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M Veeraselvam
Assistant Professor, Department of Veterinary Medicine, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Orathanadu, Thanjavur, Tamil Nadu, India

P Selvaraj
Professor, Department of Veterinary Clinical Medicine, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

S Senthil Kumar
Associate Professor and Head, Veterinary Clinical Complex, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Orathanadu, Thanjavur, Tamil Nadu, India

A Senthilkumar
Assistant Professor and Head, Farmers Training Centre, Tamil Nadu Veterinary and Animal Sciences University, Theni, Tamil Nadu, India

TMA Senthilkumar
Professor and Head Department of Wildlife Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University Vepery, Chennai, Tamil Nadu, India

C Sreekumar
Project Director, TRPVB, Directorate of Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Corresponding Author
P Selvaraj
Professor, Department of Veterinary Clinical Medicine, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

High performance liquid chromatographic estimation of concentrations of faecal cortisol in Asian elephants with foot disorders

M Veeraselvam, P Selvaraj, S Senthil Kumar, A Senthilkumar, TMA Senthilkumar and C Sreekumar

Abstract
Monitoring stress is important in the management of captive animals, especially elephants. A study was undertaken to investigate the stress induced cortisol levels in captive Asian elephants with foot disorders. In elephants, serum cortisol studies are not always possible on account of their protected species status. However, faecal samples are easily available and using them, cortisol can be estimated. High Performance Liquid Chromatography Technique (HPLC) being one of the sensitive methods, can be used for elephant studies also. A total of 42 captive elephants of Tamil Nadu were evaluated for foot health during their periodical clinical examinations; they were grouped according to the foot lesions. Fresh dung samples (0.5 gm) were collected in 5 ml of 80% methanol and faecal extraction was done. Faecal extracts were measured for cortisol metabolites by HPLC. The faecal cortisol concentrations of these captive elephants ranged from 0.083 µg/g to 0.553 µg/g. Individual mean faecal cortisol concentration did not differ significantly between the groups of elephants with and without foot disorders. The results also showed higher the faecal cortisol concentration was noticed in elephants with structural deformities and it was significantly differed when correlated with of elephants without foot disorders. This study highlights the utility of faecal cortisol metabolites as a useful non-invasive tool for assessing the well-being of captive elephants with foot diseases and disorders.

Keywords: Captive elephants, foot disorders, faecal cortisol

1. Introduction
Asian elephants were probably first tamed by the people of the Indus Valley civilization around 4,000 years ago. Since that time these elephants remained an integral part of human culture and tradition, religion, myths, history and economic life of the people of India [1]. The ancient Tamil Land is filled with evidences of utility of elephants in everyday life of those people and cultures. Elephants have adapted to a wide variety of environments, especially in captive conditions. One of the most important tasks in captive conditions is maintaining their psychological and physiological well-being. Alterations in environmental, social, physical, physiological and psychological factors under captive conditions can modify the homeostasis of individual elephants. Elephants are subjected to greater stress due to their monotonous routines, lack of interactions and being confined to small areas [2]. Such stress, if persist for a prolonged period, will result in the animal losing its adaptive value and will adversely impacted the health and longevity of the species by degrading body mass and impairing immune and endocrine systems, causing reproductive failures [3].
Many studies have revealed that the stressful situations were triggered in captive elephants by several stimuli, including the food quality and quantity, transportation, housing facilities and prolonged health issues like unhealed wound, foot diseases and disorders [6, 7]. Foot problems in captive elephants are the second biggest cause of morbidity. This is perhaps the single most important ailments among the captive Asian elephants, with as many as half of all captive elephants suffering from foot problems [4]. Elephants kept in captive enclosures with hard surfaces for four hours each day were more found to develop foot disorders including lameness, joint stiffness and osteoarthritis, when compared to those exposed to hard surfaces for 2.5 hours per day [5].
A retrospective study revealed that the chronic stress was manifested in captive elephants, especially in those with prolonged foot abnormalities and foot disorders [8]. A prolonged high concentration of cortisol was found during chronic stress and that was observed to have deleterious effects on bone formation and in accelerating bone loss [9], ultimately decreasing the individual fitness by impairing the immune and endocrine functions [3, 10].

Faecal glucocorticoid metabolites are being used as a non-invasive approach to measuring the cortisol as a marker of stress and health of elephants [11, 12]. The aim of this study was to investigate the relationship between stress induced cortisol levels, measured using HPLC, in captive Asian elephants with foot diseases and disorders.

2. Materials and Methods

2.1. Study animals

The study was carried out in 42 captive Asian elephants. Out of this, 24 temple elephants were assessed during a health and welfare campaign for elephants. Remaining 18 were privately maintained elephants, which were assessed during their periodical health examinations. All these elephants are maintained in different locations of Tamil Nadu. The age of these elephants ranged from 11 year to 64 years. Foot health of each elephant was clinically assessed during their Medical examinations and they were grouped in to two, as elephants with foot disorder and those without foot disorders. In the second group, based on the lesions presented in the foot, the elephants were also grouped as elephants with nail disorders, cuticle disorders, sole and pad disorders and those without foot disorders.

2.2. Faecal sample collection and extraction of cortisol

Fresh dung samples (0.5 gm.) were collected in the morning hours in 5 mL of 80% methanol in air tight containers and the samples were immediately shifted to laboratory for further extraction on the to avoid variations. The cortisol extraction was carried out according to Nishanth *et al.* [13] briefly, 0.5 gm of methanol stored dung samples were vortexed for 30 minutes followed by 15 minutes of centrifugation at 3500 g for 15 minutes. After centrifugation a quantity of 1 mL of supernatant was mixed with phosphate buffer saline and stored at -20 °C until further analysis.

2.3. High Performance Liquid Chromatography (HPLC) Technique for Faecal Cortisol

Faecal extracts were measured for cortisol metabolites by HPLC according to the procedure described by Ganswindt *et al.* [14] with some modification. Cortisol (Cerilliant®, Merck) was used as standard solution. Standard stock solution was prepared with a concentration of 1mg/ml methanol. From the stock solution, the concentration of 10µg, 5µg, 2.5 µg, 1µg 0.5µg, 0.5µg, 0.25µg, 0.125µg, 0.1µg, 0.05µg and 0.025 µg were prepared to setup a standard calibration; 10 µL of these concentrations was injected into HPLC and the standard calibration curve for cortisol was obtained by plotting concentrations verses mean of the peak areas obtained for their respective standard.

The extract obtained was directly passed through the HPLC Millipore filter (0.45 µ) vials. The separation of cortisol metabolites was carried out using a C-18 column (particle size 5 mm x Length 150 mm); the flow rate 1.0 ml/min was maintained at ambient temperature. An equal mixture of methanol: water was utilized a mobile phase. The peaks obtained were plotted on the standard HPLC curve and multiplied by the dilution factor to quantify the cortisol concentration. ANOVA was performed for testing differences in cortisol metabolites in elephants with different foot disorders.

3. Results and Discussions

The overall faecal cortisol concentrations of the captive elephants ranged from 83 ng/g to 553 ng/g. Mean overall faecal cortisol metabolite concentration was 243.52 ± 11.2 ng/g. Individual mean faecal cortisol concentrations did not differ significantly (F value =0.354; P=0.555) between the groups of elephants with and without foot disorders (Table 1). However, the faecal cortisol concentrations varied among different groups of elephants which were grouped based on the type of foot disorders. Elephants with structural deformities in foot were found to have the higher cortisol level of 413.33 ± 69.80 ng/g. The faecal cortisol concentration was found to be lower in elephants with nail disorders (157.75 ±25.43ng/g). Elephants with nail disorders, Sole and pad disorders, cuticular disorders and mixed disorders did not differ significantly when compared with the elephants without disorders (Table 2,3,4,6), except the elephants had structural deformities (F value =10.35; P value < 0.009) (Table 5).

Table 1: Mean faecal cortisol concentration in the elephants with and without foot disorders

Groups	Number of elephants	Mean ± SD (µg/g)	F-value	P value
Elephants with foot disorders	37	243±21.0	0.205	0.653
Elephants without foot disorders	9	222.77±26.25		

Table 2: Mean faecal cortisol concentration in the elephants with nail disorders

Groups	Number of elephants	Mean ± SD (µg/g)	F-value	P value
Elephants without foot disorders	9	222.77±26.25	3.130	0.097
Elephants with nail disorders	8	157.75±5.43		

Table 3: Mean faecal cortisol concentration in the elephants with sole and pad disorders

Groups	Number of elephants	Mean ± SD (µg/g)	F-value	P value
Elephants without foot disorders	9	222.77±26.25	0.283	0.604
Elephants with sole and pad disorders	6	196.00±47.98		

Table 4: Mean faecal cortisol concentration in the elephants with cuticular disorders

Groups	Number of elephants	Mean ± SD (µg/g)	F-value	P value
Elephants without foot disorders	9	222.77±26.25	2.718	0.121
Elephants with cuticular disorders	7	287.00±47.98		

Table 5: Mean faecal cortisol concentration of elephants with structural deformities

Groups	Number of elephants	Mean ± SD (µg/g)	F-value	P value
Elephants without foot disorders	9	222.77±26.25	10.35	0.009*
Elephants with structural deformities disorders	3	413.33±69.80		

(*p<0.01- highly significant difference)

Table 6: Mean faecal cortisol concentration of elephants with mixed disorders

Groups	Number of elephants	Mean ± SD (µg/g)	F-value	P value
Elephants without foot disorders	9	222.77±26.25	0.947	0.342
Elephants with mixed disorders	13	270.00±35.87		

Increased glucocorticoid concentrations were observed in stressful situations [15, 16] and it can be found in plasma, saliva as well as faeces and urine samples [17]. Measuring the cortisol metabolites were widely used as a biomarker of stress [18, 19] and serves as an indicator of health of animals. Non-invasive faecal cortisol analysis is a well-established tool to quantify the stress in captive and free ranging elephants [14, 20]. Captive elephants are more prone for chronic stress due to their restricted movements, lack of exercise, restricted behavioural and feeding opportunities [2, 21]. Elephants which were kept under captivity situations showed an increased probability of having foot problems [4]. Fowler and Mikota [8] observed that the chronic stress was manifested in captive elephants, especially those with prolonged foot abnormalities and foot disorders.

The present study revealed that there was an overall elevation of faecal cortisol level in captive Asian elephants with different kinds of foot disorders; but when the values of cortisol in different kinds of foot disorders were pooled together, then there were no significant differences observed between the groups of elephants with and without foot disorders. This was in agreement with Bansiddhi *et al.*, [22] who reported that elephants with moderate foot problems exhibited lower faecal glucocorticoid metabolites than those with no foot problem; such findings were observed among the tourist camp elephants in Thailand. In this study, the results showed that the faecal cortisol concentrations significantly differed among the various types of foot disorders and a higher faecal cortisol concentration was found in elephants with structural deformities in the foot. Ganswindt *et al.* [23] reported that the free ranging African elephants with physical injuries in foot and other regions of the body had also shown elevated level of cortisol concentration.

In this study, the elephants with minor nail cracks, cuticular feathering were found to had a low level of cortisol concentration and those chronically stressed elephants with persistent foot disorders were found to had higher cortisol levels; in fact, the observed elevation in cortisol was almost twice that of elephants with minor foot affections such as nail, cuticle and sole disorders. In a recent study, Bansiddhi *et al.* [22] reported that a higher faecal glucocorticoid concentration was observed in elephants with major wounds than those elephants which had no visible wounds. Elevated levels of stress hormone was found to have detrimental effects, resulting in suppression of immune response, imperfect wound healing and neuronal cell death [3]. This might be the contributing factors for delaying of wound healing noticed in elephant foot affections.

According to Panagiotopoulou *et al.* [24] foot disorders were one of the most debilitating afflictions affecting the health and well-being of captive elephants and attributed it to the increased levels of stress hormones. Prolonged higher concentration of stress hormone was known to affect the growth, immunity and reproduction of elephants [3]. Hence, periodical monitoring of cortisol levels in elephants with foot disorders is essential, as such monitoring will help in reducing the detrimental effects on the animal's health and wellbeing. This study showed that the determination of faecal cortisol metabolites can be very useful for noninvasively assessing the well-being of captive elephants with various foot diseases and disorders.

4. Conclusion

HPLC based Faecal Cortisol Assessment was found to be feasible in Elephants. Elephants with structural deformities in foot were found to have the higher cortisol level of 0.413 ± 0.121 µg/g. Faecal cortisol concentration was found to be lower in elephants with nail disorders (0.157 ±0.071 µg/g). This study helped in arriving at a HPLC based reference baseline cortisol values for captive Asian elephants of Tamil Nadu. As stress monitoring is very essential in managing the welfare and health of captive elephants, and especially those with various foot disorders, the HPLC based Faecal Cortisol Monitoring shall be incorporated as part of the Periodical Veterinary Assessment Programmes in Captive Elephant Health Management Programmes.

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