ISSN 0103-8478

NON-RADIOMETRIC IMMUNOASSAYS [FLUOROIMMUNOASSAY (FIA) AND FLUOROMETRIC ENZYME IMMUNOASSAY (FEIA)] WITH RADIOIMMUNOASSAY (RIA) FOR EVALUATION OF ADRENAL FUNCTION IN NORMAL AND HYPERCORTISOLEMIC DOGS

MÉTODOS DE IMUNOENSAIO NÃO-RADIOMÉTRICOS [FLUOROIMUONOENSAIO (FIE) E ENZIMAIMUNOENSAIO (EIE)] E O RADIOIMUNOENSAIO (RIE) NA AVALIAÇÃO DA FUNÇÃO ADRENAL DE CÃES NORMAIS E CÃES COM HIPERADRENOCORTICISMO

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SUMMARY

Non-radiometric immunoassays offer many advantages over radiometric assays, such as higher stability of kit compounds and absence of potential hazardous effects for users and environment. The comparison of cortisol measurements by fluoroimmunoassay (FIA) and fluorometric enzyme immunoassay (FEIA) with radioimmunoassay (RIA) in adrenal function evaluation of normal (n=50) and hypercortisolemic dogs (n=12) was proposed. Serum concentrations of cortisol were measured in basal conditions and 8 hours after dexamethasone (DEX) suppression (0.01mg/kg/IV). All our reference values were based on the 5th and 95th percentile. The values for basal cortisol of healthy dogs were 0.20 to 2.35µg/dℓ for FIA, 0.30 to 5.39µg/dℓ for FEIA, and 0.65 to 4.64µg/dℓ for RIA. After DEX suppression the values were ≤0.87µg/dℓ, ≤0.30µg/dℓ and ≤ 0.80µg/dℓ for FIA, FEIA and RIA, respectively. In hypercortisolemic dogs, the values of cortisol (mean ± SD) in basal and post-DEX conditions were 2.71 ± 0.41µg/dℓ and 1.73 ± 1.15µg/dℓ for FIA, 7.05 ± 2.85µg/dℓ and 4.93 ± 2.26µg/dℓ for FEIA, and 4.80 ± 1.43µg/dℓ and 3.52 ± 1.08µg/dℓ for RIA. Statistically significant differences ($p<0.05$) between the normal and the hypercortisolemic groups (Kruskal-Wallis test) were observed in the three methods, and between basal and post-DEX values (Wilcoxon test) using RIA and FEIA methods but not with FIA. Cortisol determinations by FEIA and RIA methods at DEX suppression test showed 100% of sensitivity and specificity for the diagnosis of hyperadrenocorticism in dogs. The results demonstrate that serum cortisol concentrations measurements by FEIA is a suitable alternative to the traditional RIA method for adrenal function evaluation in dogs.

***Key words**: fluoroimmunoassay, enzyme immunoassay, radioimmunoassay, dogs, hyperadrenocorticism*

RESUMO

Os métodos de dosagem hormonal por técnicas não-radioativas apresentam inúmeras vantagens sobre os que utilizam radioisótopos como marcadores de hormônios ou anticorpos. Dentre tais vantagens, incluem-se maior meia-vida útil dos

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Recebido para publicação em 18.07.00. Aprovado em 11.07.01

reagentes por maior estabilidade dos compenentes, inexistência de riscos de contaminação radioativa, tanto pessoal quanto ambiental. Para avaliar a aplicação destes novos métodos na prática da endocrinologia clínica de pequenos animais, comparamos o método de radioimunoensaio (RIE) aos métodos alternativos fluoroimunoensaio (FIE) e enzimaimunoensaio (EIE) na avaliação da função adrenal canina. Para tanto, padronizaram-se os níveis de cortisol em cães normais (n=50) e em cães com hiperadrenocorticismo (n=12), sob condições basais e oito horas após supressão com dexametasona (DEX) (0,01mg/kg IV). Os valores do grupo de animais controle compreendidos entre os percentis 5 a 95 foram estabelecidos como normais. Os valores de cortisol basal foram de 0,20 a 2,35µg/dℓ para FIE, 0,65 a 4,64µg/dℓ para RIE e de 0,30 a 5,39µg/dℓ para EIE. Após supressão com DEX, os valores do grupo controle foram <0,87µg/dℓ para FIE, <0,80µg/dℓ para RIE e 0,30µg/dℓ para EIE. Nos cães com hipercortisolismo, os valores de média e desvio padrão dos níveis de cortisol em condições basais e pós-supressão com DEX foram de 2,71 ± 0,41µg/dℓ e 1,73 ±1,15µg/dℓ (FIE), 7,05 ± 2,85µg/dℓ e 4,93 ± 2,26µg/dℓ (EIE), e 4,80 ± 1,43µg/dℓ e 3,52 ± 1,08µg/dℓ(RIE), respectivamente. Os resultados obtidos pelos três métodos (RIE, FIE, EIE) foram comparados, de forma a se averiguar a sua confiabilidade para o diagnóstico do hiperadrenocorticismo endógeno, no que tange às suas características de sensibilidade e especificidade. Desta forma, na avaliação da função adrenal, os métodos de EIE e RIE apresentam desempenhos semelhantes, considerados ideais (ambos com 100% de sensibilidade e especificidade). Concluiu-se que o método de EIE, para avaliação da função adrenal, mostrou-se tão adequado quanto o tradicional método de RIE para as determinações de cortisol.

***Palavras-chave**: fluoroimunoensaio, enzimaimunoensaio, cães, radioimunoensaio, hiperadrenocorticismo.*

INTRODUCTION

Canine hyperadrenocorticism is a common endocrine disorder caused by the excessive cortisol secretion by the adrenal cortex. Approximately 85% of hypercortisolemic dogs have excessive adrenocorticotropic hormone (ACTH) secretion from the pituitary gland, resulting in bilateral adrenocortical hyperplasia. The other 15% have adrenocortical tumors, which autonomously secrete excess amounts of cortisol (MACK & FELDMAN, 1990; FELDMAN & NELSON, 1996). Diagnosis of canine hyperadrenocorticism is based on history, physical examination, results of routine laboratory tests (CBC, serum biochemical analysis, and urinalysis) along with serum cortisol determinations from specific pituitary-adrenal function tests, such as the low-dose dexamethasone suppression test (KAPLAN ***et al.***, 1995).

Accurate measurements of serum cortisol have been made by radioimmunoassay (RIA) since the 1970s. However, recently developed non-radiometric immunoassays offer many advantages over RIA kits: (a) they share the characteristics of specificity, sensitivity and speed of the isotopic techniques; (b) the assay compounds are stable for relatively long periods and there are no health hazards for users and environment and (c) special training and licenses associated with the use of radioactive material are not required (GIEGEL ***et al.***, 1982; SOINI & KOJOLA, 1983; LÖVGREN ***et al***.; 1984; ALBERTSON, 1990; PETTERSON, 1993).

The purpose of this study is the comparison of cortisol measurements by fluoroimmunoassay (FIA) and fluorometric enzyme immunoassay (FEIA) with radioimmunoassay (RIA) in the evaluation of adrenal function in normal and hypercortisolemic dogs.

MATERIAL AND METHODS

Dogs- Fifty healthy dogs (25 males and 25 females) from the Police Force kennel were used as normal control (**Group N**). Other 12 dogs were referred to the Veterinary Hospital of the University of São Paulo, with clinical signs of hypercortisolism. Preliminary routine laboratory results and adrenal evaluation, combined with positive response to the therapy with mitotane were compatible with hyperadrenocorticism diagnosis (**Group HC**). Both groups were submitted to the low-dose dexamethasone suppression test (baseline cortisol measurements and 8 hours after 0.01mg/kg of dexamethasone/IV).

Cortisol measurements- Serum cortisol concentration was determined by commercial kits of radioimmunoassay[a] (RIA), fluoroimmunoassay[b] (FIA) and fluorometric enzyme immunoassay[c] (FEIA), according to the manufacturer's instructions. All samples were analyzed in duplicate. The classic RIA method (I^{125} labelled), which uses rabbit polyclonal antibodies, detected concentrations down to 0.21µg/dℓ, with inter and intra-assay coefficients of variation which were < 8.3%. Cortisol determinations by FIA method, a solid phase time-resolved fluoroimmunoassay based on the competitive reaction between europium-labelled and non-labelled cortisol for a limited amount of binding sites on cortisol specific mouse monoclonal antibodies. The assay detected cortisol concentrations down to 0.20µg/dℓ, with inter and intra-assay coefficients of variation < 3.2%. The FEIA cortisol measurements, also based on competitive reaction by rabbit policlonal antibodies between the sample cortisol and the labelled cortisol with calf intestinal alkaline phosphatase (that will produce fluorescence when it reacts with the appropriate substrate), resulted in sensitivity level of 0.3µg/dℓ and inter and intra-assay coefficients of variation < 6.3%.

Statistical analysis- The results of the 2 groups (normal and hypercortisolemic dogs) were compared by non-parametric statistical analysis, including the Kruskal-Wallis, Wilcoxon and Friedman tests. Sensitivity and specificity were also calculated for basal and post-DEX measurements, for each one of the three immunoassays evaluated.

RESULTS

The results are shown in tables 1 and 2 and in figures 1 and 2. Reference values for basal cortisol of healthy dogs on the 5th and 95th percentile were: 0.20 to 2.35µg/dℓ for FIA, 0.30 to 5.39µg/dℓ for FEIA, and 0.65 to 4.64µg/dℓ for RIA. After dexamethasone suppression the values on the 95th percentile were ≤ 0.87µg/dℓ for FIA, ≤ 0.30µg for FEIA and ≤ 0.80µg/dℓ for RIA. The Wilcoxon test showed a statistically significant difference ($p<0.05$) between the basal and after dexamethasone suppression cortisol values for all methods in Groups N and HC, except for FIA in Group N ($p>0.179$). The Kruskall-Wallis test showed that the three methods are able to differentiate normal from hypercortisolemic dogs, for baselines as well post-DEX values. The Friedman test showed that the values of cortisol are statistically different among the three methods, either for basal or post-DEX results. Sensitivity and specificity of 100% of the dexamethasone suppression test for the diagnosis of hyperadrenocorticism in dogs was achieved with cortisol determinations done by both FEIA and RIA methods, when compared Groups N and HC.

DISCUSSION

Measurement of serum or plasma cortisol concentrations by the low-dose dexamethasone suppression test has been one of the most useful diagnostic tools for the diagnosis of canine hyperadrenocorticism with sensitivity values between 95 and 100% (FELDMAN & NELSON, 1996; KAPLAN ***et al.***, 1995; GUPTILL ***et al.***, 1997). A low dose of dexamethasone injection provides enough negative feedback in normal dogs to suppress pituitary ACTH secretion and therefore reduces cortisol concentration within 2 to 3 hours to a less than standard value set by each laboratory. Suppression in normal dogs persists for at least 8 hours and often for several days (FELDMAN & NELSON, 1996; GUPTILL ***et al.***, 1997). All dogs selected for the Group HC, together with their history and clinical signs, exhibited previous results of adrenal function evaluation by the low-dose dexamethasone suppression test which were compatible with endogenous hyperadrenocorticism (according to our reference values). Moreover, they presented positive response to the treatment with mitotane.

Non-radioactive methods of immunoassay have some advantages over RIA because they do not require the handling of radioactive materials. Assay compounds remain stable for relatively long periods, special training and licenses associated with the use of radioactive material are not required and the health hazards are much less likely. (GIEGEL ***et al.***, 1982; SOINI & KOJOLA, 1983; LÖVGREN ***et al.***; 1984; ALBERTSON, 1990; PETTERSON, 1993. Very limited information is available on the measurement of cortisol concentrations by the modern non-isotopic immunoassays methods in dogs, and the few reported studies focused on the iodothironines (PARADIS ***et al.***, 1996; THORESEN ***et al.***, 1996) or canine prolactin (HOIER & JENSEN, 1996). To the authors' knowledge, no published data are available on the determination of cortisol concentrations by FIA or FEIA in the canine serum (baseline or after dexamethasone administration values).

The results show that measurements of serum cortisol concentrations by FIA, RIA or FEIA in the low-dose dexamethasone suppression test are able to differentiate normal from

Table 1 - Results of serum cortisol (µg/dl) concentrations before and 8h after dexamethasone suppression (0.01 mg/kg IV) measured by fluoroimmunoassay (FIA), radioimmunoassay (RIA) and fluorometric enzyme immunoassay (FEIA), in normal dogs (Group N - n=50) and in dogs with hyperadrenocorticism (Group HC - n=12).

		Group N		Group HC	
		Mean ± S D	*p*	Mean ± S D	*p*
FIA	Basal	0.41 ± 0.66		2,71 ± 1,41	
	Post DEX	0.25 ± 0.27	*0.179*	1,73 ± 1,15	*0.005*
RIA	Basal	1.44 ± 1.25		4,80 ± 1,43	
	Post DEX	0.58 ± 0.11	*<0.001*	3,52 ± 1,08	*0.007*
FEIA	Basal	1.26 ± 1.65		7,05 ± 2,85	
	Post DEX	< 0.30	*<0.001*	4,93 ± 2,26	*0.003*

(Wilcoxon's test)

Table 2 - Comparison of sensitivity and specificity of basal cortisol and cortisol 8 hours after dexamethasone suppression as determinated by fluoroimmunoassay (FIA), radioimmunoassay (RIA) and fluorometric enzyme immunoassay (FEIA).

ADRENAL FUNCTION						
	CORTISOL BASAL			CORTISOL POST DEX		
	FIA	RIA	EIA	FIA	RIA	EIA
Sensitivity	66.7%	66.7%	66.7%	75.0%	100%	100%
Specificity	96.0%	96.0%	96.0%	96.7%	100%	100%
FP(1)	2	2	2	1	0	0
FN (2)	4	4	4	3	0	0

(1) FP: false positive; (2) FN: false negative

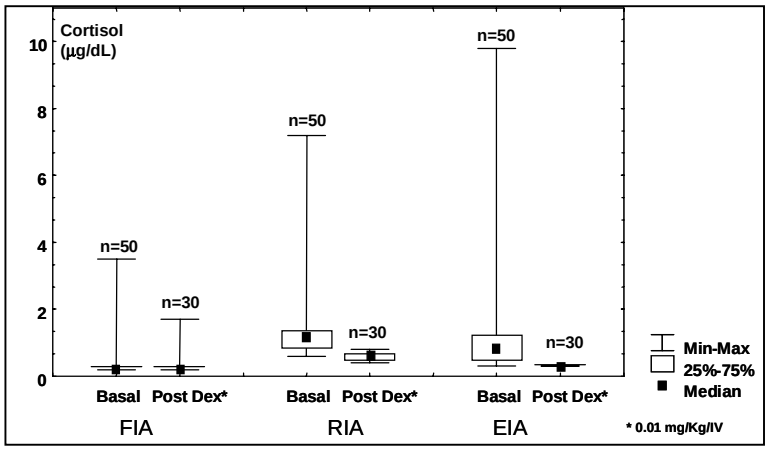

Figure 1 - Basal serum cortisol concentrations and 8 hrs after dexamethasone (Dex) in normal dogs (Group N), measured by fluoroimmunoassay (FIA), radioimmunoassay (RIA) and fluorometric enzyme immunoassay (FEIA).

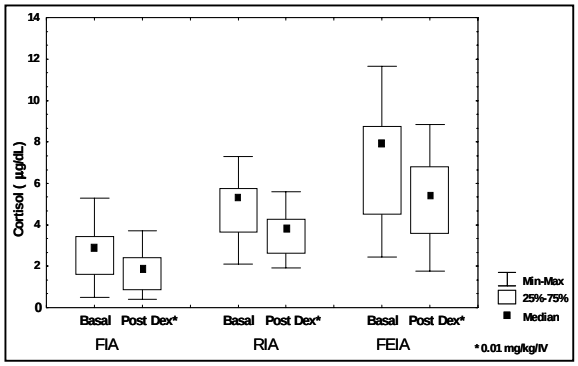

Figure 2 - Serum cortisol concentrations in 12 dogs with hyperadrenocorticism (Group HC), basal and 8 hrs after dexamethasone (Dex), measured by fluoroimmunoassay (FIA), radioimmunoassay (RIA) and fluorometric enzyme immunoassay (FEIA).

hypercortisolemic dogs. On the other hand, baseline and post DEX FIA cortisol values were not different in the normal group. FIA cortisol values were the lowest in both groups when compared to RIA and FEIA methods. This fact probably interfered with the sensitivity and specificity of the FIA method. The FEIA method showed similar results to RIA regarding sensitivity and specificity (both with 100% on post-DEX values), when compared to the groups of normal and hypercortisolemic dogs.

In this paper the reference values for cortisol levels were established by the three methods, in basal conditions and after low dose dexamethasone suppression. The authors conclude that serum cortisol measured by FEIA may be a valuable alternative to the traditional radioactive method.

ACKNOWLEDGMENTS

We are thankful to the command of the Police Force of the state of São Paulo for their permission to use their dogs as the normal group and to the veterinarians and technicians at the Veterinary Hospital and the Hormonal Analysis Laboratory for their support.

SOURCES AND MANUFACTURERS

[a]Gamma Coat [125I] Cortisol RIA Kit, INCSTAR, USA
[b]AUTODELFIA Cortisol Kit, Wallac, Finland
[c]Stratus Cortisol Fluorometric Enzyme Immunoassay, Dade, USA.

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