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HAIR CORTISOL LEVELS DETERMINED AT DIFFERENT BODY SITES IN THE NEW ZEALAND WHITE RABBIT

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Abstract: This study was designed to determine hair cortisol levels in the New Zealand White (NZW) rabbit and to examine possible differences in the cortisol levels of hair samples collected from different body regions in stable environmental conditions. The experiment was performed on eight 18 month-old female NZW rabbits. All animals were shaved to collect hair samples from 26 different body regions. Hair cortisol levels were determined by the RIA method. The mean hair cortisol concentration for the 26 samples in the 8 animals was 2.12±0.05 pg/mg (mean±standard error). This study reveals individual hair cortisol distributions in the 8 animals (P<0.001) and no statistical differences (P>0.05) in hair cortisol levels among the different body sites in each of the animals.

Key Words: rabbit, hair, cortisol.

INTRODUCTION

Rabbits are generally considered very sensitive to external stimuli as they are easily frightened. Any disturbance such as a loud noise will induce a rapid cascade of endocrine secretions regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Cabezas *et al.*, 2007). HPA activity has been used to assess stress and welfare in farm animals (Mormède *et al.*, 2007). Cortisol is an important hormone of the HPA axis and a key hormone in the physiological response to stress.

In controlled laboratory conditions, rabbits secrete cortisol according to a circadian rhythm peaking in the afternoon and reaching its lowest levels at 06:00 h. This cycle is approximately 12 h out-of-phase with respect to the human glucocorticoid circadian rhythm and consistent with the fact that the rabbit is a nocturnal animal (Szeto *et al.*, 2004).

Considering that the rabbit is an animal very sensitive to stressful conditions affecting cortisol production and that cortisol has a circadian rhythm, the determination of this hormone in blood or other biological tissues is difficult, since several samples are needed for a daily average value.

Cortisol is usually determined in samples of blood (Szeto *et al.*, 2004; de Prada *et al.*, 2007; Cabezas *et al.*, 2007), faeces (Szeto *et al.*, 2004), urine (Walker *et al.*, 2007), milk (Gygax *et al.*, 2006) and saliva (Negrao *et al.*, 2004). Cortisol measurements over a longer period of time, weeks or months, can be taken in hair samples. Hair has long been used in toxicology, forensic science, doping control and other fields as a biological matrix for the detection of environmental agents, drugs or toxins (Pragst and Balikova, 2006). Recently, Raul *et al.* (2004) demonstrated the detection of endogenous concentrations of cortisol and cortisone in human hair. This matrix was

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found to be a potential biological marker for chronic stress (Kalra *et al.*, 2007). The collection of this biological material is simple, non-invasive and the sample does not decompose like other body fluids or tissues (Balikova, 2005).

Incorporation models of this hormone consider that cortisol enters the hair shaft during hair growth (the anagen phase) through passive diffusion from blood capillaries present on the basement membrane (Pragst and Balikova, 2006; Steudte *et al.*, 2011).

Cortisol measurement in hair samples has been described using validated methods in humans (Sauvé *et al.*, 2007), rhesus monkeys (Davenport *et al*., 2006), dogs and cats (Accorsi *et al.*, 2008) and cows (Comin *et al.*, 2011; del Rosario González-de-la-Vara *et al.*, 2011). As is well known, hair cortisol is the result of a process of cortisol accumulation and incorporation from the plasma over a period of several days to a few weeks (Yamada *et al.*, 2007). Thus, measuring hair levels of cortisol provides a "retrospective picture" of its prior accumulation.

In studies in which hair was compared to other biological sources, a positive correlation was detected between a change in HPA activity and hair cortisol (Kalra *et al.*, 2007; Yamada *et al.*, 2007; Kirschbaum *et al.*, 2009). Thus, measuring hair cortisol in rabbits could help the farmer, veterinarian or researcher to monitor stress and well-being in these animals.

The aim of the present study was to determine hair cortisol levels in the rabbit, while also addressing the most appropriate way to collect hair as well as possible differences in cortisol levels in hair samples collected from different body areas.

MATERIALS AND METHODS

The research protocol was approved by the Animal Ethics Committee of the Centre for Coordination and Development Projects and Equipment of the University of Trieste (Italy).

Animals and hair samples

The rabbits included in this study were purchased from the rabbitry Harlan s.r.l., San Giovanni al Natisone, Italy. The experiment was performed on 8 New Zealand White (NZW) female rabbits aged 18 mo. The rabbits were brought to the animal breeding facility of the Centre for Coordination and Development Projects and Equipment and kept in stable environmental conditions. They were housed in individual cages, provided with water and food *ad libitum* and kept in a temperature-controlled environment (22-24°C). The rabbits were all in good health and had not been handled previously.

Five months after the arrival of the rabbits, hair samples were carefully taken using clippers from the 26 body sites detailed in Table 1 and stored in dry tubes at room temperature until analysis. At the time of sample collection, hair lengths were measured for each body region.

To avoid stress and in compliance with current animal welfare legislation, samples were collected during routine farm activities.

Hair cortisol assay

Hair strands were washed in 5 mL isopropanol, as suggested by Davenport *et al*. (2006), and hair cortisol extracted according to the method described by Koren *et al.* (2002) with some modification. Approximately 60 mg of trimmed hair was placed in a glass vial along with 3 mL of methanol. The vials were incubated at 37°C for 18 h. Next, the liquid in the vial was evaporated

Table 1: Sampling sites, corresponding codes, and hair lengths (mean±SE) recorded in the 8 rabbits.

Body region	Hair length cm	Code	Body region	Hair length cm	Code
Frontal	1.78±0.03	A	Costal (right side)	3.03±0.03	N
Intermandibular	1.76±0.05	B	Costal (left side)	3.04±0.05	O
Right ear	1.01±0.06	C	Abdominal	3.01±0.05	P
Left ear	1.01±0.05	D	Sacral	3.03±0.06	Q
Neck (right side)	3.04±0.04	E	Right thigh	3.03±0.04	R
Neck (left side)	3.05±0.06	F	Left thigh	3.04±0.06	S
Right brachial-triceps-antebrachial	2.18±0.05	G	Right crural-tarsal	2.47±0.06	T
Left brachial-triceps-antebrachial	2.19±0.06	H	Left crural-tarsal	2.48±0.06	U
Right metacarpal-phalangeal	1.52±0.05	I	Right metatarsal-phalangeal	1.47±0.03	V
Left metacarpal-phalangeal	1.51±0.05	J	Left metatarsal-phalangeal	1.46±0.04	W
Thoracic (right side)	3.05±0.05	K	Tail	2.78±0.03	X
Thoracic (left side)	3.06±0.05	L	Inner right thigh	3.03±0.05	Y
Sternal	3.04±0.05	M	Inner left thigh	3.04±0.04	Z

to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.6 mL of phosphate-buffered saline (PBS) 0.05 M, pH 7.5.

Hair cortisol was measured using a solid-phase microtitre RIA procedure. In brief, a 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with anti-rabbit γ-globulin serum raised in a goat, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4°C. The plate was washed twice with RIA buffer, pH 7.4 and incubated overnight at 4°C with 200 µL of the anti-cortisol serum diluted 1:12000. The rabbit anti-cortisol antibody used was obtained from Biogenesis (Poole, UK). Cross-reactivities of this antibody with other steroids are: cortisol 100%, corticosterone 1.8% and aldosterone<0.02%. After washing the plate with RIA buffer, standards (5-300 pg/well), a quality control extract, the test extracts and tracer (Hydrocortisone (Cortisol, [1,2,6,7-^{3}H (N)]-), Perkin-Elmer Life Sciences, Boston, MA, USA) were added and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µL scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). Intra-assay and inter-assay coefficients of variation were 3.6% and 9.8%, respectively. The assay sensitivity, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 1.23 pg/well.

Statistical analysis

Statistical analysis was performed using free R software version 2.14.1. The normality of data distribution was tested using the Shapiro-Wilk test. Scheirer-Ray-Hare, a non-parametric test, was applied as suggested by Dytham (2003) to the hair cortisol data; sampling area and rabbit were considered as the fixed factor and block respectively. The Mann-Whitney U non-parametric test with Bonferroni adjustment was used as post hoc test as suggested by Daniel (1978).

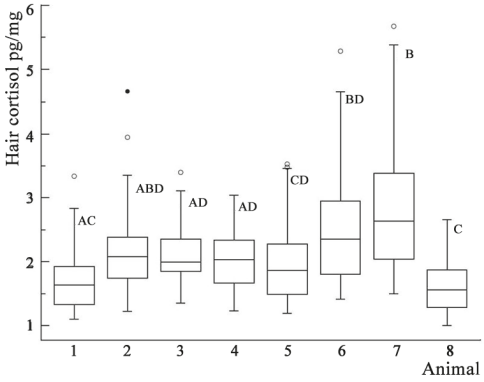

Figure 1: Hair cortisol levels (data for the 26 body areas/rabbit) measured in the 8 rabbits. Plots represent the median (horizontal lines), first and third quartiles (boxes) and the minimum and maximum cortisol values (whiskers). ° suspected outlier; • outlier; A,B,C,D: rabbits not sharing letters differ significantly at $P<0.001$.

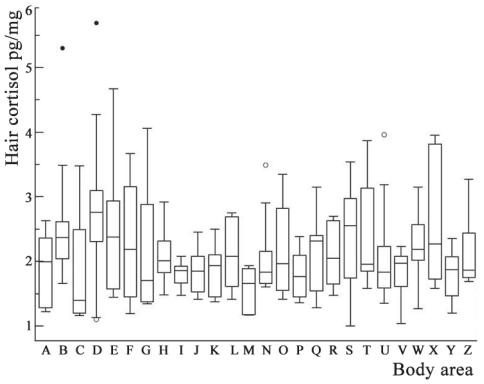

Figure 2: Hair cortisol levels determined at the 26 body sites in the 8 rabbits. Plots represent the median (horizontal lines), first and third (boxes) and the minimum and maximum of cortisol values (whiskers). ° suspected outlier; • outlier.

RESULTS

Cortisol levels were determined in 208 hair samples collected from 26 body areas in 8 female rabbits. The lengths of hairs collected from the different areas (Table 1) differed significantly ($P<0.001$).

The mean hair cortisol concentration for the 26 samples in the 8 animals was 2.12±0.05 pg/mg (mean±standard error). Minimum and maximum values were 1.00 and 5.69 pg/mg, respectively, and the median was 1.96 pg/mg.

Statistical analysis revealed significant differences of hair cortisol level in the 8 animals ($P<0.001$). Hair cortisol levels recorded in the 8 rabbits are shown in Figure 1.

Cortisol levels measured in the different body areas did not differ significantly ($P>0.05$). The hair cortisol levels measured at the 26 body sites in the 8 rabbits are provided in Figure 2.

DISCUSSION

In this study, we determined individual distributions of hair cortisol levels in 8 rabbits. During the study period, all animals were housed and kept under the same conditions and all were of comparable age and size. The individuality of values shown by each animal indicates that the response of an organism to a similar stimulus involves a different level of HPA activity. Individual variation in the HPA axis has been well documented in humans and attributed to genetic factors in twin and family studies (Linkowski *et al.*, 1993; Inglis *et al.*, 1999). Genetic factors (Désautés *et al.*, 2002), but also the efficiency of corticosteroid receptors (DeRijk *et al.*, 2002), may influence the bioavailability of corticosteroid hormones.

Our study revealed that rabbits kept in stable environmental conditions showed no significant differences in hair cortisol levels among several different body sites.

In humans, Pragst and Balikova (2006) reported different growth rates of hair at different anatomical sites, the different duration of the 3 growth stages and different final shaft lengths. Compared to other body regions, human scalp hair has the longest anagen phase. Anagen length generally determines hair length and is crucial in the incorporation of hormones into hair.

No different final shaft lengths for the same body area were observed among rabbits but in each rabbit final shaft length varied significantly among the body sites.

Hair cortisol concentration failed to vary among the different body areas. This means that throughout the study period, in the absence of stressful stimuli that could activate the HPA axis, hairs showed similar cortisol concentrations regardless of length.

The use of hair samples to determine cortisol levels avoids problems related to blood sampling, such as stress due to animal handling or circadian variations. Measuring hair cortisol in rabbits could help the farmer or veterinarian monitor the stress and well-being of their animals. In intense rabbit breeding programmes, animals are subjected to numerous stress factors such as poor environment quality, density or space. Such stressors could affect HPA axis activity and the welfare of the animals and thus farming profitability. Indeed, it is known that hormones such as glucocorticoids secreted in response to generalised stress can increase animal susceptibility to disease, often by initiating immunosuppression (Sevi, 2009), and affect reproductive processes through their actions on the hypothalamus-pituitary-ovary axis (Boiti, 2004).

Measuring hair cortisol in rabbits could also be useful to evaluate individual resilience and stressful conditions and ensure the welfare of rabbits used as animal models for scientific research purposes.

In conclusion, the hair cortisol concentrations reported in this study represent levels determined in the absence of HPA stimulation. In such stable conditions, scarce differences were observed in hair cortisol levels between different body areas. This work provides useful baseline data for future studies designed to assess the effects of stress on cortisol levels.

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