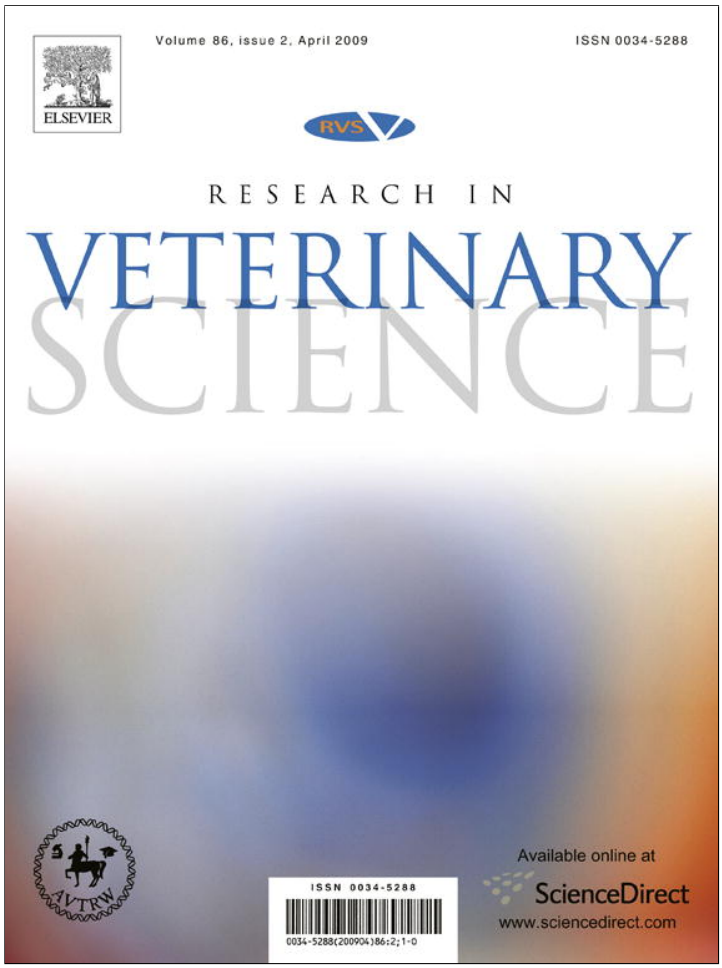

Research in Veterinary Science 86 (2009) 223–229

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Diurnal ACTH and plasma cortisol variations in healthy dogs and in those with pituitary-dependent Cushing's syndrome before and after treatment with retinoic acid

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ARTICLE INFO

Article history:
Accepted 30 June 2008

Keywords:
Cushing's disease
Pituitary-dependent hypercortisolism
Retinoic acid
ACTH diurnal rhythm
Cortisol diurnal rhythm

ABSTRACT

Daytime variations in ACTH and plasma cortisol were studied in healthy dogs and in dogs with pituitary-dependent hypercortisolism (PDH), before and after treatment with retinoic acid. In control dogs ACTH showed a higher concentration at 8.00 AM and between 2.00 and 6.00 PM, with the lowest concentration registered at 10.00 AM ($p < 0.05$ vs. 8.00 AM and 2.00 PM and $p < 0.01$ vs. 4.00 PM). Cortisol did not show significant differences. In dogs with PDH, ACTH was lower at 8.00 AM (ACTH: $p < 0.01$ vs. 2.00 and 4.00 PM; and $p < 0.05$ vs. 6.00 PM). The lowest cortisol concentration was registered at 8.00 AM and 8.00 PM and the highest at 4.00 PM ($p < 0.05$ vs. 8.00 AM and $p < 0.01$ vs. 8.00 PM). After treatment, the lowest ACTH concentration was registered at 10.00 AM ($p < 0.01$ vs. 2.00 and 4.00 PM). To conclude, the adrenal is desensitized in PDH possibly showing negative in diagnostic tests.

1. Introduction

It has long been documented that ACTH and cortisol are secreted following a rhythm throughout the day (circadian rhythm), which in humans and monkeys determines high values in the early morning (8.00 AM) and lower ones at night (as from 20.00 h), with the inverse being the case for rats (Ader and Friedman, 1968; Orth and Kovacs, 1998). This rhythm is synchronized by the hours of light through the suprachiasmatic nucleus, and it reflects the sleep characteristics of each species (Czeisler et al., 1991; Dallman et al., 1978). Whether the dog presents a marked rhythm as in other species is still under discussion. Kempanien and Sartin (1984) suggest that ACTH secretion in the dog is sporadic but not rhythmic nor pulsatile and therefore it is not a typical circadian rhythm.

The first studies on the rhythm of secretion of ACTH and cortisol in dogs were carried out by Egdahl (1962) and by Ferin et al. (1977), who studied the variations in cortisol after different surgical procedures both in the hypothalamus and in the hypophysis. Nevertheless, these surgical procedures caused necrosis both of the anterior and intermediate lobes of the hypophysis, and therefore the results are questioned (Engler et al., 1999). Studies on the circadian rhythm in rats and sheep and in anesthetized dogs have also been carried out, with opposite results because anaesthesia affects the noradrenalin regulation of the hypothalamus–hypophysis–adrenal axis (HHA) (Engler et al., 1999; Ganong, 1980; Jacobs, 1986). As a result of these findings and due to the complex nature of the regulatory mechanisms of this axis, it is difficult to study a complete 24 h rhythm in the dog, especially considering that this species rapidly enters wakefulness.

Cushing's syndrome (CS) is a frequent pathology in the dog, showing 21–23% prevalence with respect to other endocrine diseases (data from the Endocrinology Unit of the Faculty of Veterinary Sciences of the University of Buenos Aires). As in man, the main cause of CS in the dog is the adenoma of the corticotroph area or corticotrophinoma, which secretes adrenocorticotropic hormone (ACTH), receiving the name of Cushing's syndrome or pituitary-dependent hypercortisolism (PDH). In the individual with PDH, the circadian rhythm is either inverted or lost (Liu et al., 1987; Turek, 1994; van der Berg et al., 1995). It has been proven that the frequency of ACTH secretion is not altered, rather the length and amplitude of the wave are increased (Orth et al., 1988; Orth and Kovacs, 1998; Dahia and Grossman, 1999). The treatments for PDH that have been most described and used (both in man and dogs) are the partial or total surgical removal of the hypophysis or radiation of the adenoma (Brada, 1993; Meij et al., 2002), or the use of drugs to inhibit steroid genesis or to destroy the adrenal gland (Castillo et al., 1986; Sonino and Boscaro, 1999; Kintzer and Peterson, 1991; Komanicky et al., 1978). Although synthesis of cortisol is controlled, an increase in ACTH has been observed because the inhibitory effect on the corticotroph is missing (Castillo et al., 2006; Sieber-Ruckstuhl et al., 2006). It is

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doi:10.1016/j.rvsc.2008.06.006

unknown whether the ACTH rhythm is normalized on receiving treatment for PDH with drugs that act on the pituitary. The effects of retinoic acid (RA) have recently been studied for treating PDH in dogs (Castillo et al., 2006), obtaining control of the disease. It has been described that RA inhibits the synthesis of pro-opiomelanocortin (POMC), and therefore also inhibits ACTH, inducing apoptosis of the tumour with a consequent reduction in size (Kang, 2000; Merino and Hurlé, 2003; Páez-Pareda et al., 2001). Taking these effects of RA into account, the diurnal rhythm of ACTH and cortisol was studied in healthy dogs and those with PDH, both before and after treatment with RA. The aim was to see if the rhythm was normalized.

2. Materials and methods

2.1. Population under study

2.1.1. Control group

Six clinically healthy dogs were used (4 females and 2 male; 2 cocker spaniel and 4 mongrels), aged between 4 and 9 years, of normal weight and kept under stress-free conditions (de-wormed, fed twice daily with a balanced commercial diet, and kept in a familiar habitat). Routine laboratory examinations (hematology and general biochemistry) gave normal results, as did the urine cortisol/creatinine ratio and ACTH plasma concentration (samples taken between 2:00 and 4:00 PM, according to previous observations). After this, the diurnal rhythm was studied (see Section 2.4).

2.1.2. Cushing group

Five dogs with PDH (3 female and 2 male, all mongrels), aged between 5 and 12 years were sent to the Endocrinology Unit of the Teaching Hospital at the Faculty of Veterinary Science of the University of Buenos Aires. PDH diagnosis was carried out according to the following criteria: presence of at least 4 clinical signs characteristic of the disease in the dog (Ling et al., 1979); urine Cortisol/creatinine basal ratio (C/CR) greater than 70 nmol/L followed by inhibition with high doses of dexamethasone to confirm the hypophysis as the origin of the disease (Galac et al., 1997); increase in plasma ACTH and evidence of the pituitary adenoma using magnetic nuclear resonance imaging (MNRI) contrasted with gadolinium. Clinical signs common to the dogs of this group were: polydipsia–polyuria, polyphagia, prominent abdomen, obesity, anestrous in all four females, non-elastic thinner skin. No dog had alopecia. As previously reported, RA (isotretinoin 9-cis [RA 9-cis]) was administered as a treatment in the evenings, during 6 months, at a dose of 2 mg/kg/day (Castillo et al., 2006). ACTH (sample obtained between 2.00 and 4.00 PM) and C/CR were determined before and immediately after the last day of treatment and before studying the diurnal rhythm.

2.2. Diagnosis of Cushing's disease

The PDH diagnosis protocol (Endocrinology Unit, Faculty of Veterinary Sciences of the University of Buenos Aires) was based on (1) an increase of urine clearance of cortisol (evaluated through the cortisol/creatinine ratio in urine, C/CR) with inadequate high concentrations of plasma ACTH, and (2) a reduction of the C/CR to more than 50% of the basal value of C/CR previously obtained, according to Rijnberk et al. (1988) and Galac et al. (1997) after oral administration of 0.1 mg/kg of dexamethasone every 8 h. Confirmation of the pituitary adenoma was obtained through nuclear magnetic resonance imaging (NMRI), with sections every 2 mm. To evaluate the appearance of the adenoma, the sagittal section was taken through the middle line, classifying the tumours into intra-sellar ($\leqslant$5 mm) and extra-sellar ($\geqslant$5.5 mm) according to the phases described by Asa and Ezzat (1998) and validated by our previous observations in normal dogs and dogs with PDH.

2.3. Hormone measurements

Plasma ACTH concentration was measured by means of the immunoradiometric assay (IRMA) using an available commercial kit (Nichols Advantage ACTH Assay, Nichols Institute Diagnostics, Bad Vilbel, Germany) previously validated for dogs in our laboratory. The intra-assay and inter-assay coefficients of variation for ACTH were 3% and 6.8%, respectively.

Urine cortisol was measured by means of radioimmunoassay (RIA), using a commercial kit (DPC Corporation, San Diego, California, USA). The urine cortisol was expressed as a ratio of urine cortisol to creatinine (measured in Metrolab Autoanalizer Merck, Germany, according to the manufacturer's indications). The inter- and intra-assay coefficients of variation for cortisol were 8% and 5%, respectively.

Creatinin (measured by the automated kinetic method, BioSystems®, Metrolab Autoanalizer Merck, Germany) inter- and intra-assay coefficient of variation was 1.5% and 5.3%, respectively.

2.4. Diurnal variation: extraction times for plasma ACTH and cortisol

For the study, all dogs were housed in a calm, acclimatized environment, without external noises and with water ad libitum. They had access to an outside garden for exercise and for their physiological needs whenever they needed it. To moderate the stress of a prolonged fast, the last meal prior to the study was given at 12.00 PM. Dogs from both groups were brought by their owners (who accepted to participate in the experience) and, in order to get used to the place, were housed there with their owners 1 h prior to the study. Sample extractions were carried out wherever the dog chose to get comfortable, minimizing in this way the stress provoked by holding them down.

Blood extractions were carried out in both groups every two hours, starting at 8.00 AM and until 8.00 PM for a total of 12 h (samples: 8.00, 10.00 and 12.00 AM, and 2.00, 4.00, 6.00 and 8.00 PM). The blood samples (3 mL) were collected with chilled plastic syringes, containing EDTA, and then centrifuged immediately after collection and frozen (−80 °C) until their processing.

2.5. Statistical analysis

ANOVA–Kruskal–Wallis test followed by the Dunn test were used to analyse the intra- and inter-group results, comparing every two hours within the same group and the controls and the Cushing group before and after treatment. The Mann–Whitney test was used to compare results obtained every two hours within the Cushing group, before and after treatment. In the Cushing group, ACTH (prior to the timetable study) and RC/C (before and after treatment with RA 9-cis) were compared using the Mann–Whitney test. Results are expressed as median and interquartile ranges, with a significance level set at $p < 0.05$. Individual ACTH and plasma cortisol variations are shown using a scatter plotted graph, where each symbol corresponds to a dog.

2.6. Ethical approval

The study was approved by the Ethics Committee of the Faculty of Veterinary Sciences of the University of Buenos Aires and by the Secretaría de Ciencia y Técnica (Secretariat of Science and Techniques) of the said University (UBACyT; Project V045) in fulfilment of the national laws on experiments with animals. Dog owners gave their signed consent for the participation of their animals in this study.

3. Results

As in a previous study (Castillo et al., 2006), dogs treated with AR 9-cis showed clinical recovery: absence of polydipsia–polyuria, polyphagia, decrease in weight and size of abdomen, improvement of skin thickness and elasticity and return to oestrus in 3 of the treated females. ACTH and RC/C levels after treatment were significantly lower than those before treatment (ACTH $p < 0.01$, RC/C $p < 0.01$), showing no differences with the control group (Table 1). With regard to the RMNI study, 3 dogs had extra-sellar adenomas (6.5 mm, 8 mm and 12 mm), with the rest being intra-sellar (5 mm and 4 mm). After treatment with RA 9-cis, the pituitary image was normal in 3 dogs, decreased in size in one and did not vary at all in the dog with the 12 mm adenoma (Table 1).

3.1. Behaviour of the dogs during the study

Once acclimatized to their surroundings, the dogs were calm and alert. After the first extraction (8.00 AM), dogs in both groups either lay down and slept or remained resting until 2.00 PM, with only brief interruptions for the remaining stipulated extractions or to go out to the garden for their physiological needs (especially those belonging to the Cushing group) to then return to rest again. Control dogs and those of the Cushing group (after treatment) were more active after midday. Prior to treatment, the animals spent more time resting and only showed activity between 4.00 and 8.00 PM.

3.2. Diurnal variations of ACTH and cortisol within groups

3.2.1. Control group

Within the group, ACTH medians were significantly different ($p < 0.05$) (Fig. 1a). The highest value was observed at 8.00 AM, and then decreased significantly between 10.00 AM and 12.00 AM ($p < 0.05$ for 10.00 AM vs. 8.00 AM and 2.00 PM; $p < 0.01$ for 10.00 AM vs. 4.00 PM; $p < 0.05$ for 12.00 AM vs. 8.00 AM). As from 2.00 PM, levels increased significantly and, after peaking at 4.00 PM, were maintained almost on a plateau until 8.00 PM, without significant differences between these times. The highest ACTH value was observed at 4.00 PM and the lowest at 10.00 AM.

Regarding cortisol (Fig. 2b), no significant differences were observed between medians. A variation of cortisol, following that of ACTH, can be appreciated when comparing each sampling time (highest concentrations at 8.00 AM and between 2.00 and 6.00 PM). Nevertheless, no differences were observed between the times studied. A decrease in cortisol concentration was observed at 8.00 PM. It is evident that each dog shows individual variation of this hormone throughout the day.

The increase of ACTH in the afternoon, followed by cortisol, coincides with the greater activity shown by the dogs.

3.2.2. Cushing Group

Before treatment, ACTH showed significant differences ($p < 0.001$). Contrary to the control group, low concentrations were observed during the morning when compared to those of the afternoon, with the lowest concentration observed at 8.00 AM ($p < 0.01$ vs. 2.00 and 4.00 PM; $p < 0.05$ vs. 6.00 PM), with no differences with the concentrations at 10.00 and 12.00 AM. At these times, significant differences were observed with the afternoon hours ($p < 0.05$ for 10.00 AM vs. 2.00 PM; $p < 0.01$ for 10.00 AM vs. 4.00 PM and $p < 0.05$ for 12.00 AM vs. 4.00 PM). The highest ACTH pre-treatment value was observed at 4.00 PM, and the lowest at 8.00 AM.

Cortisol also showed different secretion when compared to the controls. Significant differences were observed between intragroup medians ($p < 0.01$). Lowest concentrations were observed at 8.00 AM and 8.00 PM and the highest at 4.00 PM ($p < 0.05$ vs. 8.00 AM; $p < 0.01$ vs. 8.00 PM). The pronounced decrease in cortisol at 8.00 PM is noteworthy (showing no significant difference with 8.00 AM values). Individual variations in cortisol throughout the day were more marked than those of the control group, with one case where the values were always low with respect to the rest of the dogs studied. The greater activity shown by the dogs coincided with the moment of highest cortisol concentration.

After treatment with RA 9-cis, both ACTH and cortisol patterns were similar to that of the control group (Figs. 1a and b). Intragroup median values differed significantly for ACTH ($p < 0.01$) with no differences being observed for intragroup cortisol median values, the latter being the same as those observed in the control group. Similar to that seen in control dogs, ACTH concentration values after treatment were lowest at 10.00 AM and at 12.00 AM ($p < 0.01$ for 10.00 AM vs. 2.00 and 4.00 PM; $p < 0.05$ for 10.00 AM vs. 6.00 PM; $p < 0.05$ for 12.00 AM vs. 2.00, 4.00 and 6.00 PM), and highest at 4.00 PM, even though no significant differences were observed between afternoon timetables. Physical activity in these dogs was the same as that in the control group.

3.3. Secretion of ACTH and cortisol: comparison between controls and PDH dogs before and after treatment

Median values of ACTH (Fig. 2a) showed significant differences between the control group and PDH before and after treatment ($p < 0.0001$ and $p < 0.01$, respectively). When comparing sampling times, the control group showed higher ACTH values at 8.00 AM with respect to the same time in PDH dogs before treatment ($p < 0.01$) and lower values at 2.00 and 4.00 PM ($p < 0.05$ vs. same times). No significant differences were found between these sampling times when comparing the control group to the PDH group after treatment with RA 9-cis.

Significant differences were observed between median ACTH values ($p < 0.0001$) when comparing sampling times between PDH dogs before and after treatment. ACTH values before treatment were lower at 8.00 AM ($p < 0.05$) vs. 8.00 AM after treatment and were higher at 4.00 PM ($p < 0.01$) vs. 4.00 PM after treatment.

Cortisol median values (Fig. 2b) showed significant differences between the control group and the PDH group before treatment ($p < 0.01$), but no differences between the control group and PDH group after treatment. When comparing sampling times, the PDH group before treatment showed higher concentrations at 4.00 PM

Table 1
ACTH, C/CR and pituitary tumour size (using RMNI) in control dogs and in those with PDH before and after treatment

	Control	PDH	
		Before treatment	After treatment (6 months)
ACTH (pmol/L)	15.6 (14.4–17.5)	23.1[a] (20–30.5)	15.3 (13–15.3)
RC/C	42.5 (28.5–52.5)	109[b] (92.5–136)	45 (37.5–49)
Size of adenoma (mm)			
#1		8	5
#2		12	12
#3		6.5	0
#4		5	0
#5		4	0

Control group $n = 6$; PDH group (before and after treatment with RA 9-cis) $n = 5$.
Values expressed as median and interquartile ranges. Mann–Whitney test.
Size of adenoma (sagittal slide) measured in each dog. Normal pituitary aspect is expressed as "0 mm".
[a] ACTH: $p<0.01$ PDH pre-treatment vs post RA and vs control.
[b] C/CR: $p<0.01$ PDH pre-treatment vs post RA and vs control.

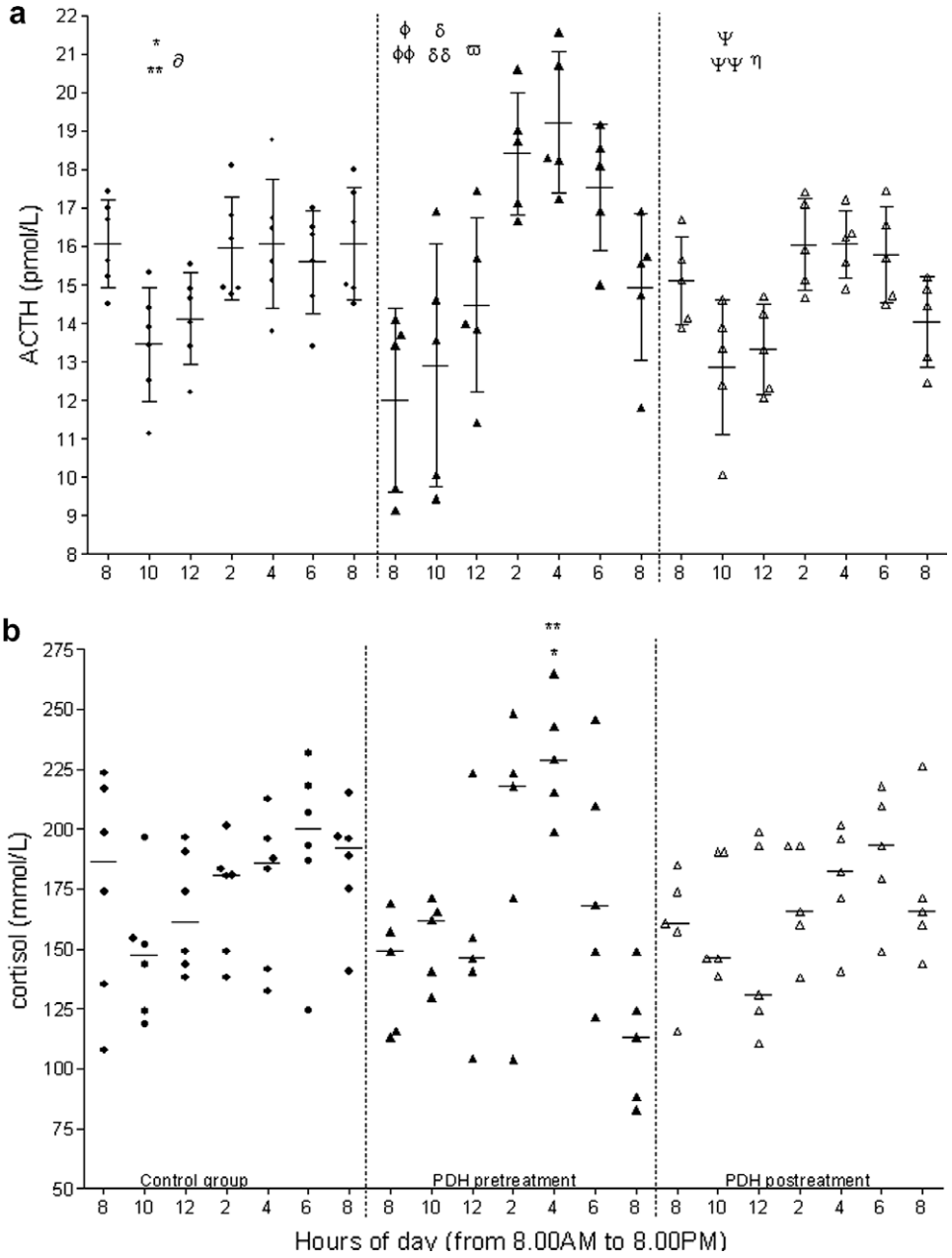

Fig. 1. Diurnal variation within groups of ACTH (a) and of plasma cortisol (b) in healthy dogs and in dogs with PDH before (full triangles) and after (open triangles) treatment with 2 mg/kg/day of RA-9-cis. (a) ACTH: controls $^{*}p < 0.05$: 10.00 AM vs. 8.00 AM and 2.00 PM; $^{**}p < 0.01$: 10.00 AM vs. 4.00 PM; $^{\sigma}p < 0.05$: 12.00 AM vs. 8.00 AM. PDH before treatment: $^{\phi\phi}p < 0.01$: 8.00 AM vs. 2.00 and 4.00 PM and $^{\phi}p < 0.05$: 8.00 AM vs. 6.00 PM; $^{\delta}p < 0.05$: 10.00 AM vs. 2.00 PM and $^{\delta\delta}p < 0.01$: vs. 4.00 PM; $^{\varpi}p < 0.05$: 12.00 AM vs. 4.00 PM. PDH after treatment: $^{\psi\psi}p < 0.01$: 10.00 AM vs. 2.00 and 4.00 PM, $^{\psi}p < 0.05$ vs. 6.00 PM; $^{\eta}p < 0.05$: 12.00 AM vs. 2.00, 4.00 and 6.00 PM. (b) Plasma cortisol: PDH before treatment $^{*}p < 0.05$: 4.00 PM vs. 8.00 AM and $^{**}p < 0.01$: 4.00 PM vs. 8.00 PM. Values expressed as median and inter-quartile ranges. ANOVA-Dunn's test.

vs. the control group ($p < 0.01$) and lower concentrations at 8.00 PM ($p < 0.05$) vs. the control group at the same time.

Comparing between PDH groups before and after treatment, cortisol median values showed significant differences ($p < 0.01$), with pre treatment values at 8.00 PM being lower than values at the same timetable after treatment ($p < 0.05$).

4. Discussion

RA 9-cis controlled PDH, as previously described (Castillo et al., 2006) and as evidenced in the values of ACTH and RC/C and also in the decrease observed in the size of the adenoma in 4 out of 5 dogs. Regarding the study on the diurnal variation of ACTH and cortisol, both showed variations, although not as marked as those described in humans and rats. These variations accompanied the behaviour (rest or activity) of the healthy dogs during the study. When resting, both ACTH and cortisol values decreased, to then increase when active. The value observed at 8.00 AM in these animals can be explained by the fact that they were active and excited by the transfer and adaptation to a new environment. The lower concentrations of both hormones between 10.00 and 12.00 AM were obtained in resting relaxed dogs, after they had explored their environment. After midday, ACTH, accompanied by cortisol, increases and maintains a plateau until 8.00 PM, coinciding with moments of activity. These variations match reports made by Kempanien and Sartin (1984) and Orth et al. (1988), where ACTH secretion was sporadic and not rhythmic. ACTH behaviour shows

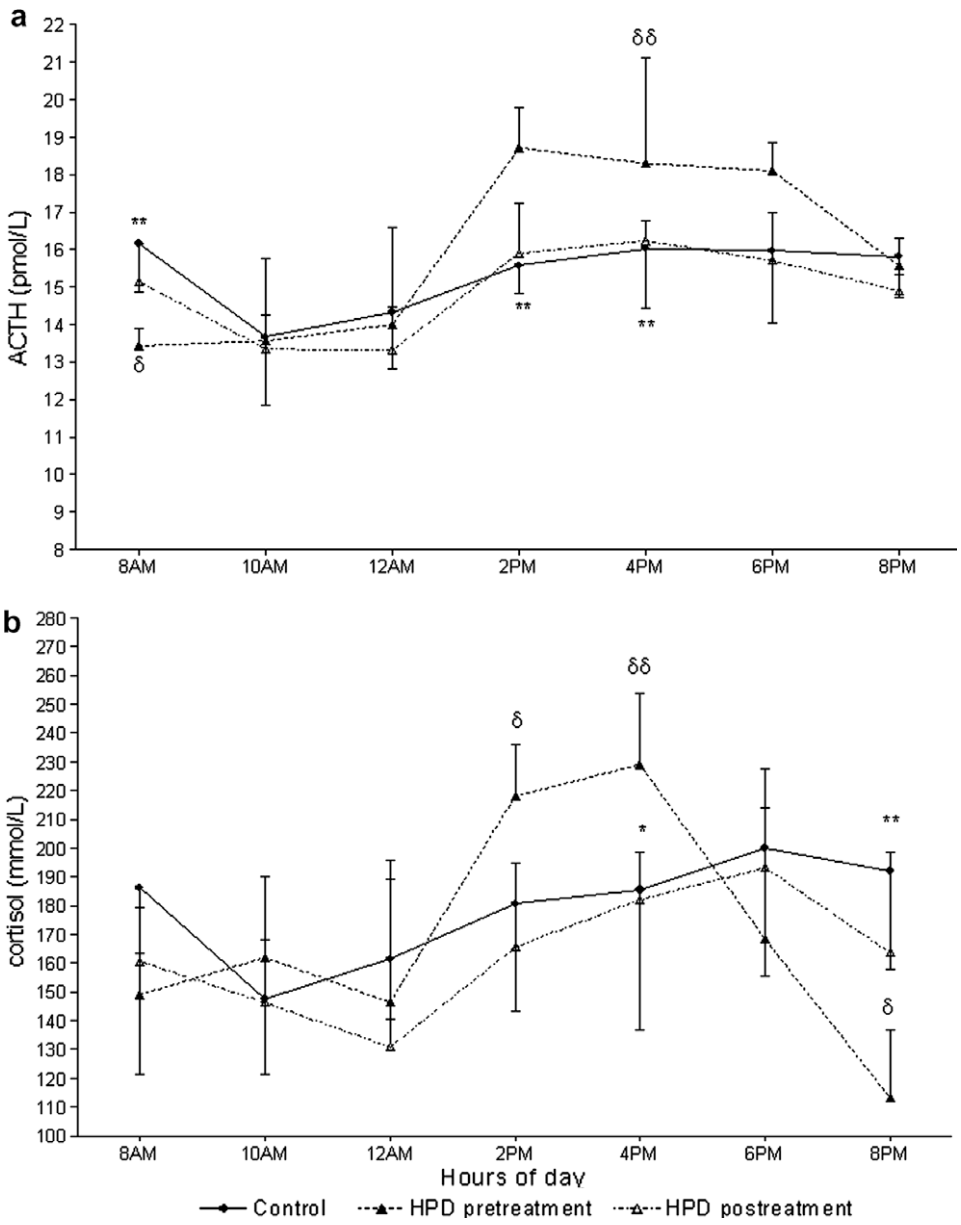

Fig. 2. Comparison of the diurnal variation of ACTH (a) and plasma cortisol (b) between the control group and PDH group before and after treatment with RA 9-cis (2 mg/kg/day). Note that in the PDH group after treatment, ACTH and cortisol behaviour is similar to the control group. In the PDH group before treatment, after a peak at 4 PM, there is a marked decrease in cortisol (8.00 PM). (a) ACTH: Control vs. PDH before treatment: $^{**}p < 0.01$ vs. 8.00 AM and vs. 4.00 PM; $^{*}p < 0.05$ vs. 2.00 PM. PDH before treatment vs. after treatment: $^{\delta}p < 0.05$ vs. 8.00 AM and $^{\delta\delta}p < 0.01$ vs. 4.00 PM. (b) Cortisol: Control vs. PDH before treatment: $^{**}p < 0.01$ vs. 8.00 PM; $^{*}p < 0.05$ vs. 4.00 PM. PDH before treatment vs. after treatment: $^{\delta}p < 0.05$ vs. 8.00 PM. Values expressed as median and inter-quartile ranges. Comparisons made between every hour of controls vs. PDH before and after treatment were carried out using ANOVA-Dunn test; comparisons made between each hour of PDH before and after treatment were carried out using Mann–Whitney test.

individual variation and would depend on the specific moment being experienced by each dog.

Population cortisol did not present significant variations throughout the day, similar to that observed in studies in rats (Buijs et al., 1997). Nevertheless, individual variations for each animal were observed in response to specific external stimuli, in the case of this study, the moment of venopuncture or their greater physical and psychic activity after midday. This was similar to what has been reported by Ader and Friedman (1968) in one of their first studies. This behaviour of ACTH and cortisol in normal dogs can be explained by the fact that the hypothalamus–hypophysis–adrenal axis (HPA) shows two different types of activation: one that depends directly on the suprachiasmatic nucleus (SCH), essential for maintaining the energy balance responsible for the circadian variations of the hormones. The other activation depends on the stress from the stimuli received (Dallman et al., 1978; Moore and Eichler, 1972; Watts et al., 2004). ACTH, followed by cortisol, decreases during rest. In this aspect, the lesser or no effect of light on the SCH would determine the decrease observed in ACTH between 10.00 and 12.00 AM (Sage et al., 2001). On the other hand, the rhythm of secretion of cortisol and ACTH shows certain autonomy, being able to function independently (Ottenweller et al., 1978; Vinson et al., 1994; Wood et al., 1982a). Autonomous innervations of the adrenal gland play an important role in the rhythm of cortisol, its dissociation from the rhythm of ACTH and from the response of the adrenal cells to this hormone (Buijs et al., 1997; Dijkstra et al., 1996). Experiments carried out in hypophysectomized dogs show that cortisol still maintains a secretion rhythm

although lacking in ACTH stimulus (Engeland and Gann, 1989). ACTH rhythm is maintained throughout the day in sheep without splenic innervations, despite having low or no cortisol concentrations (Engeland, 1998). These adrenal innervations would be one of the reasons why cortisol does not present significant differences throughout the day and why it is not an exact reflection of ACTH variations (Jasper and Engeland, 1994). On the other hand, Nicholson et al. (1985) and Wood et al. (1982a,b) have demonstrated, both in rats and in dogs, that the adrenal gland shows changes in its sensitivity to ACTH stimulus throughout the day. This change in sensitivity would involve the diurnal variation in blood flow that the adrenal gland receives (Jones et al., 1990; L'Age et al., 1970). Therefore, the autonomous nervous system and the variations in sensitivity of the gland would result in cortisol secretion not being completely parallel to ACTH variations.

It is clear that in dogs with PDH, the ACTH and cortisol patterns of secretion are altered. Strikingly, concentrations of both hormones are low in the morning (particularly at 8.00 AM when compared to controls), increasing slowly throughout the day until reaching a maximum between 4.00 and 6.00 PM to then decrease significantly. This happens despite the state of either rest or activity of the dogs. Although it cannot be stated that the circadian rhythm is inverted in dogs, as has been described in other species, it is evident that there are changes in the way these hormones are secreted, particularly cortisol. Kaneko et al. (1980, 1981) and more recently Young et al. (2004) have described that the adrenal gland becomes desensitized when subjected to repeated and increasing ACTH stimuli, until it stops responding to this hormone. Bradbury et al. (1991) have reported a decrease in cortisol in the evening in situations of chronic stress, similar to our observations in the dogs with PDH. In previous work from van der Berg et al. (1995), the authors report that in Cushing patients there would be evidence of a progressive decrease in response of the adrenal gland to ACTH. Our findings in dogs coincide with these authors, in agreement with the pronounced decrease observed in cortisol concentrations observed in the evenings. This could be because of a greater dissociation between the adrenal–pituitary-autonomous system and/or an alteration of the regulation by the SCH accompanied by an increase in blood flow to the adrenal gland in the individuals with PDH. It would be interesting to analyse how cortisol would vary at night, if it follows the same pattern as during the day or if at night there is more cortisol secretion because of a greater sensitivity and response of the adrenal gland to ACTH, as has been described by Carnes et al. (1989) and Cascio et al. (1987).

Therefore in PDH, due to a greater exposure to ACTH in the timetables studied combined with a greater blood flow and light variations inherent to the times of day or because of the environment, the result would seem to be a loss of sensitivity of the adrenal gland to ACTH stimulus. Cortisol secretion decreases, resulting in the lower concentrations observed in the morning and evening. This would also explain the 10–12% false negatives we have observed when stimulating dogs with PDH with 25 UI/EV of synthetic ACTH. Once sensitivity is recovered and in the presence of a new secretion of ACTH, the adrenal gland would hyper-respond to the increased stimulus., Apparently moments of greater ACTH secretion would seem to occur, followed by a greater response by the adrenal gland and a resulting increase in cortisol and moments of lower sensitivity to ACTH and low cortisol, even in the presence of high concentrations of ACTH, as was observed in this study. After treatment with RA 9-cis, secretion characteristics tend to normalize, as they become similar to those of the healthy controls. This is a clear indication that when treating the ACTH producing adenoma and regularizing the secretion of this hormone, the pituitary–adrenal-autonomous nervous system complex and the regulation by the SCH would once again be in harmony. This has not been studied previously because when using drugs that affect the adrenal cortex the changes in ACTH and cortisol cannot be evaluated.

In conclusion, the dog would seem to manifest diurnal changes according to its physical and psychic activity, with the afternoon secretion being the highest for ACTH. Therefore, this must be taken into account when using this hormone to diagnose the difference between PDH and adrenal neoplasia. Cortisol measured in blood would not have diagnostic value, taking into account the desensitizing of the adrenal cortex to ACTH in Cushing's disease, when carrying out the stimulation test with synthetic ACTH and getting a negative result despite a clinical suspicion of the disease.

Acknowledgement

This study was carried out with a grant given by UBACyT (Project V045).

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