Australian Journal of Basic and Applied Sciences, 6(13): 154-158, 2012
ISSN 1991-8178

Non-Invasive Monitoring Of Fecal Cortisol Metabolites Level In Free-Ranging Asiatic Elephants In Response To Stress Due To Environmental Factors

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Abstract: In the present study we determined the efficacy of the measurement of fecal cortisol levels in fifteen female adult free-ranging elephants of three different protected wildlife regions of Tamilnadu State, India during dry seasons (February- June, 2010) were examined. Fresh dung samples were collected in a sterile container with 80% methanol solution and brought to the laboratory and the samples were frozen at -20^{o}C until the extraction procedure. Using ELISA, the mean values of cortisol concentration from the examined dung samples were 158ng/g, 153ng/g and 26.4ng/g of feces from Mudumalai wildlife sanctuary, Anamalai wildlife sanctuary and Sathyamangalam-Erode forest divisions respectively. These data showed the determination of fecal cortisol level can be very useful and an appropriate technique for monitoring adrenal activity in wildlife and as a complement to behavioral, physiological, and pathological studies.

Key words: Free-ranging elephants, Wildlife Sanctuary, protected areas, Cortisol, stress.

INTRODUCTION

Elephants are the largest land mammals and most primeval mega herbivore with evolutionary history of more than 60 million years. The word elephant root in Latin is with two words **"ele"** meaning arch and **"phant"** meaning huge. In short it's a **"Huge Arch".** The world's single largest populations of Asian elephants are seen in Nilgiris - Eastern Ghats region of southern India.

Many conservation measures aim in the control of poaching and new threats to wildlife in India; but on the other hand, many infectious diseases and clinical problems taking upper hand in many of the wild animals are always given less significance than they actually deserve. Wild animals are more prone for the stress compared to their domestic counterparts, as they are not used to human intervention. Animal welfare has increasing significance in the current periods and absence of chronic stress is one of the prerequisites for welfare of elephants. A potential indicator of animal welfare is the absence of stress. A package of hormones like ACTH, glucocorticoids, catecholamines, prolactin etc. is involved in the stress response.

During the past 20 years, measuring steroid hormone metabolites in fecal samples has become a widely appreciated technique, because it has proved to be a powerful, noninvasive tool that provides important information about an animal's endocrine status (adrenocortical activity and reproductive status). However, although sampling is relatively easy to perform and free of feedback, a careful consideration of various factors is necessary to achieve proper results that lead to sound conclusions (Palme, 2005). Noninvasive measures of physiological stress have a wide array of applications for conservation biology, wildlife management, animal husbandry, behavioral ecology and biomedicine. In order to assess stress indirectly in wild migrating elephants, the fecal cortisol is generally estimated in many species, including the mega herbivores under study. Considering all these, in order to add more essence to the conservation biology, the present work may act as a tool for successful conservation in India.

MATERIALS AND METHODS

Fresh dung Samples from fifteen adult female free-ranging elephants collected were subjected to through mixing of 2-3 central portions of freshly voided dung materials of each elephants from the free-ranging wildlife regions because Schwarzenberger *et al.* (1996) opined that the steroids might be unevenly distributed in the fecal balls of elephants, horses and swine but the steroid concentrations in feces exhibited a similar pattern to those in plasma. Each dung sample collected aseptically from free-ranging elephants was placed in sterile containers with 80% methanol solution and brought to the laboratory and then the samples were frozen at -20^{o}C until the extraction procedure using ELISA technique.

A. Extraction Of Steroid From Feces:
Vortexing (Non-Boiling) Extraction Method:

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The vortexing extraction method was used for extraction of glucocorticoid in fecal samples stored as per the procedure quoted by Wasser *et al.* (2000)

0.6 g well-mixed wet feces was placed in a capped tube containing 2.00 ml 90% methanol, vortexed for 30 minutes and then the tubes were carefully centrifuged for 20 minutes at 2500 rpm. The supernatant material was diluted in PBS and stored at -80 ºC for subsequent use.

B. Enzyme Linked Immunosorbent Assay (ELISA):

ELISA Kit specific to cortisol (UBI Magiwell Enzyme Immunoassay Cortisol Catalog No. SH-101) and the kit comprised the followings:

- Microwell strips Anti- cortisol IgG coated wells.
- Enzyme conjugate cortisol conjugated to horseradish peroxidase (HRPO)
- Reference standard set Standards containing 0, 1, 3, 10, 30 and 60 µg of cortisol for every one dL solution.
- Low control Value range between 1.5-3.0 µg/dL
- High control Value range between 20-40 µg/dL
- TMB solution Buffer solution containing hydrogen peroxide and TMB.
- Wash concentrate (to be diluted to 1 Lw/ distilled water)
- Washing Buffer (100x dilution) prepared by adding 2ml concentrate into 198 ml of distilled water.
- Stop solution 2N HCl.

Assay Procedure:

- Before beginning the test, all specimens and reagents were brought to the room temperature and mixed well.
- The desired numbers of coated wells were first secured in the holder.
- 10µl of standards, controls and serum samples were dispensed into the appropriate wells and labeled accordingly. One well for the blank was required (in which no standards or enzyme conjugate is to be added.)
- 100 µl of Cortisol-enzyme conjugate was then added into the wells.
- The mix of solutions was allowed to incubate at room temperature for 60 minutes.
- After this period, the incubation mixture was removed and the wells were then rinsed with washing buffer 5 times.
- Then, 100 µl of TMB solution was added into each of the wells including the blank well.
- The mix was allowed to incubate at room temperature for 30 minutes.
- The reaction was then stopped by adding 50 µl of 2N HCI to each of the wells.
- The OD was then read at 450 nm in a microwell reader.
- The resulting absorbencies were recorded and cortisol concentrations were subsequently calculated.

3.5.2.2.3 Calculation of Cortisol Concentration:

The index was calculated as follows:

- A/Ao x 100 for standards, controls and elephant samples. (A is the absorbance of each standards, control or sample and Ao was the average absorbance of the replicated of 0 µg/ dl cortisol standard.)
- The concentration (X) of each reference standard was plotted against its A/ Ao x 100 index (Y) on a logit- log paper. Through the mean of the duplicate point, a point to point line was drawn, subsequently.
- The value of concentration of cortisol was obtained in the samples from the standard curve.

Table 1: Absorbance Value For Standard Curve (Elisa)

Absorbance at 450 nm (A)	Cortisol Concentration (µg/dL) (X)	A/AO X 100 % (Y)
1.295	0	100
1.278	1	98.65
0.942	3	72.46
0.849	10	65.56
0.361	30	27.84
17.720	60	17.72

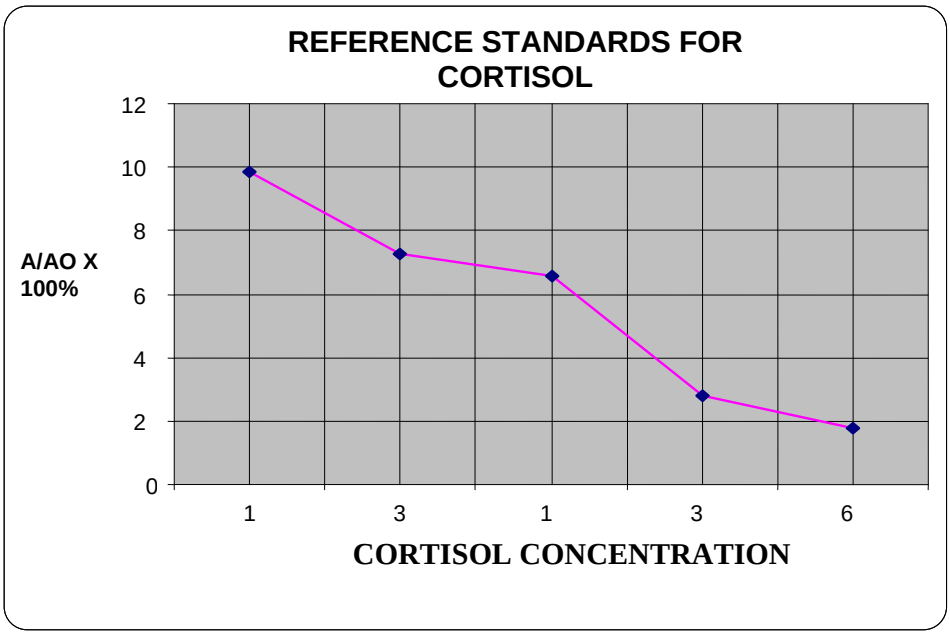

Fig. 1:

Table-2: Sample Values For Cortisol Concentration

Mudumalai Wildlife Sanctuary (n=5)		Anamalai Wildlife Sanctuary (n=5)		Sathyamangalam-Erode Forest Divisions (n=5)	
A/AO X 100 % (Y)	Cortisol Concentration (ng/g)	A/AO X 100 % (Y)	Cortisol Concentration (ng/g)	A/AO X 100 % (Y)	Cortisol Concentration (ng/g)
51.43	150[a]	41.31	190[a]	74.05	31[b]
40.93	200[a]	54.29	140[a]	88.34	13[b]
77.53	230[a]	57.37	130[a]	75.68	25[b]
65.64	100[a]	39.45	190[a]	92.12	13[b]
63.94	110[a]	62.47	115[a]	69.27	50[b]

Mean: 158ng/g Mean: 153ng/g Mean: 26.4ng/g

Values bearing the same superscript don't differ significantly

RESULTS AND DISCUSSION

Using the cortisol kit and ELISA reader, standard curve was obtained (Fig.1) and different concentration of cortisol in µg/dL in the processed dung samples obtained from elephants of all the three free-ranging regions were presented along with the absorbance valves at 450 nm in (Table 2). Among the dung samples collected from the free-ranging wildlife regions, the mean value of cortisol concentration in the dung samples of Mudumalai wildlife sanctuary was 158ng/g of feces and the mean value was 153 ng/g of feces in samples obtained from Anamalai wildlife sanctuary and the mean value was 26.4 ng/g of feces in samples obtained from Sathyamangalam-Erode forest divisions. Similarly individual values of cortisol in the fifteen dung samples were also furnished (Fig.2).

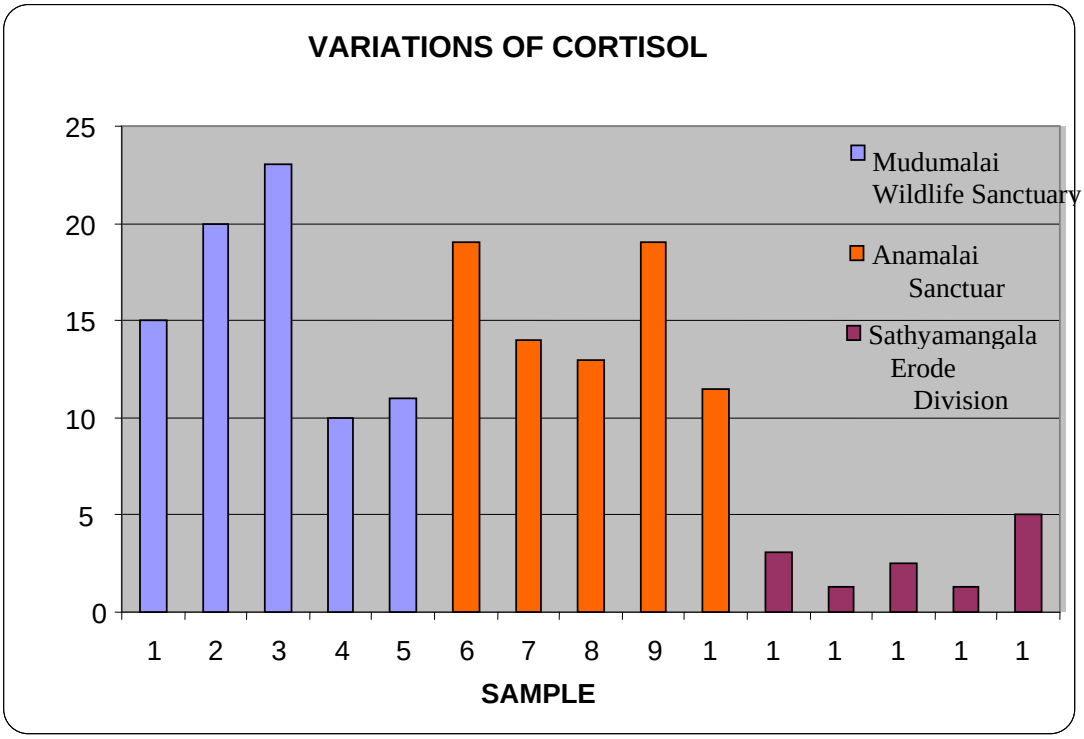

Fig. 2:

Throughout the study, well mixed fecal sample was obtained during every time of sampling to avoid the possible intra-sample variations in the fecal hormone concentrations and such a kind of sampling was in agreement with the reports presented by Wasser *et al.* (1996). Usage of 80% methanol for preservation was in agreement with the reports furnished by Mostl and Palme (2002) who made highest recoveries of hormones pertaining to stress. Usage of ELISA technique as carried out in this study to estimate faecal cortisol was recommended by Palme (2005) and Rehbinder and Hau (2006). The standard curve was obtained (Fig. 2) for cortisol level and there were no significant variations between the cortisol levels of dung samples from Mudumalai Wildlife Sanctuary and Anamalai Wildlife Sanctuary, unlike the values of dung samples obtained from Sathyamangalam-Erode forest divisions (Table 2). This difference might be attributed to the less number of human interference and change in agro-climatic condition to Sathyamangalam-Erode forest divisions, when compared mainly with Mudumalai Wildlife Sanctuary and also with Anamalai Wildlife Sanctuary. Additionally variations in the availability of water resources, food-availability in dry-seasons of the year, competitions from livestock, environmental factors, activity-patterns of adrenal glands etc. might be accounted as the contributing factors for such differences in the mean values of cortisol. It becomes noteworthy to mention that the front-line hormones to overcome stress situation were the glucocorticoids and catecholamines and these hormones were determined not only as parameters of adrenal activity but also as parameters of disturbances. The linkage between estimation of cortisol and stress in animal populations was emphasized by Morato *et al.* (2004) and Sheriff *et al.* (2010). Since stress or minute disturbances could not be quantified practically, it becomes a need to estimate the biochemical or endocrine parameters for the detection of disturbances. Measurement of fecal glucocorticoids, as done in this study was quoted to detect accurately the long-term stressors (Bayazit, 2009).

The stress causing factors or disturbing factors need to be identified especially in Mudumalai Wildlife Sanctuary and Anamalai Wildlife Sanctuary and those factors have to be minimized to the possible extent. This might help in the long-term conservation of the free-ranging elephants in the dry-season of the year. Periodic monitoring of the samples not only for evidence of parasitic infections but also for the level of fecal cortisol need to be carried out in a systemic manner, in the free-ranging regions.

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