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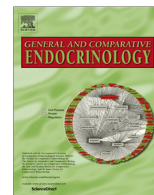

Influence of external factors on hair cortisol concentrations

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ABSTRACT

Measuring hair cortisol has attracted interest as a long term parameter for chronic stress evaluation. However, some studies support the hypothesis that locally produced cortisol, originating from the hair follicle or skin cells, affects concentrations in the hair. In an animal model the influence of different treatments (extensive brushing, administration of a hyperemising fluid that enhances blood circulation or a synthetic glucocorticoid) on the local cortisol production of hair was evaluated. Therefore eight sheep were sheared and the area of the skin surface of the back was quartered, with three quarters being daily subjected to a certain treatment and one quarter remaining untreated. The skin areas were sheared again after three weeks and cortisol concentrations of all wool samples were determined by immunoassay. Systemic cortisol concentrations were additionally monitored with faecal samples, indicating a significant decline in concentrations of glucocorticoid metabolites between week 1 and 2 or 3, respectively. We found no significant difference in hair cortisol concentrations between fields before treatment ($p = 0.310$). Comparing matched fields before and after treatment, we found no significant differences in wool cortisol concentrations for fields treated with hyperemising fluid as well as for the control fields ($p = 0.329$, $p = 0.097$). Hairs exposed to either extensive brushing or dexamethasone fluid had significantly higher immunoreactive cortisol concentrations after three weeks of treatment ($p = 0.016$, $p = 0.01$). We therefore advise cautious interpretation when measuring hair cortisol concentrations as a parameter for chronic stress, because external factors may have a significant influence on the results.

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1. Introduction

The activity of the hypothalamic-pituitary-adrenocortical (HPA) axis can be frequently monitored to assess stress levels and therefore the well-being of animals (Möstl and Palme, 2002). In stressful situations elevated glucocorticoid concentrations can be found in plasma and saliva, as well as in urine or faeces, where their metabolites can be quantified. The latter represent integrated steroid concentrations over a certain time period (Palme, 2012). The increased production of stress hormones is crucial to overcome challenging situations. This is generally beneficial for the individual, but in case of long-lasting systemic elevation of stress hormones, symptoms of allostatic overload appear (McEwen, 2004). Chronic stress affects, for example, immune competence, reproduction, metabolism and behaviour in animals (Moberg, 2000). To monitor long-term changes of the HPA axis activity the hair is gaining interest as a non-invasive sample material, which is thought to reflect integrated cortisol concentrations over a period of several months. In contrast to other sample materials, cortisol concentrations in the hair are not influenced by circadian rhythmicity or other factors that induce short-term elevation of HPA axis activity. Hair analysis is well known from forensic science and toxicology, where the benefit of getting retrospective information that reaches back months to years is valued (Pragst and Balikova, 2006). Also, in the field of veterinary medicine, the measurement of steroids in hair offers some great benefits, such as the non-invasive sample acquisition and easy storage at room temperature. First validation studies that compared repeated salivary samples of rhesus macaques during a period of two weeks to hair samples gained twelve weeks later indicated a positive correlation between the two sample materials (Davenport et al., 2006). Since then, the measurement of mainly cortisol in hair samples is widely used to identify chronic stress situations in animals (Russell et al., 2012). Furthermore, studies indicate a diagnostic benefit of hair cortisol measurement, for example when determining

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hyperadrenocorticism in domestic animals, characterized by high amounts of systemic cortisol (Ouschan et al., 2013). The mechanism of incorporation of the hormones into the hair shaft is not fully investigated yet. Diffusion from blood during the growth period of the hair shaft might be a possibility, although some authors have suggested that hair cortisol may in part originate from cortisol excreted in sweat or sebum (Russell et al., 2014; Cone, 1996).

An *in vitro* study (Ito et al., 2005) using organ-cultured human scalp follicles suggests an intrafollicular, autonomous cortisol synthesis. Also, Keckeis et al. (2012) point out possible local production of cortisol as they found only little (if at all) amounts of injected, labelled ^{3}H-cortisol in the hair of guinea pigs, but high amounts of un-labelled cortisol. A recent study, investigating sheep inoculated with different strains of *Dichelobacter nodosus*, suggests local production and/or metabolism of glucocorticoids in the hair follicles of the affected hind legs (Stubsjøen et al., 2015). Such a local cortisol synthesis could hamper hair cortisol concentrations as a parameter for evaluating long-term stress.

The present study aimed to evaluate local cortisol production *in vivo*, comparing different external factors that may cause changes to intrafollicular synthesis using sheep as an animal model.

2. Material and methods

2.1. Animals, housing conditions, diet and handling

Eight white, adult alpine mountain sheep (*Ovis orientalis aries*) were selected from a flock, which was held in a barn with a roaming area. They were examined clinically and only healthy sheep attended the study. The ones most affectionate towards humans were separated, marked with an animal marking crayon (numbers 1–8), and prepared for the study by getting them used to the persons and procedures involved during a preparation phase of three weeks. This approach was used to facilitate baseline systemic cortisol levels at the beginning of the study. The group was mixed-sex including three female and five castrated males between three to five years old. One week prior to commencement of the study the sheep were rehoused in an indoor stable to get them used to the new environment (two or three sheep together in a stable-box). Straw was used for the bedding. During the study period all animals received the same diet and the stables were cleaned daily.

The study was discussed and approved by the institutional ethics committee in accordance with GSP guidelines and national legislation (07/05/97/2014).

2.2. Study design and treatments

One day prior (day 0) to the start of the treatment period, the back of each sheep was visually quartered and in each quarter, an area of approximately 10 × 10 cm was sheared as near to the skin as possible. A buffer area of 10 cm was left between the four fields to avoid mutual influence of the other treatment quarters (Fig. 1).

During the experimental period of 21 d, the sheep were exposed to the treatments twice daily, with a minimum of 6 h between treatment cycles. On field 1 (see Fig. 1), a medium hardness brush was used to simulate mechanical irritation (20 strokes per treatment) to presumably increase wool cortisol levels. For synthetic glucocorticoid treatment (field 2) *Solutio Cordes Dexa N*® (ICHTHYOL-GESELLSCHAFT Cordes, Hermanni & Co. GmbH & Co. KG, 22335 Hamburg, Germany) fluid was diluted 1:4 with 40% isopropanol resulting in a dosage of 0.5 mg dexamethasone/10 mL solution (dosage based on (Abraham et al., 2009)). Dexamethasone was chosen as it was expected to reduce local cortisol concentrations as well as to show minimal cross-reactions in the cortisol assay. On field 3 a hyperemising fluid (“*Kwizdas Restitutionsfluid*”; Kwizda Pharma GmbH, Vienna, Austria; Ingredients: Aqua, Alcohol Denat., Urtica Dioica Extract, Ammonia, Ethyl Ether, Camphor, Rosmarinus Officinalis Leaf Oil, Limonene) was used. This solution was expected to increase wool cortisol levels as it enhances blood circulation of the skin and is mainly applied to horses to support recovery of tendons. Of each solution 10 mL were applied dropwise with a syringe on field 2 and 3 respectively, avoiding rubbing and therefore mechanical stimulation. The fourth sheared field remained untreated (control). The treatment fields on the sheep were rotated to reduce the likelihood for posture- or location-dependent influences (see Fig. 1). Two sheep received the equivalent treatments on the same body sites, to prevent loss of data in case of exclusion of an animal. So each treatment was applied on every sheep, but on different body sites; only two sheep each received the same composition of treatment and location. On day 22 all fields were sheared again and the regrown wool was kept for sample preparation. Faecal cortisol metabolite (FCM) levels were determined to monitor systemic stress.

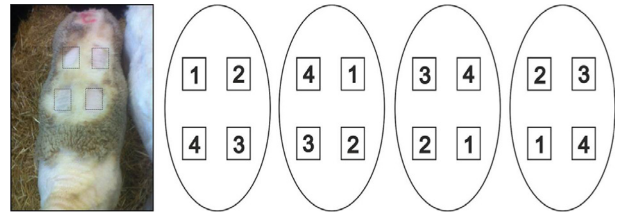

Fig. 1. Dorsal view of sheared, untreated quarters with a buffer area between the test fields (left picture). Two sheep received the equivalent treatments (1: brushing, 2: dexamethasone fluid, 3: Kwizda’s hyperemising fluid, 4: untreated control) on the same body sites (only one of each pair is pictured here schematically). Among the sheep pairs the treatment fields were rotated, to guarantee that each treatment was applied on everybody site.

2.3. Sample collection and steroid extraction

2.3.1. Faecal samples

Faeces were collected after spontaneous defecation, attempting to gather a sample per day from each animal. Number of samples per animal varied from three to seven samples per week. Faeces was collected into a plastic vial and immediately stored at −20 °C until steroid extraction. A total of 0.5 g of wet, well homogenized faeces per sample were weighed and extracted with 5 mL of 80% methanol. Each sample was vortexed on a multivortexer for 30 min and then centrifuged at 1500×g for 15 min (Palme et al., 2013). A 0.5 mL aliquot of the supernatant was transferred into a 1.5-mL plastic tube (Biorad, Hemel Hempstead, UK) and stored at −20 °C until analysis.

2.3.2. Wool samples

Wool samples were taken with a commercially available sheep wool clipper on day 0 and 22 of the experiment. The clipper was cleaned with 80% methanol in between each sample. The wool samples were stored in a paper bag and frozen at −20 °C until sample preparation. From each sample, two portions of 250 mg wool were weighed in a glass vial and manually cleaned from remaining straw and dirt particles. Washing procedure and extraction protocol followed Ouschan et al. (2013) with slight modifications due to the specific character of sheep wool. For removing wool lanolin and other lipids from the surface, 7 mL of n-hexane were added per sample. After shaking each sample on a hand vortexer for a minute the wool was transferred into a new vial, avoiding transferring the sunken dirt particles. The dried wool was cut into small fragments

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