Short communication

Hair or salivary cortisol analysis to identify chronic stress in piglets?

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Abstract

Hair cortisol might better represent chronic stress than salivary cortisol in piglets. To test this hypothesis, 24 female, 7-day old piglets were allocated to two groups and artificially reared. The piglets in the stressed group were exposed to overcrowding (0.10 m^2/piglet) and frequent mixing with unfamiliar piglets until the age of 28 days. The control group remained in an unchanging group at a density of 0.29 m^2/piglet. After 3 weeks, stressed animals had gained significantly less weight (median, here and throughout, 7.58 kg) than the control animals (6.43 kg; $P = 0.021$). Additionally, hair from the stressed group contained significantly higher cortisol concentrations (87.29 vs. 75.60 pg/mg hair; $P = 0.005$), whereas salivary cortisol concentrations did not significantly differ between groups (0.30 vs. 0.25 µg/dL saliva; $P = 0.447$). Weight gain and hair cortisol concentrations were significantly correlated ($P = 0.036$, $r = -0.430$), but neither of these parameters were correlated with salivary cortisol concentrations ($P = 0.929$, $r = 0.019$ and $P = 0.904$, $r = 0.026$, respectively).

Keywords: Chronic stress; Cortisol; Hair; Pig; Saliva

Monitoring chronic stress is of value in assessing animal welfare and in searching for factors that could limit animal performance and/or increase susceptibility to infectious diseases (de Groot et al., 2001). Cortisol is predominantly used as a biomarker to assess chronic stress, as it is released upon activation of the hypothalamic-pituitary-adrenal axis (Chrousos et al., 1988). However, interpreting cortisol concentrations in biological fluids, such as saliva, has certain constraints, since they are influenced by various factors including a circadian rhythm (Ruis et al., 1997). Furthermore, cortisol concentrations might rise in response to an acute stressor and could therefore merely present a snapshot of an animal's physiological state (Cook et al., 1996). Since cortisol concentrations in hair accumulate over time (Koren et al., 2002; Casal et al., 2017), we hypothesised that this parameter might be a better indicator of chronic stress than salivary cortisol concentrations.

To test this hypothesis, 24 female piglets (Belgian Landrace × Piétrain), born from eight litters, were transported from a local farm to the University of Antwerp at the age of 4 days (August/September 2016). They were housed in commercial brooders (Rescue Decks, S and R Resources LLC) and reared on milk formula (BIGGILAC PL+, AVEVE), which was provided ad libitum. These piglets were litter-matched, and randomly assigned to either the control (n = 8) or the stressed group (n = 16) by handpicking ear tag numbers from a bag. The latter group was exposed to three stressors: overcrowding, mixing with unfamiliar piglets and deprivation of environmental enrichment (de Groot et al., 2001). These animals were housed at a density of 0.10 m^2/animal, which is below the legal minimum of 0.15 m^2/piglet (< 10 kg; 2001/88/EC)[1]. On 32 randomly selected occasions, piglets from the stressed group were randomly allocated to be mixed between brooders by selecting ear tag numbers from a bag. Environmental enrichment was not provided to piglets in the stressed group. The control piglets were housed in stable

[1] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32001L0088 (Accessed 13 August 2019).

groups at a density of 0.29 m^2/animal with balls and ropes as environmental enrichment. Animals were observed twice a day to document behaviour, body condition, lesions, and faecal composition. In case of any deviation from normal behaviour, body condition, the presence of lesions or the presence of diarrhea a veterinarian would be consulted. Since all animals remained in good health this was not necessary. Humane endpoints were determined a priori (parameters that would lead to euthanasia: not standing up when startled, body condition score of one, pale oral mucosa and capillary refill time of gingiva longer than 3 s, or body temperature below 36°C) but were not reached. Prior to commencement, all experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (Approval date 4 May 2016; Approval number: 2016-41) and were in accordance with the European Directive (2010/63/EU)[2].

Piglets were weighed at the start (7 days old) and at the end of the experiment (28 days old). Saliva was collected at day 28 between 8:30 am and 9:30 am by allowing piglets to chew on a synthetic cylindrical collection pad (Micro·SAL, Oasis Diagnostics; Shirtcliff et al., 2015). All specimens were stored at -80 °C until further analysis. Cortisol concentrations were determined in duplicate in a single assay using a commercially available cortisol saliva ELISA (IBL-International) validated for pig saliva (Thomsson et al., 2014). To determine cortisol accumulation in hair during the experiment, the dorsum (approximately 35 cm x 10 cm) of each piglet was shaved with clippers at day 7 to set the baseline. Mechanical forces resulting in scratching and rubbing that could elevate cortisol concentrations occur relatively infrequently in this region (Salaberger et al., 2016). At day 28, the dorsal region was shaved again. The collected hairs were washed twice for 3 min with 10 mL isopropanol to remove dust and sebum

[2] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063 (Accessed 13 August 2019)

(Davenport et al., 2006). After 5 days of drying, specimens were ground using a mortar and pestle. Because hair cortisol concentrations are known to vary between different regions of the back, the entire specimen was homogenised (Casal et al., 2017). From this specimen, 50 mg was taken and added to 1.8 mL methanol. After incubation for 24 h, the specimens were centrifuged for 15 min at 1500 *g* (Davenport et al., 2006). From the supernatant, 1.3 mL was lyophilised and resuspended in 300 µL of phosphate buffered saline. Cortisol concentrations were determined using the same ELISA described above. Intra-assay coefficients of variation for both cortisol assays were <5%.

For weight gain and salivary and hair cortisol concentration analysis, a mixed model was applied to identify potential differences between both groups. Sow was included as a random factor. In order to meet normality and/or homoscedasticity assumptions, data from hair and salivary cortisol concentrations were log transformed. A nonparametric Spearman's assay was conducted to identify possible correlations between the three parameters. All data were analysed using JMP Pro 12 (SAS Institute) and reported as medians and $25^{th}/75^{th}$ percentiles; $P \leq 0.05$ was considered statistically significant. Power analysis confirmed that, for the parameters studied, the chosen sample size resulted in power >80%.

Median weight gain for the control group over the 21-day study period (7.58 kg; 6.79 kg/8.22 kg, $25^{th}/75^{th}$ percentiles respectively, here and throughout) was significantly higher than that of the stressed group (6.43 kg; 5.94/7.20 kg; $P = 0.021$; Fig. 1). Salivary cortisol concentrations at day 28 in the control group (0.25 µg/dL saliva; 0.21/0.39 µg/dL saliva) did not differ from the stressed group (0.30 µg/dL saliva; 0.27/0.51 µg/dL saliva; $P = 0.447$). In two animals, salivary cortisol concentrations were atypically high. Most likely this was due to an acute response to a stressful stimulus, rather than chronic stress. Of note, the correct timing

for salivary sampling could be a point of discussion. It is possible that differences between groups might be greater if sampling occurs when baseline cortisol concentrations are low, i.e. in the evening. However, the circadian rhythm of cortisol does not mature until 20 weeks (Ruis et al., 1997), making the identification of daily baseline cortisol concentration in young animals unpredictable. Additionally, a previous study has demonstrated that decreased welfare leads to a blunted circadian rhythm (de Jong et al., 2000), making the determination of the optimal timepoint for sampling problematic. As a result, the identification of chronic stress in pigs requires a cortisol measurement technique that is less sensitive to short-term fluctuations. In our study, in contrast to salivary cortisol concentrations, hair from stressed pigs contained higher concentrations of cortisol (87.29 pg/mg hair; 78.55/99.61 pg/mg hair) than hair from control animals (75.60 pg/mg hair; 69.95/78.42 pg/mg hair; $P = 0.005$), although there was a wide range of hair cortisol concentrations in the stressed group (range, 64.26 - 107.45 pg/mg hair). This variation might be explained by differences in susceptibility to stress because of different coping styles (Koolhaas et al., 1999).

The concentrations found in this study are higher than the previously reported values of older boars (Casal et al., 2017). Although concentrations of free cortisol are higher in boars and barrows than in gilts, these concentrations decrease with age (Ruis et al., 1997). This possibly explains why the cortisol concentrations detected in our 4-week-old gilts were higher. Nevertheless, the effects of gender, age and breed on hair cortisol concentrations should be further investigated. Additionally, it is important to emphasise that cortisol accumulation during the last days of the experiment was still present in the hair roots located in the skin. Since the mean depth of the hair follicles was 1.32 ± 0.04 mm and the estimated hair growth rate/month is 10.01 ± 0.24 mm, it would have taken 4 days before this cortisol accumulation was measurable in hair. However, the effect of the stressors was large enough to be detected, since

cortisol concentrations in hair correlated significantly and negatively with weight gain (P = 0.036, r = -0.430). Neither of these parameters correlated significantly with cortisol concentrations in saliva (P = 0.904, r = 0.026 and P = 0.929, r = 0.019, respectively). Nevertheless, salivary specimens taken over a longer time period might have correlated with weight gain and hair cortisol concentration in stressed piglets (Casal et al., 2017).

Despite inter-individual variations, there was a significant negative correlation between hair cortisol concentrations and weight gain in stressed piglets. This was not the case for salivary cortisol concentrations and salivary and hair cortisol concentrations were not correlated at the end of the 28-day study period. Hair cortisol concentrations could be used to identify chronically stressed piglets at a group level.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig. 1. A. Four-week-old piglets exposed to three stressors (overcrowding, mixing with unfamiliar piglets and privation of environmental enrichment) gained significantly less weight after 3 weeks compared to control piglets. B. At day 28, cortisol concentrations in saliva were not significantly different between the two groups. C. The stressed group had significantly higher values of hair cortisol compared to their control littermates. D. A nonparametric Spearman's assay indicated that weight gain and hair cortisol concentrations correlated significantly, while neither of these parameters correlated significantly with salivary cortisol. Control group (n = 8); stressed group (n = 16). Significant differences (linear mixed models, $P \leq 0.05$) are indicated by an asterisk and a line. For each group the median (thick line), the 25^{th} and 75^{th} percentiles (thin lines) and the 5^{th} and 95^{th} percentiles (dotted lines) are shown.