Journal of Veterinary Behavior 30 (2019) 92–95

Contents lists available at ScienceDirect

Journal of Veterinary Behavior

journal homepage: www.journalvetbehavior.com

Bovine Research

Comparing hair cortisol concentrations from various body sites and serum cortisol in Holstein lactating cows and heifers during thermal comfort zone

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ARTICLE INFO

Article history:
Received 11 July 2018
Received in revised form
5 November 2018
Accepted 10 December 2018
Available online 16 December 2018

Keywords:
serum cortisol
hair cortisol
Holstein cows
heifers
various body sites

ABSTRACT

Measuring cortisol in hair has recently been established as a reliable physiological index to evaluate chronic stress conditions in humans and many animal species. The aim of this study was to determine serum cortisol and the concentrations of hair cortisol from various body sites of lactating Holstein cows and heifers during thermal comfort zone. Forty-seven multiparous lactating Holstein cows (average milk yield 37.5 ± 2.3 kgd^{-1}, days in milk = 110 ± 47 days, body weight = 623 ± 75 kg) and 23 Holstein heifers (10-12 months of age, body weight = 258 ± 20 kg) were used in this study. Blood samples were collected on days (d) 0, 30, and 60 of the study. Hair samples were harvested from the forehead, withers, and rump sites of the animals. Data for temperature-humidity index (average THI = 69.3 ± 1.2) represented the threshold environmental conditions during the experiment. Serum cortisol concentration was higher on day 0 of the study and lower on day 30 and day 60 of sampling in lactating Holstein cows and heifers ($P < 0.05$). However, no differences in serum cortisol concentrations were observed in the second (day 30) and the third (day 60) samples in heifers and lactating cows ($P < 0.05$). No differences were found in hair cortisol concentrations among various sampling sites in lactating cows and heifers ($P > 0.05$). Conclusions drawn indicate that the distribution of cortisol into the hair shaft does not depend on hair sampling sites, and so that of the hair cut for cortisol analysis can be collected from any of the three body sites.

Introduction

Hair cortisol (HC) concentration is believed to be less invasive than blood and saliva cortisol particularly during sample collections compared with other matrices. Given that cortisol passively and gradually diffuses from blood to growing hair follicles, it represents the prolonged stress exposure in ruminants (Burnett et al., 2014; Ghassemi Nejad et al., 2017a,b). It is suggested that sweat and sebum secretion amounts may affect HC values because these secretions may be varied if samples are harvested from different body sites (González-de-la-Vara Mdel et al., 2011) particularly during various environmental conditions. In addition, Burnett et al. (2014) reported a higher HC level in tails compared to shoulders and hips of Holstein cows. However, hair samples from the tails are not recommended in other studies because the contaminations from feces may profoundly affect the HC levels (Davenport et al., 2006; Ghassemi Nejad et al., 2014), particularly in cattle because their tails are constantly in touch with feces. Thus, hair growth rate, sex, and anatomical sites were suggested as sources of HC variations (Burnett et al., 2014). This is inconsistent with another study by Fourie et al. (2016) who reported no differences in HC concentrations between hair collected from the base of tails and shoulders or thighs and shoulders of yellow baboons.

Moreover, HC differentiation in hair from various sampling sites can be hypothesized because of different factors including different cattle (e.g., heifers vs. lactating cows), different growth rate of hair

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https://doi.org/10.1016/j.jveb.2018.12.007
1558-7878/

(Fourie et al., 2016), and varying blood flow circulations in different body sites. Furthermore, Comin et al. (2012) reported no significant differences in HC among samples taken from eight different body sites of white rabbits. Results of this investigation regarding possible differences in HC concentrations from various body sites can help researchers to nominate a proper body sites for HC analysis. To the author's knowledge, there has not been a study reporting HC from various body sites and serum cortisol when the comparison is made within the same farm and in both lactating cows and heifers. Therefore, this study was designed to determine HC concentrations from various body sites and serum cortisol of lactating Holstein cows and heifers.

Materials and methods

Experimental design and animals

The experimental procedure and methods were approved by The Animal Welfare and Ethics Authority of Kangwon National University, Chuncheon, Republic of Korea. Forty-seven multiparous lactating Holstein cows (days in milk [DIM] = 110 ± 47 days, milk yield = 37.5 ± 2.3 kgd^{-1}, body weight = 623 ± 75 kg) and 23 Holstein heifers (10-12 months of age, average body weight = 258 ± 20 kg) were used in this experiment. Lactating cows were chosen to be at their highest production levels (average 37.5 ± 2.3 kgd^{-1}) based in the DIM. All animals were housed in a covered industrial farm, having the same environmental conditions and free access to water. The barn where the heifers and lactating cows were separated, however, was along the same corridor. There were seven pens with capacity of seven cows in each pen. All cattle were housed in a roofed shelter equipped with concrete pens confined with metal and bedded with dry manure (4.5 m^2 per cow, 4 m^2 per heifer). Metal automatic roofs were used as cover ceilings in the barn where the roofs were open during the day when there was no rain. Cattle were fed in mangers and each pen was equipped with separate water through. Feed, based on the farm management system, using automated feeder, was offered three times daily at 08:00 hour, 13:00 hour, and 20:00 hour. Water was available *ad libitum*.

Blood and hair samples collection

Blood was collected an hour before offering mid-day feed by jugular venipuncture (Vacuette, greiner bio-one ltd. Kremsmünster, Austria) in vacutainer tubes (Kovax-syringe, Korea vaccine Co. Ltd., Seoul, Korea) at 12:00 hour on days (d) 0, 30, and 60. After collection, the serum was obtained by centrifugation (2500 × g for 15 minutes) and then placed in storage tubes (−20°C) and was used for cortisol analysis (radioimmunoassay method). All the animals were subjected to hair harvesting using commercially available clippers (Pro HS-303, Hasung electronics Co. Ltd., Seoul, Korea) at the same time of the day (13:00 hour) twice at enrollment (day 0), and the end (after 2 months) of the experiment from three different body sites including forehead, withers (right side), and rump (right side). The same sites were subjected to hair cut to harvest the regrown hair. The sampling sites were chosen by dividing the body into the frontal, mid, and distal regions. The criteria for choosing the body sites were accessibility, being clean (no fecal contamination), and ease of hair sample collections, and therefore, could represent the whole body of the animals. Only the first 3 cm of hair shaft were used from all individuals; however, heifers showed a higher hair growth (approximately 3 cm) than lactating cows (approximately 1.5 cm). The color of hair samples was all black. Hair samples (250 mg) were weighed into labeled 10 mL sterilepolypropylene tubes (GC Fuji PLUS, Radiopaque reinforced, GC Corporation, Tokyo, Japan) and twice washed in 5 ml isopropanol for 3 minutes to remove exogenous contaminants, sweat, and sebum (Davenport et al., 2006), and air dried for 7 days in a clean protected hood (Ghassemi Nejad et al., 2017a,b). Once dry, the samples (50 mg) were minced into fine pieces with surgical scissors. Minced samples were weighed and placed into a 2 mL microcentrifuge tube. Then 1 mL of methanol was added to each microcentrifuge tube, and tubes were incubated at room temperature for 24 hours with slow rotation to extract steroid hormones. After extraction, samples were spun for 30 seconds in a microcentrifuge, and a 0.6 mL aliquot of the methanolic extract was added to a new tube and dried at 38°C. This volume was chosen to avoid contamination of the supernatant with hair particles. The dried extracts were reconstituted with 0.4 mL of phosphate buffer provided in the assay kit as described by Davenport et al. (2006) for analyzing cortisol concentration. Finally, the dried extracts were subjected to analysis by an enzyme immunoassay kit (Salimetrics, high sensitivity salivary cortisol, enzyme immuneassay kit, no. 1-3002, State College, PA, USA, 16803) according to the manufacturer's recommendation.

Temperature and humidity index (THI)

The experiment was conducted in the spring season where temperature and humidity falls into thermal comfort zone of Holstein cows in Korea. The mean temperature (°C) and relative humidity (% RH) of the area where cattle were housed were obtained from the Korean meteorological administration. The following equation was applied to calculate THI:

$$\mathrm{THI} = (1.8 \times t + 32) - (0.55 - 0.0055 \times \mathrm{RH}) \times (1.8 \times \mathrm{t} - 26).$$

where, t is the air temperature (°C) and RH is the relative humidity (%). The THI values obtained indicate the following: <71 = absence of heat stress; 72 to <79 = mild heat stress: 80 to <89 = moderate heat stress and 90 and above = severe heat stress (Armstrong 1994); 68 < THI < 71 were considered as the absence of heat stress.

Average THI in the experimental farm was 69.3 ± 1.3 throughout the experiment calculated, which defined thermal comfort zone (threshold) conditions.

Data for serum cortisol were analyzed using the ANOVA procedure of SAS (version 9.0; SAS institute Inc., Cary, NC). Statistical analysis for HC was carried out using the ANOVA procedure of SAS (version 9.0; SAS institute Inc., Cary, NC) based on completely randomized design. Duncan's multiple range test was used to compare the differences between treatments means. All the data in figure bars are reported as the sample mean ± the standard error of mean. The intra-assay and inter-assay coefficients of variations were 3.15 and 9.35, respectively. The normality of the data for its distribution was tested before the application of the final comparison by SAS. F-tests were performed to check for equality of variances of HC concentrations. Statistical differences were considered significant at $P < 0.05$.

Results and discussion

Serum cortisol concentration ($ng\ mL^{-1}$) was higher ($P < 0.05$) on day 0 of the study and lower ($P < 0.05$) on day 30 and day 60 of sampling in both lactating Holstein cows and heifers (Figure 1). However, no differences were observed in second (day 30) and third (day 60) samples within and between heifers and lactating cows ($P < 0.05$).

Peak of serum cortisol may vary from time to time, depending on the physiological status of cows such as during pregnancy and postpartum. In the present study, lactating cows during early

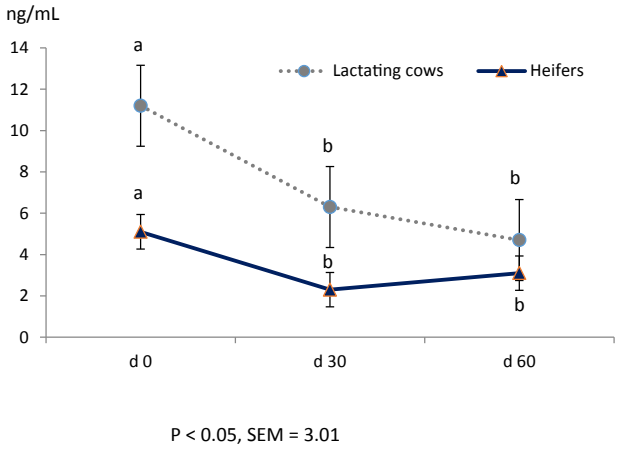

Figure 1. Serum cortisol concentrations (ng mL^{-1}) in lactating Holstein cows and heifers.

lactation were used. Higher stress conditions due to high milk yield were expected. The heifers, however, were not pregnant before the experiment; thus, lower stress conditions could be expected. In addition, the presumptive adaptation may explain the fall of serum cortisol concentration in the following sampling on day 30 and day 60. It also could be suggested that with time passing, the early lactating period drops to mid-lactating period, which is less stressful due to lower milk production. This may result in lower serum cortisol concentration that obtained in this study at day 30 and day 60.

These data show the baseline of HC levels in different body locations in both heifers and lactating cows. The differences of cortisol levels between heifers and high production lactating cows were found to be interesting and were shown in this study. Thus, this study shows the importance of baseline HC levels in absences of environmental stressors such as heat and cold. No differences were found in HC concentrations (pg mg^{-1}) obtained from various sampling sites ($P > 0.05$, Figures 2 and 3) in both lactating cows and heifers; however, the values for cows were higher ($P < 0.05$) than those for heifers in each region. In a previous study, Ghassemi Nejad et al. (2017a) reported a difference in HC of dairy Holstein cows and heifers with different coat colors; however, the authors in that study only reported the HC from foreheads of the animals but not between various body sites. They also observed that HC in Holstein heifers had lower concentrations compared to lactating cows on the

Figure 2. Hair cortisol concentrations (pg mg^{-1}) from various sampling sites in lactating Holstein cows.

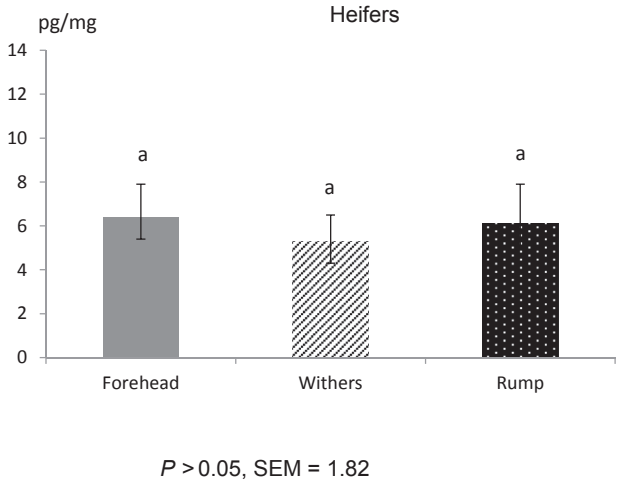

Figure 3. Hair cortisol concentrations (pg mg^{-1}) from various sampling sites in Holstein heifers.

same farm under same heat stress conditions. However, no differences in serum cortisol concentrations within lactating Holstein cows and heifers were observed (Ghassemi Nejad et al., 2017a). In addition, variability across body regions in HC concentrations has been reported in humans (Sharpley et al., 2010), grizzly bears (Macbeth et al., 2010), chimpanzees and orangutans (Carlitz al., 2014, 2015), and horse manes and tails (Duran et al., 2017). Moya et al. (2013) reported higher HC levels collected from tails of beef cattle, which is not applicable in our study. Likewise, it has been always suggested to choose the sampling location from the cleanest body sites of the animals and thus less exposure to fecal contamination. Therefore, the forehead, wither, and rump body sites of the animals have been chosen in the present study. Duran et al. (2017) evaluated HC concentrations in two locations horse's bodies including manes and tails. They found greater HC in the manes compared with the tails. They did not mention a reason behind this result but suggested to collect hair from the tails. The reason can be attributed to the blood flow and hair growth rate, which have been previously reported as sources of HC variations (Fourie et al., 2016) and is substantially different in the tails and the manes. However, in the present study, the tails of lactating cows and heifers were not subjected to evaluate HC because of fecal contamination. Tallo-Parra et al. (2017) used calves to find out the effects of administration of ACTH on HC levels. Calves and young heifers usually show higher HC than mature cows (González-de-la-Vara Mdel et al., 2011) when they are new born because of higher stress levels before acclimation to the new environment. However, once the habitation to the new environment is completed, production levels play a more important role to increase stress with dairy cattle. This is consistent with the obtained result when lactating cows showed higher HC and serum cortisol than do heifers. They found no differences in HC levels between forehead and hip, which is in line with our result. In another study, Fourie et al (2016) reported no differences in cortisol concentrations between hair samples collected from the base of tails and shoulders or thighs and shoulders of yellow baboons. Higher hair growth rate in heifers compared to lactating cows when they are exposed to additional stress because of high milk production may explain the relatively lower HC in heifers; however, finding the meticulous reason(s) may need further investigation. These results are in line with the findings by Fourie et al. (2016) in calves and Comin et al. (2010) in white rabbits who observed no significant differences in HC among samples taken from eight different body sites. High producing

lactating cows during the postpartum period are more susceptible to stressful conditions such as add-up production stress and environmental stress (Ghassemi Nejad et al., 2015; West 2003). This may increase cortisol production in serum and consequently in hair compared with heifers that have no additional due stress. Regardless of variance among individual heifers, crossbred Holstein-Friesian heifers demonstrated lower values of HC than that of pure ones (Peric et al., 2013).

In contrast, Sharpley et al. (2010) reported higher HC in humans for samples taken from forearms compared with those from lower legs, suggesting a localized HC response. Burnett et al. (2014) reported that hair from the tails of Holstein cows had relatively higher levels of cortisol than the shoulders and the hips locations.

The correlations between the two variables of serum cortisol and HC were not applicable because of different characteristics of the matrices. In addition to the fact that the characteristic of each matrix (hair vs. serum) is different and few samples of blood are not enough to make such comparison, the method of analyzing hair and serum cortisol is different. Besides, the serum cortisol is characterized as ng per milliliter or similar units because it is a liquid, whereas the HC can be reported as pg per mg of hair or similar units that represent the solid values. The aforementioned facts may explain why the correlations between these two variables were not applicable.

Hair cortisol concentrations may provide precise and reliable data because concentration changes with a positive correlation to stressors (Russell et al., 2012). Moreover, measurements of HC in cattle could help the farmers to monitor stress and well-being of the animals by evaluating individual resilience to stressors (Ghassemi Nejad et al., 2014; Comin et al. (2012)). The correlations between the two variables of serum cortisol and HC were not applicable because of different characteristics of the matrices. In addition to the fact that the characteristic of each matrix (hair vs. serum) is different and few samples of blood are not enough to make such comparison, the method of analyzing hair and serum cortisol is different. Besides, the serum cortisol is characterized as ng per milliliter or similar units because it is a liquid, whereas the HC can be reported as pg per mg of hair or similar units that represent the solid values. The aforementioned facts may explain why the correlations between these two variables were not applicable.

Conclusions

It is concluded that HC concentration does not vary from various body sites. Thus, the hair for cortisol analysis can be collected from any of the three locations based on the ease of collection and the restraint and treatment facilities for animals.

Acknowledgments

This work was carried out with the support of the Cooperation Research Program for Agriculture Science and Technology Development (grant number: PJ010209), Rural Development Administration, Republic of Korea. The cortisol analysis, however, was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (Project number: 2018051321, ID: 2018R1D1A1A02051321). The authors are grateful to Jason Park (Rosetta Stone English Institute, Chuncheon, Republic of Korea) and Dr. Morteza Ghaffari (Dept. of Animal Science, University of Alberta, Canada) for their help in professional editing of the article.

Ethical considerations

The experimental procedure and methods were approved by The Animal Welfare and Ethics Authority of Kangwon National University, Chuncheon, Republic of Korea.

Conflict of interest

The authors certify that there is no conflict of interest regarding the material discussed in this article.

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