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Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor

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ABSTRACT

A combination of salivary biomarkers measured at the same time, reflecting the different systems that are involved in the stress mechanism, could be the best tool for its evaluation. In this study, changes in a panel of salivary biomarkers of stress and immunity including chromogranin A (CgA), IgA, cortisol, testosterone, haptoglobin (Hp) and C-reactive protein (CRP), were evaluated. A total of 14 (7 control and 7 test) crossbred Duroc × (Landrace × Large White) males of 190 days of age were used for this experiment. The stress mechanism was evaluated after applying a psychosocial stressor model in 7 pigs, based on isolation and regrouping. Our results show that after of isolation, there was a significant ($P < 0.05$) increase in salivary CgA and IgA. However, after regrouping, there was a significant increase ($P < 0.05$) in salivary cortisol, testosterone and CgA. Salivary Hp and CRP concentrations did not significantly change after applying this stress model. This panel of salivary biomarkers could be used as a practical and non-invasive tool for reflecting the activity of different physiology systems involved in the stress response.

1. Introduction

Saliva sampling has the advantage of being non-invasive as it causes minimal stress during collection, thus making it an ideal tool for evaluating stress physiology in pigs (Muneta et al., 2010; Soler et al., 2013a). Unlike blood sampling methods, it does not cause additional stress, which could be a confounding factor in stress models. Furthermore, in contrast to the urine or faeces samples, repeated sampling over short time intervals can be carried out, which facilitates ongoing animal monitoring (Escribano et al., 2012a).

Stress can be defined as a state of threat to homeostasis, caused by psychological, environmental or physiological stressors (Chrousos and Gold, 1992). A complex system of physiological and behavioural responses is initiated under conditions of stress, which is known as the adaptive stress response (Johnson et al., 1992) and serves to re-establish the threatened body equilibrium. The main tools used to quantify stress in pigs include: direct behavioural observation (Smulders et al., 2006) and quantification of the adaptive responses to stress by means of "biomarkers" (Cook et al., 1996; Muneta et al., 2010; Escribano et al., 2013). Although widely used, the methods of observation can have some potential for error or misinterpretation in some situations due to the potential subjective interpretation of the animal behavioural response or to the fact that the wellbeing of an animal might be impaired, even when signs of stress are not obviously visible (Hart, 2012). For this reason, the quantification of physiological responses to a stimulus by biomarkers could be a complement to behavioural observation methods.

Several potential salivary stress biomarkers have been identified and used, in an effort to produce objective tools to evaluate the different pathways of the stress response. Salivary cortisol can indicate activity of the hypothalamic pituitary–adrenal (HPA) axis in response to different stressors in pigs (Cook et al., 1996; Merlot et al., 2011). The hypothalamus pituitary gonadal (HPG) axis produces the release of testosterone from the gonads and the adrenals (Rivier and Rivest, 1991) and salivary testosterone levels are strongly correlated with free serum testosterone levels in humans (Arregger et al., 2007; Cardoso et al., 2011). Chromogranin A (CgA) is co-released with catecholamines from the adrenal medulla (Tony, 2003) and, in humans, it has been quantified in saliva as an alternative to catecholamines, as the latter are considered to be poor markers of acute changes in sympathoadrenal medullary (SAM) activity (Schwab et al., 1992; Kennedy et al., 2001). Recent studies have suggested that testosterone (Escribano et al., 2014) and CgA (Escribano et al., 2013) might be salivary biomarkers of the stress response in pigs, reflecting HPG and SAM activity, as suggested in humans (Gallina et al., 2011; Schoofs and Wolf, 2011).

Salivary immunoglobulin A (IgA) can be increased by both parasympathetic and sympathetic nerve system (Carpenter et al., 2000; Allgrove et al., 2008). The IgA expression has been related to stress in the pig saliva (Muneta et al., 2010), in addition, antigen-specific secretory IgA can be used to assess the immune status (Escribano et al., 2012b). On the other hand, although the exact linkage between the acute phase response and the stress response is not known, it has been hypothesized that acute phase proteins (APPs) can be induced directly

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or indirectly (through cytokines) by metabolites released through the SAM and/or HPA pathway (Aninat et al., 2008; Gruys et al., 2005; Murata et al., 2004). In pigs, measurement of salivary haptoglobin (Hp) and C-reactive protein (CRP) correlates well with serum concentrations, and saliva sampling is considered to be a reliable non-invasive alternative to blood sampling (Gutiérrez et al., 2009a,b).

The hypothesis of this study is that these salivary biomarker responses may be of different magnitudes and kinetics, depending on the axis affected by the stressor. A recent study (Ott et al., 2014) indicated that different types of stressors produced different physiological stress responses in pigs, and therefore, the inclusion of various salivary biomarkers in stress evaluation seems to be useful. Thus, a combination of salivary biomarkers measured at the same time, reflecting the different systems that are involved in the stress mechanism would be the best tool for its evaluation. However, to the author's knowledge, there are no studies where biomarkers for all four pathways have been used simultaneously to study the stress response in pigs. In addition, the methodological information in relation with the importance of measurements of the total protein and volume of the saliva samples (flow rate) is lacking in most of the papers published using salivary biomarkers in pigs.

Our objective was to study a panel of salivary markers of stress, including CgA, IgA, cortisol, testosterone, Hp and CRP, to evaluate the four different physiological systems (HPA, HPG, SAM and immune system) which may take part in the stress mechanism, after applying a psychosocial stressor model in pigs, based on isolation and regrouping. An additional objective was to study the possible influence of the dilution of saliva in the analytes measured by adjustment of the concentration of each salivary biomarker to total protein or flow rate.

2. Materials and methods

2.1. Animals and housing

A total of 14 non-castrated crossbred Duroc × (Landrace × Large White) males were used for the experiment. The procedures were conducted on a high sanitary/health-status farm in the southeast of Spain under general commercial housing, feeding and husbandry conditions conforming to the European Union Guidelines (Directive 2010/63/EU[1]). The pigs had access to a nutritionally balanced diet (commercial dry diets) and water ad libitum (from nipple drinkers). The pen size employed during this experiment was 2.73 × 2.85 m (7.78 m^2) and they had slatted-floors. At the beginning of the growing period (56 days of age), 14 pigs were housed in slatted-floor pens (1.1 m^2 of surface per pig) in two groups (test and control) of seven animals in each group until the start of the experiment (190 days of age). All animals were subjected to a clinical examination prior to, and throughout, the study and no clinical signs of disease were detected. Furthermore, the concentrations of salivary CRP and Hp obtained in the present study were always lower than the internal reference cut-off values of the laboratory for diseased age-matched animals (Gutiérrez et al., 2009c).

2.2. Experimental design

5 days before the beginning of the experimental period, all animals were accustomed to human contact and saliva collection methodology. The experimental design was divided into three periods: pre-stressor, isolation and regrouping. Each day, during the three periods, saliva samples were collected at 1100 h in both the test and control groups. Two additional saliva samples were collected at 30 min after isolation and at 30 min after regrouping. In the study of isolation, the behaviour of each animal was measured each day after sampling, based on the ethogram of behaviour used by Ruis et al. (2001). This ethogram includes behaviours of exploring, defecation/urination, inactive (sleeping, lying, sitting and standing), ingestive (feeding and drinking), vocalizing and walking. Behavioural data were expressed in percentages of all (total) behavioural observations (except for vocalizing, which could coincide with other behaviours). The animals were recorded in their home pens during 30 min and then the behavioural observations of each animal was analysed, by a same person, at 1 min intervals on recorded images (every day and always between 1100 and 1130 h). This timing of the observation period was selected to facilitate the daily work. In this form, there was always someone to check that the record was done correctly and this fact for them posed no disturbance in their daily lives. The normal behaviour occurring after regrouping of pigs is aggression with physical injuries (Coutellier et al., 2007) thus producing an acute stress situation between animals. Therefore, the accumulation of skin lesions in pigs is a way to provide a proof that pigs have shown the expected reaction and behaviour after applying of the regrouping stress model. The use of recording lesions was identified by Turner et al. (2006) as a potential means of rapidly assessing this individual aggressiveness of pigs when mixed under commercial conditions. Lesions caused by aggression were assessed on individual pigs the day immediately before and all days after regrouping. For purposes of the assessment, the body of each animal was divided into 3 zones per side: head, neck and shoulder, and the remaining parts of the body, using a modified method of Hodgkiss et al. (1998) and used by Li and Johnston (2009). Each zone received an injury score based on the number of scratches on it. The scoring method used followed that of Gonyou et al. (1988) which was 0 = no scratches; 1 = 1 to 3 scratches; 2 = 4 to 6 scratches; 3 = more than 6 scratches. The injury scores on the left and right side of the body were summed to obtain a total score for the head, the neck and shoulders, and the remaining parts of the body as well as a total score for each animal. The minimal score for each pig was 0, and the maximal score was 18 (6 zones times maximum score of 3). The injury score was assessed always at the same day time and by one person to keep the scores consistent throughout the experiment.

The pre-stressor period lasted 5 days (days −1d to −5d; Fig. 1 and 2). Test (n = 7) and control (n = 7) pigs remained in the same housing conditions during this period (1.1 m^2 of surface per pig). On day 6 of this period, the baseline samples of isolation (0dI) for test and control groups were obtained. For the isolation procedure, 7 pigs were relocated to a new barn where each one in the same room was isolated into individual pens (7.78 m^2 each), where they had olfactory and auditory, but not physical neither visual contact with the other pigs. From day 0 of isolation (0dI; Fig. 1 and 2) until day 5 of isolation (5dI; Fig. 1 and 2) the test pigs were kept in this experimental condition. On day 6 of this period, the baseline samples of regrouping (0dR) for test and control groups were taken. The regrouping period started after the isolation period. Test pigs were regrouped and remained in these conditions for 3 days (day 0 of regrouping, 0dR, to day 3 of regrouping, 3dR; Fig. 1 and 2), returning to their original group with the initial conditions (1.1 m^2 of surface per pig). The overall isolation and regrouping procedures took about 3 min.

The control pigs remained in the same housing conditions during the experimental period. The temperature of the pens at these sampling time-points was 21.2 °C ± 0.8 (mean ± SD) and animals were maintained during the experimental period with natural lighting. Pigs of both groups were slaughtered at the end of the experiment in the local slaughterhouse. Procedures involving animal handling were approved by the University of Murcia Ethics Committee and followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe, ETS Number 123).

2.3. Saliva collection and salivary analysis

Saliva was collected from all animals using saliva collection tubes (Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany) containing a

[1] See: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF.