 2.8 years, and mean weight was 9.8 kg), a short term of trilostane administration group (TS group) (n = 8, 3 males and 5 females, mean age was 1.5 years, and mean weight was 9.8 kg), a long term of trilostane administration group (TL group) (n = 3, 2 males and 1 female, mean age was 1.4 years, and mean weight was 10.0 kg). Protocols for all experiments involving the use of dogs were approved by the Bioethics Committee at Nippon Veterinary and Life Science University.

2.2. Trilostane administration

In the trilostane administration group, adrenocortical function was inhibited by the administration of trilostane (DESOPAN®Tab.; Mochida Pharmaceutical, Tokyo, Japan). Trilostane was capsuled at a dosage of 5 mg/kg body weight and administered orally twice a day (10 mg/kg/day) with meals every day for 8 weeks (TS group) and 16 weeks (TL group). The dosage was established from several reports of trilostane [8–12,18–21]. During the administration period, the general condition of dogs was monitored.

2.3. Endocrine tests and sample collection

Blood samples for hormone measurements were collected from the jugular vein and transferred to ice-chilled tubes containing EDTA and plain serum. Plasma and serum were separated by centrifugation at 4 °C for 15 min and stored at −80 °C until assayed.

The adrenocorticotropic hormone (ACTH)-stimulation test was performed by collecting blood samples for measurement of the cortisol concentration at 0 and 60 min after the intravenous administration of 0.25 mg of synthetic ACTH (Cortrosyn®; Daiichi Sankyo, Tokyo, Japan) [22].

The corticotropin releasing hormone (CRH)-stimulation test was performed by collecting blood samples for measurement of the ACTH concentration at 0 and 30 min after the intravenous administration of 1.5 µg ovine corticotropin-releasing factor (Peptide institute, Inc., Osaka, Japan) per kg body weight [23,24].

In the trilostane administration group, the ACTH-stimulation test was performed prior to trilostane administration, every week until 8 weeks during the trilostane administration, and every 2 weeks after 8 weeks (TL group). The CRH-stimulation test was performed prior to trilostane administration, at 30 (4 weeks) days, 58 (8 weeks) and 86 (12 weeks) days (TL group), and after completion of the trilostane administration period. All ACTH- and CRH-stimulation tests were performed at 4 h after the administration of the morning dose of trilostane.

2.4. Hormone determination

Serum cortisol concentrations were measured using a competitive immunoassay (Immulite® Cortisol; Diagnostics Products Corporation, Los Angeles, USA) as described previously [25]. The intra-assay coefficients of
variation (CV) were 8.8 and 5.8% at cortisol levels of 74 and 524 nmol/l, respectively. The inter-assay CV were 10.0 and 6.3% at cortisol levels of 74 and 524 nmol/l, respectively. The sensitivity of the assay was 0.25 nmol/l.

Plasma ACTH concentrations were measured using a solid-phase, 2-site chemiluminescent enzyme immunometric assay (Immumlite® ACTH; Diagnostic Products Corporation, Los Angeles, USA) in duplicate as described previously [26,27]. The intra-assay coefficients of variation were 9.6 and 4.9% at ACTH levels of 5.3 and 221.8 pmol/l, respectively. The inter-assay CV were 8.8 and 5.1% at ACTH levels of 5.8 and 248.9 pmol/l, respectively. The sensitivity of the assay was 1.1 pmol/l. In cases that exceeded the measurement range (>278 pmol/l), samples were diluted using a special diluent (Diagnostics Products Corporation, Los Angeles, USA).

2.5. Magnetic resonance imaging

Food was withheld from the trilostane group for 18 h prior to MRI. Following the intramuscular administration of 0.25 mg droperidol (DROLEPTAN®; Daiichi Sankyo, Tokyo, Japan) per kg body weight, anesthesia was induced by intravenous administration of 7 mg propofol (Rapinovet®; Schering-Plough Animal Health, Tokyo, Japan) per kg body weight and maintained by inhalation of isoflurane (Escain®; Merck, Osaka, Japan) and oxygen.

The MRI of the pituitary was performed using a 1.5 T superconducting magnet (VISART; Toshiba Medicals, Tokyo, Japan). T1-weighted transverse images were made before and after an intravenous bolus injection of 0.1 mmol contrast medium (Omniscan®, gadodiamide-hydrate (Gd); Daiichi Sankyo, Tokyo, Japan) per kg body weight. With the dogs in sternal recumbence, transverse scans of the skull base from the rostral clinoid processes to the dorsum sellae using the spin-echo method, which obtained a 350-ms repetition time (TR), a 15-ms echo time (TE), and 2.0-mm thick consecutive slices. After Gd-T1-weighted images of the pituitary were obtained, T1-weighted imaging of the adrenal gland was performed prior to trilostane administration. With the dogs in supine position, coronal scans of the adrenal glands were obtained perpendicular to the spinous process using the field-echo method, which obtained a TR of 200 ms, a TE of 6.8 ms, and 5.0-mm thick consecutive slices.

In each dog, the pituitary height was measured on the image using the largest cross-section of the pituitary. In order to enable adjustments for the size of the dog, the edges of the brain were traced on this image and the enclosed area was calculated by the computer according to the method used in a study on CT [28]. From the height of the pituitary and the area of the brain obtained in the Gd-T1-weighted image, the pituitary height/brain area (P/B) ratio was calculated, as previously described [28]. Pituitaries with a P/B ratio of more than 0.31 × 10⁻² mm⁻¹ were considered to be enlarged. The MRI of the pituitary was performed prior to trilostane administration and every 2 weeks during the trilostane administration.

2.6. Tissue samples

Each dog was euthanized by an intravenous injection of an overdose pentobarbital solution following the CRH-stimulation test in the control group and after the administration of trilostane in the trilostane administration group. The pituitary and bilateral adrenal glands were carefully harvested and weighed before being fixed in 4% paraformaldehyde for histological examination or stored at −80 °C for RNA extraction. Each pituitary was cut at the median sagittal plane and each adrenal gland was cut twice transversely, midway between the indentation of the phrenicoabdominal vein and each pole [29].

2.7. Histological examination

Samples for histological examination were dehydrated and embedded in paraffin. Sections were cut at 2 μm. One section was stained with hematoxylin and eosin (HE), and adjacent sections of pituitaries were subjected to immunohistochemical staining by standard enzyme antibody techniques using a monoclonal mouse antibody to synthetic ACTH₁⁻₃₉ (Dako Japan, Kyoto, Japan) [30].

2.8. Pituitary and adrenal morphometry

Pituitary and adrenal morphometry were performed with the “Image J” software version 1.36 (http://rsb.info.nih.gov/ij/). In each dog, the area and numbers of ACTH-positive cells in the anterior pituitary gland were measured. For pituitary morphometry, the anterior pituitary gland was divided equally into five regions between the dorsal end and the ventral end of the pituitary gland in the median section, and two fields each were randomly selected from the five regions [30]. The number of cells and the numbers of ACTH-positive cells per field of the anterior pituitary gland were measured in 10 fields at 400× magnification for each dog, and the ratio of ACTH-positive cells were calculated.

In the adrenal glands, the width of the zona glomerulosa (ZG), zona fasciculata, and zona reticularis were
determined at 10 different places. The number of cells per field of ZF and ZR were measured in 10 fields at 400× magnification for each dog.

2.9. RNA extraction and reverse transcription

Total RNA was extracted from pituitaries and adrenal glands in tubes containing 2 ml Trizol (Invitrogen Japan, Tokyo, Japan). The tissues were disrupted using a standard homogenizer. For purification of total RNA, samples were treated with RNasey Mini Kit (QIAGEN, Tokyo, Japan). The yield of RNA was quantified by measuring the optical density of a sample diluted to 1:20 at 260 and 280 nm. 0.5 μg of total RNA of pituitary and adrenal gland were then reverse transcribed with the SuperScript™ III first-Strand Synthesis SuperMix (Invitrogen Japan, Tokyo, Japan). Reverse transcribed samples were diluted to 20 μl in DEPC treated water (Invitrogen Japan, Tokyo, Japan).

2.10. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR analysis for proopiomelanocortin (POMC) and ACTH receptor (ACTH-R) mRNAs were performed using the Applied Biosystems 7500 Sequence Detections System (Applied Biosystems Japan, Tokyo, Japan). Sequences of the canine POMC and ACTH-R were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Corresponding primer pairs (forward and reverse) for the Sybmerge system, as listed in Table 1, were designed using the NCBI database (http://www.ncbi.nlm.nih.gov/) and used according to methods described in the literature [31,32]. The PCR reaction was performed in MicroAmp Strio Tubes (Applied Biosystems, Tokyo, Japan) with an end-volume of 20 μl. A master mix containing 1.0 μl reverse transcribed RNA, 10 μl SYBR GREEN PCR Master Mix, 0.4 μl (10 μmol/l) of respective forward and reverse primer, and 8.2 μl DEPC treated water per reaction was prepared. The PCR reaction was divided into three stages: (1) 2 min at 50°C; (2) 2 min at 95°C, and (3) 15 s at 95°C, and 60 s at 60°C. Stage 3 was repeated 40 times. All samples were assayed in duplicate. The specificity and the size of the PCR products were assessed by adding a melt curve at the end of the amplifications and a single melting curve peak was observed. Data were quantitatively analyzed using a serial dilution of control samples included in each reaction to produce a standard curve. To normalize the expression level of each gene in each sample, the expression level of three commonly used reference genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta glucuronidase (GUS), and ribosomal protein S5 (RPS5)) was determined for each sample. The most stable reference gene for normalization of the relative concentrations of mRNA was identified using the GeNorm Visual basic application for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm/) [33]. We found that GAPDH was the most stably expressed reference gene. The relative expression of each gene in each sample was calculated by dividing by that of GAPDH.

2.11. Statistical analysis

All results were presented as median and range. In the trilostane administration group, changes in serum cortisol concentration, plasma ACTH concentration, and P/B ratio were analyzed by two-way ANOVA followed by Tukey–Kramer’s post hoc tests. Regarding excised tissue weights of pituitary and bilateral adrenal glands, the ratios and area of ACTH-positive cells in the anterior pituitary gland, the width of ZG, ZF, and ZR, the num-

<table>
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<th>Gene</th>
<th>Sequence (5′ → 3′)</th>
<th>Length (bp)</th>
<th>Melting temperature (°C)</th>
<th>Reference</th>
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Fig. 1. Changes in serum cortisol concentrations. Serum cortisol concentrations before (●) and after ACTH stimulation (□) during trilostane administration. The post-stimulation serum cortisol concentrations decreased significantly during trilostane administration. *, $P < 0.01$ vs. at time 0.

ber of cells per field of ZF and ZR, and the expression of POMC and ACTH receptor mRNA were analyzed by one-way ANOVA, and then differences among the means were analyzed using Tukey–Kramer’s post hoc tests. Statistical analyses were performed using Excel 2003 with the add-in software Statcel 2. Differences were considered significant when $P < 0.05$.

3. Results

Changes of serum cortisol and plasma ACTH concentrations during trilostane administration are summarized in Table 2.

3.1. Effect on serum cortisol and plasma ACTH concentrations

After the initiation of trilostane administration, post-stimulation serum cortisol concentrations decreased significantly compared to that observed before administration (Fig. 1). The basal plasma ACTH concentrations in the control group and the trilostane administration group before the administration of trilostane were 5.2 (3.3–10.8) pmol/l and 5.2 (3.3–10.1) pmol/l, respectively. The post-stimulation plasma ACTH concentrations in the control group and trilostane group before the administration of trilostane were 40.9 (19.1–66.9) pmol/l and 43.6 (22.4–75.5) pmol/l, respectively. No significant difference was observed in the basal plasma ACTH concentrations and the plasma ACTH concentrations after CRH-stimulation between the control and trilostane groups. After the initiation of trilostane administration, both the basal plasma ACTH

<table>
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<th>Serum cortisol and plasma ACTH concentrations during trilostane administration.</th>
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<td>4 weeks</td>
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<td><strong>Cortisol (nmol/l)</strong></td>
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<td>Basal concentration</td>
<td>38.6 (27.6–107.6)</td>
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<td>Post-ACTH concentration</td>
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<td><strong>ACTH (pmol/l)</strong></td>
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<tr>
<td>Basal concentration</td>
<td>5.2 (3.3–10.1)</td>
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<tr>
<td>Post-ACTH concentration</td>
<td>543.4 (316.6–894.1)</td>
</tr>
</tbody>
</table>
| nd = not determined.
concentrations and the plasma ACTH concentrations after CRH-stimulation increased significantly compared to those before trilostane administration (Fig. 2). Finally, the plasma ACTH concentrations at 16 weeks increased significantly compared to that at 12 weeks (P < 0.05).

3.2. P/B ratio

Before the administration of trilostane, the P/B ratio was 0.269 (0.256–0.288; × 10⁻² mm⁻¹) in the trilostane administration group. During the administration of trilostane, the P/B ratios showed a significant increase after trilostane administration (Figs. 3 and 4). The P/B ratios at every 2 weeks were 0.288 (0.261–0.330; 2 weeks), 0.314 (0.264–0.332; 4 weeks), 0.329 (0.282–0.354; 6 weeks), 0.336 (0.294–0.368; 8 weeks), 0.342 (0.321–0.353; 10 weeks), 0.331 (0.321–0.355; 12 weeks), 0.348 (0.321–0.356; 14 weeks), and 0.350 (0.323–0.389; 16 weeks), respectively. Finally, the increase of the P/B ratios at 16 weeks increased significantly compared to that at 8 weeks (P < 0.05).

3.3. Tissue weights

The weight of the pituitary was 55 (50–70) mg, 90 (70–120) mg, and 120 (110–130) mg in the control, TS, and TL group, respectively. The weight of the right adrenal gland was 65 (0.41–0.83) g, 1.49 (1.24–3.14) g, and 1.92 (1.91–1.96) g, and left adrenal gland was 0.60 (0.42–0.84) g, 1.39 (1.11–2.88) g, and 1.63 (1.52–1.88) g in the control, TS and TL group, respectively. The weights of the pituitary and bilateral adrenal glands were significantly higher in the TS and TL group than in the control group (pituitary, and right and left adrenal gland, P < 0.01, respectively). The weight of the pituitary of the TL group was also significantly higher than that of the TS group (P < 0.05).

3.4. Pituitary and adrenal morphometry

The ACTH-positive cell area in the control, TS, and TL group were 78.54 (58.37–228.95) μm², 93.66 (72.03–253.22) μm², and 107.87 (78.87–289.77) μm², respectively. The ACTH-positive cell areas were significantly (P < 0.01) greater in the TS and TL group than in the control group. The ACTH-positive cell areas of the TL group were also significantly greater than that of the TS group (P < 0.01) (Figs. 5 and 6). The ACTH-positive cell ratios were 22.2 (18.0–28.2)%, 28.7 (24.2–38.1)%, and 32.7 (32.1–38.9)% in the control, TS, and TL group, respectively. The ACTH-positive cell ratio of the TS and TL group were significantly (P < 0.05) higher than that of the control group.

The MR images prior to trilostane administration revealed no abnormalities in size and shape of the bilateral adrenal glands in all dogs. The width of ZG in the control, TS, and TL group were 0.132 (0.100–0.234) mm, 0.146 (0.112–0.329) mm, and 0.148 (0.108–0.305) mm, respectively. The width of ZF in the control in the control, TS and TL group were 0.786 (0.560–1.719) mm, 1.839 (0.726–3.229) mm, and 1.607 (1.147–2.863) mm, respectively. The width of ZR in the control in the control, TS and TL group were 0.403 (0.257–0.888) mm, 0.858 (0.288–1.986) mm, and 1.101 (0.688–1.739) mm, respectively. The width of ZG, ZF, and ZR were significantly (P < 0.01, respectively) greater in the TS and TL group than in the control group. The number of cells per field of ZF in the control, TS, and TL group were 158.0 (114–194) cells, 110 (72–180) cells, and 107 (94–141) cells, respectively. The number of cells per field of ZR in the control, TS, and TL group were 260 (188–394) cells, 209 (151–289) cells, and 204 (160–235) cells, respectively. The number of cells of ZF and ZR were significantly lower (P < 0.01, respectively) in the TS and TL group than in the control group (Fig. 7).

3.5. Levels of POMC and ACTH receptor mRNA expression

The levels of POMC mRNA expression in the TS and TL group were about 3.3-fold (median 3.48, range
Fig. 3. Changes in pituitary gland observed by Gd-T1-weighted transverse images. Gd-T1-weighted transverse images of a dog in the TS group before (A) and after 8 weeks of trilostane administration (B). Before trilostane administration, pituitary gland height is 4.4 mm and P/B ratio is 0.273. After 8 weeks of trilostane administration, pituitary gland height is 5.6 mm and P/B ratio is 0.339. Gd-T1-weighted transverse images of a dog in the TL group before (C) and after 16 weeks of trilostane administration (D). Before trilostane administration, pituitary gland height is 4.7 mm and P/B ratio is 0.261. After 16 weeks of trilostane administration, pituitary gland height is 6.8 mm and P/B ratio is 0.389. Bar = 10 mm.

Fig. 4. Changes in P/B ratios during the trilostane administration. P/B ratio of more than $0.31 \times 10^{-2}$ mm$^{-1}$ is considered to be pituitary enlarged. a, $P<0.01$ vs. time 0. b, $P<0.01$ vs. 2 weeks. c, $P<0.01$ vs. 4 weeks. d, $P<0.05$ vs. 4 weeks. e, $P<0.01$ vs. 6 weeks. f, $P<0.05$ vs. 8 weeks.

2.09–4.45) and 4.0-fold (median 4.53, range 2.63–4.85) greater than that in the control group ($P<0.01$). The levels of ACTH-R mRNA expression in the TS and TL group were about 2.5-fold (median 2.14, range 1.52–4.35) and 2.7-fold (median 2.76, range 2.40–3.18) greater than that in the control group ($P<0.05$) (Fig. 8).

4. Discussion

Cortisol secretion from the adrenal cortex is regulated by the HPA axis, and a negative feedback mechanism functions to control cortisol concentrations in vivo [34,35]. Several reports have shown that the inhibition of cortisol secretion in dogs with Cushing’s disease by mitotane treatment suppressed negative feedback, and may accelerate the growth of pituitary adenoma
Fig. 5. Immunohistochemical staining of the anterior pituitary gland using anti-ACTH antibody. The ACTH-positive cells in the control (A, B), TS (C, D), and TL group (E, F). Bar = 100 μm (A, C, E) and 50 μm (B, D, F).

Fig. 6. Comparison of the ACTH-positive cell area and cell ratio in the anterior pituitary gland. *, $P < 0.05$ vs. control group (CT). **, $P < 0.01$ vs. CT. #, $P < 0.01$ vs. TS group.

Fig. 7. Comparison of the width and numbers of cells in the adrenal cortex. Hematoxylin and eosin staining of a section from the adrenal gland (A). Left: TS group; right: control group. The adrenal cortex is enlarged in the TS and TL group. Bar = 1.0 mm. The width of the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) (B). The number of cells per field of ZF and ZR (C). **, \( P < 0.01 \) vs. control group. #, \( P < 0.01 \) vs. TS group.

Fig. 8. Comparison of the relative levels of POMC mRNA and ACTH receptor mRNA expression. The relative levels of proopiomelanocortin (POMC) mRNA expression in the pituitary gland and ACTH receptor (ACTH-R) mRNA expression in the adrenal gland. *, \( P < 0.05 \) vs. control group (CT). **, \( P < 0.01 \) vs. CT.
In humans, a number of reports demonstrated the occurrence of Nelson’s syndrome, in which pituitary adenoma growth was accelerated, resulting from the disappearance of cortisol secretion and negative feedback that occurred after bilateral adrenalectomy in Cushing’s disease patients [39–46]. Therefore, Nelson’s syndrome was re-evaluated recently [47–49], and this phenomenon caused by trilostane and mitotane in humans has been termed “chemical Nelson’s syndrome” [50]. We have experienced some PDH dogs in which large pituitary adenomas were found after trilostane treatment. These dogs were diagnosed with PDH without diagnostic imaging, such as CT or MRI, and treated by trilostane. However, several months later, the doses of trilostane needed increased or neurological signs were observed. However, assessing the potential risk in PDH dogs treated with trilostane is difficult. Whether changes similar to those observed in Nelson’s syndrome appear in dogs with Cushing’s disease is controversial, and no studies have investigated the effect of cortisol inhibition by trilostane on the normal pituitary corticotroph. In this study, we investigated the effect of trilostane on the pituitary–adrenal axis by inhibiting adrenal cortex function through the daily administration of trilostane in healthy Beagle dogs.

The dose of trilostane in the trilostane administration group was determined based on reports of the duration of action and its use in PDH dogs [7–12,18–21]. In addition, the doses tended to increase as the therapy continued [8,9,11,12,21]. Since the duration of trilostane is less than 20 h [14], we attempted to suppress cortisol secretion of the adrenal cortex continuously by the twice daily administration in this experiment. The ACTH stimulation test revealed that cortisol secretion was sufficiently suppressed during trilostane administration, and adverse effects such as lethargy, anorexia, vomiting and diarrhea were not observed. With regard to ACTH secretion from the pituitary, the CRH-stimulation test showed that ACTH concentrations sequentially increased remarkably during the trilostane administration, which suggests an increased production and secretion of ACTH at the pituitary corticotroph. This may be attributable to the suppressed negative feedback due to the decreased cortisol secretion from the adrenal cortex induced by trilostane administration. Indeed, increased plasma ACTH concentrations have also been observed in PDH dogs treated with trilostane [18,19].

MRI during the trilostane administration revealed that the pituitary increased in size during the period of trilostane administration. After 4 weeks of treatment, evaluation using the \( P/B \) ratio, a common marker for the size of the pituitary in dogs [28], revealed that the \( P/B \) ratio was greater than 0.31, which was judged to indicate an enlarged pituitary. Immunohistochemical analysis revealed that there was a significant change in the cytoplasmic areas of corticotrophs and the ratio of corticotrophs to all cells in the anterior lobe of the pituitary in the TS and TL group compared with the control group, and, furthermore, the cytoplasmic areas of corticotrophs in the TL group were greater than that of the TS group. In addition, the weight of the pituitary after the end of trilostane administration showed a significant increase in the TS and TL groups. These results suggest that hypertrophy and hyperplasia of the corticotroph may account for the enlargement of the pituitary, and that the longer cortisol secretion is suppressed, the size of the pituitary may become larger with increasing the plasma ACTH concentrations.

Monoclonal expansion of the corticotroph may induce adenomatous formation [51], but the hyperplasia of the corticotrophs observed in this study was not monoclonal. In humans and rats, adrenalectomy and chronic increased secretion of CRH has been shown to result in corticotroph hyperplasia and adenomatous formation [52–54]. However, adenomatous formation of the corticotroph in dogs has not been examined. In this study, since cortisol secretion was inhibited, the production and secretion of ACTH were increased through reduced negative feedback, although adenomatous formation was not observed in the trilostane administration group. Nevertheless, hypertrophy and hyperplasia of the corticotroph and enlargement of the pituitary were observed, probably resulted in reduced negative feedback. Although CRH, which stimulates the production and secretion of ACTH from the corticotrophs, was not measured in the present experiment, an increase in ACTH concentrations and POMC mRNA expression suggests that CRH secretion from the hypothalamus was increased in the trilostane administration group. In humans with Cushing’s disease, CRH secretion from the hypothalamus is believed to increase after the adrenalectomy [49]. In regards to dogs with Cushing’s disease, no study has examined whether CRH concentrations are increased by the suppressed secretion of cortisol. However, if CRH concentrations are also increased by the suppressed secretion of cortisol in dogs with Cushing’s disease, the risk of occurrence of chemical Nelson’s syndrome may become high.

After the end of trilostane administration, the level of ACTH-R mRNA expression was increased, and the size of the bilateral adrenal glands was increased in the trilostane administration group. The ACTH-R is observed principally in cells of the adrenal cortex and...
signals in the ZF to synthesize and secrete cortisol. In many species, ACTH has been reported to up-regulate the level of ACTH-R mRNA and the number of ACTH binding sites in adrenocortical cells [55–58]. Moreover, in murine adrenal glands, a high level of in vivo ACTH is a major factor up-regulating ACTH-R gene expression, while low circulating ACTH may not be a substantial factor for ACTH-R gene expression [58]. With regard to ACTH-R mRNA expression, the remarkably increased ACTH may result in an up-regulation of ACTH-R mRNA expression in the trilostane administration group. Histological analysis using HE staining revealed that each zona of the adrenal cortex was thickened in the TS and TL groups. In addition, an enlargement of cytoplasmic areas and a decrease in cell number per area were observed in ZF and ZR, where cortisol was produced and secreted, suggesting the appearance of hyperplastic changes. Two potential causes for these changes in the adrenal cortex were considered. First, since the ACTH-R mRNA expression and serum ACTH concentrations were raised, stimulation of the adrenal cortex by ACTH was increased. This may lead to hypertrophy and hyperplasia in the cells of the adrenal cortex. Second, the mechanism of action of trilostane may be involved. With inhibition of steroid synthesis in the adrenal cortex, precursors of steroids may accumulate in the cells, which may lead to hypertrophy in the adrenal cortex [59,60]. Although the precursors of steroids were not measured in this study, trilostane administration has been reported to increase precursors of steroids in dogs, humans, and guinea pigs [13,19,61]. Despite the reports that necrosis was observed in the adrenal cortex of the PDH dogs treated with trilostane [59,62], no such changes were observed in the adrenal cortex in the TS and TL groups in this study.

It is not possible to conclude, based on the present experiments using healthy dogs, that similar changes observed in the pituitary corticotroph in the trilostane group will be observed in dogs with Cushing’s disease. However, the negative feedback of cortisol on ACTH secretion from the pituitary is presumed to function even in dogs with Cushing’s disease. The dexamethasone suppression test revealed that cortisol secretion is not suppressed by low-dose dexamethasone (0.01 mg/kg), but is suppressed by high-dose dexamethasone (0.1 mg/kg) due to negative feedback in most dogs with Cushing’s disease [63–65]. Moreover, a correlation was found between the size of the pituitary gland and resistance to dexamethasone, and the dexamethasone resistance was associated with high plasma ACTH concentrations in dogs with Cushing’s disease [28]. Considering these findings, negative feedback by cortisol may occur in dogs with Cushing’s disease, and ACTH secretion may be suppressed by negative feedback by cortisol in the corticotroph adenoma as well.

Since changes in the HPA axis during the trilostane administration were examined in this study, it remains to be clarified how enlargement of the pituitary and hyperfunction of the pituitary corticotroph by suppression of cortisol secretion would change after cessation of the trilostane administration. Taking into account the pharmacology of trilostane, a competitive enzyme inhibitor, these changes may return after cessation of administration. Clinically, however, it is difficult to end trilostane administration in PDH dogs, and life-time treatment is thought to be necessary.

In conclusion, due to the suppression of cortisol secretion by trilostane, reduced negative feedback induced hyperfunction of the pituitary corticotroph, which caused an enlarged pituitary due to corticotroph hypertrophy and hyperplasia in healthy dogs. Moreover, the longer cortisol secretion was suppressed, the more corticotroph hyperfunction increased and the larger the pituitary became. These changes may help to understand whether changes similar to Nelson’s syndrome appear in dogs with Cushing’s disease.

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References


44


