FAECAL GLUCOCORTICOID MEASUREMENT AS INDICATOR STRESS IN WILD CRESTED MACAQUES (*MACACA NIGRA*): THE IMPORTANCE OF VALIDATION AND SAMPLE PROCESSING TECHNIQUES

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ABSTRACT

Monitoring of physiological stress in wild and/or endangered animals living in their natural habitat can be generated via measuring faecal glucocorticoid metabolites (FGCMs) through enzyme immunoassay technique. However, a careful validation of each enzyme immunoassays (EIAs) and methodological issues such as samples preservation, and extraction procedures must be a significant concern and validated because it may influence FGCMs levels. In this present study, the aim was to carry out a biological validation to find reliable EIAs that could be used for non-invasive measurement of glucocorticoid levels in crested macaques. Moreover, we compared three different techniques of preservation: lyophilisation, oven drying and field extraction and three different solvents for extraction: 80% methanol, 90% pure ethanol and 90% commercial alcohol (isopropyl alcohol) to find the simple and practical techniques for sample processing. For hormone analysis, we collected 189 faecal samples from wild crested macaques living in the Tangkoko-Batuangus Nature Reserve, North Sulawesi, Indonesia. The results show that biological validation can be used to validate measurement of faecal glucocorticoid which 3α,11β-dihydroxy-etiocholanolone was the most reliable EIAsfor this species. Different preservation and solvent extraction were not significantly influenced levels of FGCMs ($p>0.05$). In conclusion, Our study highlights the importance of biological validation of FGCMassays and presents practical techniques for the non-invasive monitoring of physiological stress in crested macaques.

Keywords: Crested macaque, enzyme immunoassay, preservation, solvent extraction, glucocorticoid.

Introduction

The crested macaques (*Macaca nigra*) are the most endangered endemic macaque from Sulawesi which the situation of this species is extremely threatened and has been categorized as critically endangered (IUCN Red List 2009).The population having declined mainly due to habitat loss, illegal logging and hunting. A recent field survey conducted by Palacios *et al*. (2012) reported that population densities of crested macaques in Tangkoko average about 45 individuals/km^2 which is estimated declining 35% compared to the former studies (Sugardjito *et al*. 1989; Rosenbaun *et al*. (1998). Therefore, a study on stress in highly endangered species such as crested macaques (*Macaca nigra*) is an important aspect of conservation biology.

Nowdays, assessment of physiological stress of wild and/or endangered animals living in their natural habitat can be generated via measurement of fecal glucocorticoid metabolites (FGCMs). This technique gives several advantages which may provide an accurate assessment of stress without the bias of capture-induced increases in glucocorticoids due to they do not disturb the animal (Goymann 2005; Möstl *et al*. 2005). Faecal of most vertebrate species contain metabolized forms of all major steroid hormones (i.e.,progestins, oestrogens, androgens, and glucocorticoids), which are secreted into the gut via bile (Möstl & Palme 2002). These faecal metabolites can be measured with hormone assay techniques, but the right metabolites must be determined first. Therefore, it is essential to validate hormone assay techniques based on faecal samples before applying the technique to a given species in order to generate meaningful and accurate results (Tauma & Palme 2005; Heistermann *et al*. 2006; Heistermann 2010; Murray *et al*. 2013).

In addition to the validation, preservation and extraction of samples have to be considered when measuring steroid hormones from faeces. Faecal collection in the field may affect fluctuations in steroid hormone concentrations (Khan *et al*. 2002). The best standard storage method for faecal samples is simple freezing, as this stabilizes levels of glucocorticoid metabolites (and other steroid metabolites) over long periods of time (Herring&Gawlik 2009; Palme 2005). However, many field sites have no or limited access to electricity and thus lack freezing devices. Therefore, it is necessary to conduct a study on sample processing techniques to find the best, simplest and most practical technique to preserve and extract faecal samples prior to hormone analysis.

The present study therefore was to carry out a biological validation of different glucocorticoid EIAs in crested macaques to examine reliability of the hormone assays to the response of a stress event by comparing glucocorticoid levels before (pre), during (stress), and after (post) the stress event. On the other hand, comparing different techniques of preservation and solvent extraction for glucocorticoid measurement based on the faecal samples.

Materials and Methods

Animals and Study Site

A group of crested macaques, Rambo I which are living in their natural habitat in the Tangkoko-Batuangus Nature Reserve, North Sulawesi was used in this study. Sample preparations were conducted in the hormone assay laboratory at the Faculty of Veterinary Medicine IPB. Subsequently, validation and hormone analysis was conducted in the endocrinology hormone laboratory at the German Primate Centre (DPZ) Goettingen, Germany.

Faecal Sample Collection and Extraction

Biological validation of FGCMs measurements

For the biological validation of GCM, faecal samples from six individuals which had undergone a "stressful event" were used. These six individuals had been either injured, caught in a poachers trap and released, or were severely harassed by other group members. After stress event, the individuals were directly followed to collect their faecal samples. Faecal samples

were collected opportunistically following defecation. Samples were homogenized by using a stick. Afterwards, seeds and big fibres were removed. Finally, 2-3 g of the sample were put in a tube and stored on ice until they were frozen at –20°Creturn from the forest.

Comparison different technique of sample processing

To examine the simplest and practical technique of faecal sample processing, three different techniques of faecal sample processing (freeze drying/ lyophilisation, oven drying and field extraction) were compared. Faecal samples were collected directly afterdefecation and homogenized them well by using stick. Furthermore, ±0,5 g of the faeces was placed in 5 ml 90% ethanol and extracted immediately as field extraction. The remaining faeces were divided into two portion and placed in the different tubes, one portion was dried by using a tradisional oven (oven drying) and the other portion was dried by using freeze dryer (lyophilisation). Afterthat, dried faeces were pulverized and extracted. Moreover, different solvents of extraction was tested in each technique (Fig 1).

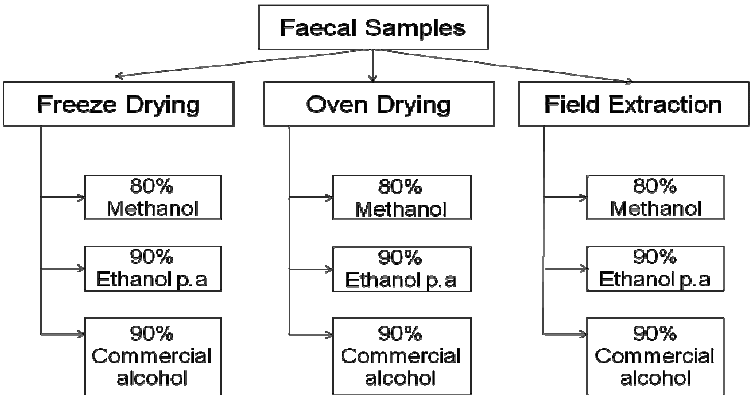

Fig 1. Scheme of preservation procedures and solvents extraction used

Validation Procedure

In order to reliably validation tests of stress hormone measurement using faecal samples, we performed an analytical validation to examine the capability and precisely of assay used for quantifying levels of FGCMs. In addition, biological validation was performed to examine whether assay used detects biologically meaningful changes in stress hormone levels reflect to the physiological events occured. For the analytical validation**,** parallelism test, assay precision were performed. For biological validation, three different glucocorticoid EIAs, cortisol,3α,11-oxo-etiocholanolone (3α,11-oxo-CM) and 3α,11β-dihydroxy-etiocholanolone (3α,11β-dihydroxy-CM) were tested.

Hormone Analysis

First faecal extracts were diluted in assay buffer. Duplicate 50 µl aliquots of faecal extracts were assayed along with 50 µl aliquots blank, zero and standard on microplates. Afterwards, 50 µl label, 50l antibody were added to each well and the mixture incubated overnight at 4°C. Following incubation, the plates were washed four times with PBS washing solution (containing 0.05% Tween 20), blotted dry, and 150 µl of streptavidin-peroxidase (S-5512, supplied by Sigma, Germany) in assay buffer was added to each well. The plates were incubated at room temperature (RT) in the dark for 30 minutes after which they were washed again four times. Substrate solution (150 µl, containing 0.025% tetramethyl-benzidine and 0.05% H_2O_2) was then added to each well. The plates were again incubated in dark at RT for 30–45 min depending on the colour change. Once the zero had taken on the expected colouration, the enzyme reaction was stopped with 50 µl 2M H_2SO_4 in each well. Finally, absorbance was measured at 450 nm on an automatic plate reader.

Results and Discussion

Analytical Validation of EIAs

Serial dilutions of extracted faecal samples gavedisplacement curves, which were parallel to the respectivestandard curves in all assays (Fig. 2 shown example profile for 3α,11β-dihydroxy-CM EIA). Similar profiles are obtained for the other EIAs, but the data are not shown. These results indicating that there were no substances in the faecal sample matrix that interferred with binding of the antibodies. Thus, the assays were capable of quantifying levels of FGCMs. For the assay precision, intra-and inter-assay coefficients of variation (%CV) of high and low value quality controls from all assays were below 10% and 15% respectively, indicating that the assays precision are acceptable (Ganswindt *et al*., 2002; Möstl *et al*., 2005).

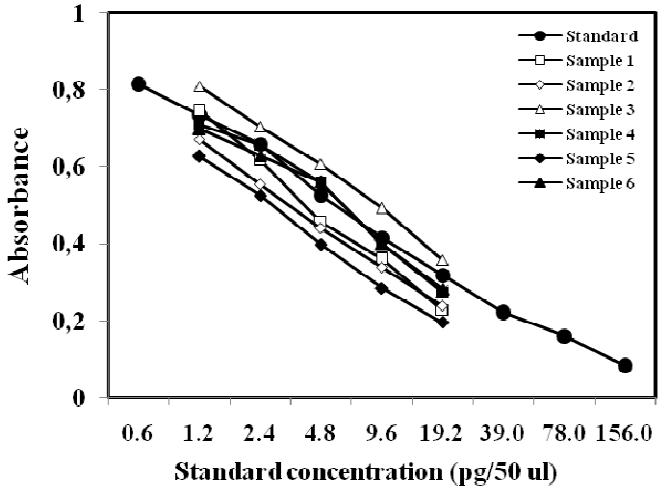

Figure 2. Parallelism test of 11β-dihydroxy-etiocholanolone assay

Although, dilution curves of extracted faecal samples gave acceptable parallelism results with standards. However, parallelism test is not a marker of specificity of the assay, but a proof of a dose–response relationship (Möstl *et al*., 2005). Therefore, it is not possible to select the best-suited glucocorticoid EIA to assess physiological stress only based on the analytical validation results. To achieve that, it is important to perform a biological validation as well by comparing level of FGCMs from individuals which had undergone a stressful event.

Biological Validation of FGCMs

To investigate the hormonal stress response after stress events experienced by animals, glucocorticoid level changes between pre-stress and stress, and stress and post-stress were compared in each EIAs (Fig. 3). In accordance with this, we found a significant change of FGCM level between pre-stress and stress, stress and post-stress in 3α,11-oxo-CM and 3α,11β-dihydroxy-CM assays, but there was not a significant difference between pre and stress in cortisol assay (Fig.3). In 3α,11-oxo-CM and 3α,11β-dihydroxy-CM assays, glucocorticoid levels are two times higher during stress compered to pre-and post-stress. However, in 3α,11β-dihydroxy-CM assay had better baseline FGCM levels (pre and post-stress) compared 3α,11-oxo-CM assay.

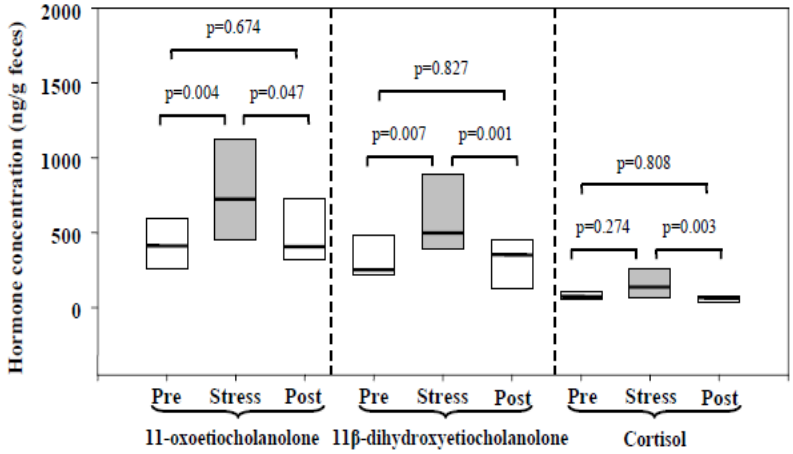

Figure 3. Levels of FGCMs, 3α,11-oxo-etiocholanolone, 3α,11β-dihydroxy-etiocholanolone and cortisol before (pre), during (stress), and after (post) a stressful event (median,1st and 3rd quartiles)

Level of FGCM through Different Technique of Preservation and Solvents Extraction

Mean FGCM levels in faeces preserved with different techniques and solvent extraction were not significantly different (p>0,05). These result indicates that a field extraction and the commercial alcohol can be used as practical tool of preservation and solvent extraction respectively in the field.

Conclusions

Our results show that biological validation can be used to examine the biological validity of glucocorticoid metabolites using faecal samples from individuals which had undergone a stressful event. On the other hand, a field extraction and commercial alcohol are the simple and practical preservation technique and solvent extraction respectively when a research conducted in the field sites that have no or limited access to electricity.

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References

Ganswindt A, Heistermann M, Borragan S, Hodges JK. 2002. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. *Zoo Biol* 21:27–36.

Goymann W. 2005. Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. *Ann NY Acad Sci* 1046: 35–53.

Heistermann M. 2010. Non-invasive monitoring of endocrine status in laboratory primates: methods, guidelines and applications. *Adv Sci Res* 1:1-9.

Heistermann M, Palme R, Ganswindt A. 2006. Comparison of different enzymeimmunoassays for assessment of andrenocortical activity in primates based on fecal analysis. *Am J Primatol* 68:257-273.

Herring G, Gawlik DE.2009. Stability of avian fecal corticosterone metabolite levels in frozen

avian feces. *J. Wildlife Manage*. 73 :1010–1013.

International Union for Conservation of Nature (IUCN). 2009. *IUCN Red List of Threatened Species,* Crested Macaques (*Macaca nigra*). http://www.iucnredlist.org [2 January 2010].

Khan MZ, Altmann J, Isania SS, Yua J. 2002. A matter of time: evaluating the storage of fecal samples for steroid analysis. Gen Comp Endocrinol 128:57–64.

Millspaugh JJ *et al*. 2001. Fecal glucocorticoidas assays and the physiological stress response in elk. *Wildl Soc Bul*: 29(3):899-907

Möstl E, Palme R. 2002. Hormones as indicators of stress. *Dom Anim Endocrinol*23: 67–74.

Möstl E, Rettenbacher ES, Palme R. 2005. Measurement of corticosterone metabolites in birds' droppings: an analytical approach. *Ann NY Acad Sci* 1046: 17–34.

Murray CM, Heintz MR, Lonsdorf EV, Parr LA, Santymire RM. 2013. Validation of a field technique and characterization of fecal glucocorticoid metabolite analysis in wild chimpanzees (*Pan troglodytes*).

Palacios JFG, Engelhardt A, Agil M, Hodges JK, Bogia R, Waltert M. 2012. Status of, and conservation recommendations for, the Critically Endangered crested black macaque *Macaca nigra* in Tangkoko, Indonesia. *Oryx* 46: 290-297.

Palme R. 2005. Measuring fecal steroids guidelines for practical application. *Ann NY Acad Sci* 1046: 75-80.

Rosenbaum B, O'brien TG, Kinnaird M, Supriatna J. 1998. Population densities of Sulawesi crested black macaques (*Macaca nigra*) on Bacan and Sulawesi: Effects of habitat disturbance and hunting. *Am J Primatol* 44:89-106.

Sugardjito J *et al*. 1989. Population survey of macaques in northern Sulawesi. *Am J Primatol* 18: 285-301.

Touma C, Palme R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of a biological validation. *Ann NY Acad Sci* 1046: 54–74