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Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker

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ABSTRACT

This study aimed to validate the use of an automated chemiluminescent immunoassay analyser for salivary testosterone measurements in growing pigs and study how circadian pattern during daytime and stress can influence its values. The test method had intra- and inter-assay coefficient of variation lower than 10%. The method showed good linearity and recovery, and detection limits were low enough to detect salivary testosterone levels. No significant differences were observed in testosterone concentrations at different sampling time, and age and gender did not influence circadian pattern. In addition, this assay was used to quantify testosterone in two models of acute stress and, in both cases, significant increases ($P < 0.01$) in salivary testosterone were detected. Therefore, the automated assay system tested for porcine testosterone determinations would be suitable for its use in saliva samples and, furthermore, salivary testosterone levels could be used as a possible marker of acute stress in pigs.

1. Introduction

Saliva can be obtained by a very simple and cheap, non-invasive and minimally stressful sampling method compared to blood, and therefore is ideal for stress studies in pigs (Muneta et al., 2010). Several potential salivary stress biomarkers have been identified in pigs, in an effort to produce objective tools to evaluate the sympathoadrenal-medullary (SAM) system (Escribano et al., 2013; Fuentes et al., 2011) and hypothalamic-pituitary-adrenal axis (HPA) (Cook et al., 1996; Escribano et al., 2012; Merlot et al., 2011) or immune system (Muneta et al., 2010; Soler et al., 2013). However, to the authors' knowledge, there are no studies about stress influence on testosterone in saliva samples of pigs.

Testosterone, a product of the hypothalamus pituitary gonadal (HPG) axis, is a circulating androgen produced by the testes in male, ovaries in female and a small amount by the adrenal gland, and the control of the production of testosterone is under the secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus. This hormone in turn stimulates the anterior pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Xue et al., 1994).

The appearance in saliva of testosterone is flow rate-independent (Arregger et al., 2007), and its levels are strongly correlated with free serum testosterone levels in humans (Arregger et al., 2007; Cardoso et al., 2011). Furthermore, saliva offers a significant theoretical advantage in relation to serum or plasma, since it contains largely the bioavailable fraction, i.e., the fraction of the total hormone that is able to exert physiological effects (Hampson et al., 2013). For these reasons and because of the non-invasive and stress-free technique of sample collection, salivary testosterone assays are being used increasingly in human research. In recent human studies, salivary testosterone has been related with the modulation of pain sensation (Choi et al., 2012), with depression and anxiety disorders (Giltay et al., 2012), and also has been associated with stress by exercise (Gatti and De Palo, 2011) with the dominance (Mehta and Josephs, 2010) and aggressive behaviour (Montoya et al., 2012; Romero-Martínez et al., 2013). In contrast, in pigs, there is only one study on salivary testosterone, which was measured in response to a lipopolysaccharide challenge (Moya et al., 2006).

Measurement of salivary testosterone concentration in veterinary species has been performed by non-automated assay such as enzyme-linked immunosorbent assay (ELISA) in pigs (Moya et al., 2006) or radioimmunoassay (RIA) in primates (Wobber et al., 2010). The use of the automated systems for analysis has several advantages: its ease of use; low repeat costs; a high throughput analysis; and the system is a fully integrated process (Escribano et al., 2012).

The objective of the current study was to validate the use of an automated chemiluminescent immunoassay analyser for salivary tes-

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tosterone measurements in pigs, and study how circadian pattern during daytime and stress can influence its values.

2. Materials and methods

All animals included in the studies were entire male and female ([*Sus scrofa domesticus*] Duroc × [Landrace × Large White]) crossbred, and the studies were conducted during the fattening period. The animals came from the experimental farm of the University of Murcia (Murcia, Spain) and were subjected to a clinical examination prior to and throughout the study, and no clinical signs of disease were detected. In addition, all procedures involving animals followed the established standards for the humane care and use of animals described in the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Directive 2010/63/EU), and were approved by the University of Murcia Ethics Committee.

2.1. Saliva collection and immunoassay procedure

Saliva was collected from all animals using saliva collection tubes (Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany) containing a sponge, as reported previously (Gutiérrez et al., 2009). Each pig was allowed to gently chew on a sponge, which was clipped to a flexible thin metal rod, over 1 min and without forcing. The sponges were then placed in the tubes and centrifuged at 3,000 × *g* for 10 min. Saliva samples were collected and stored at –80 °C until analysis.

Total testosterone concentration was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite/Immulite 1000 Total Testosterone, Siemens Medical Solutions Diagnostics, Los Angeles, CA). The assay uses a polyclonal rabbit anti-testosterone and a single reagent: alkaline phosphatase conjugated with testosterone in the buffer, with a preservative, and the incubation cycle is 60 min. We did not test for cross-reactions. The manufacturer report on testosterone cross-reaction with another androgens are: 5α-dihydrotestosterone 2.4%, androstenedione 0.8%, methyltestosterone 0.8% and 5α-androstan-3β, 17β-diol 0.4%. Cross-reaction with another glucocorticoids such as cortisol, cortisone, dexamethasone or prednisone, is not detectable. The sample volume used (120 μL) were according to the manufacturer's specification sheet (sample cup must contain at least 100 μL more than the total volume required; 20 μL minimum). All analyses were performed in duplicate.

2.2. Analytical performance of the chemiluminescent immunoassay

2.2.1. Assessment of precision

The intra-assay precision, expressed as the coefficient of variation (CV), was determined by measuring two pools of saliva samples, with different amounts of salivary testosterone, five times in a single analytical run. Each pool was prepared by mixing samples of saliva from four animals with similar concentrations (low and high concentrations) of testosterone previously quantified by the present chemiluminescent assay. These samples were obtained from male and female pigs at fattening period from the experimental farm of the University of Murcia (Murcia, Spain). The same pools were used to determine the inter-assay precision by analysis on five different days within a 15-day period. Although storage at –80 ºC of salivary samples does not affect testosterone levels during at least two years, the extra freeze thaw cycle may contribute some error to testosterone measurement (Granger et al., 2004). For this reason, the saliva samples were frozen in aliquots stored at –80 ºC, and vials were only thawed as required for each analytical run in order to prevent these possible variations as a result of repeated freeze-thaw cycles.

2.2.2. Assessment of sensitivity

The lower and upper limit of quantification calculated based on the lowest and highest testosterone concentration, respectively, that could be measured in the linear part of the calibration curve with a CV was less than 20%, and above the limit of detection in the case of the lower limit. To estimate this parameter, the high saliva sample was serially diluted in deionized water, and each dilution was analysed in five replicates in the same run.

Mean and standard deviations for the salivary testosterone concentrations of 10 replicates of the zero standard (deionized water) were calculated. The limit of detection was the mean value from zero standard samples plus two standard deviations.

2.2.3. Assessment of assay accuracy

As no gold standard assay is available to quantify testosterone in porcine saliva, the accuracy was indirectly investigated by recovery experiment and linearity under dilution as follows.

The recovery experiment was performed as previously reported (Parra et al., 2005). A sample with high testosterone concentration (967 ng/dL) was mixed with different amounts of a sample with low testosterone concentration (96.8 ng/dL) in order to achieve different sample dilutions. Therefore, the high testosterone level sample was diluted 2- (50%), 4- (25%), and 10-fold (10%) with the low testosterone level saliva sample (50%, 75%, and 90%, respectively). In addition, the low testosterone level saliva sample was diluted 4-fold (25%) with the high testosterone level saliva sample (75%). Detected and expected testosterone levels for each diluted saliva sample were compared, and the percentages of recovery were calculated.

Linearity under dilution was determined by using two porcine saliva samples with high levels of salivary testosterone serially diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64) with deionized water, and the testosterone concentrations was measured by the present chemiluminescent assay. The concentration was measured in duplicate for each dilution. Afterward, curves representing measured testosterone concentration versus salivary testosterone concentration expected were constructed.

2.3. Evaluation of circadian pattern of salivary testosterone during daytime in growing pig and effect of gender and age

The experiment was conducted in a high sanitary/health-status farm in the Southeast of Spain in the month of April. A total of 40 animals were randomly selected for inclusion in the experimental procedure. Pigs were housed in groups of 10 animals per pen: pens 1 and 2 housed 17- and 21-week-old non-castrated males, respectively; pens 3 and 4 comprised 17- and 21-week-old females, respectively. Animals were housed under general commercial housing, with feeding and husbandry conditions conforming to European Union Guidelines (Directive 2010/63/EU), and were given ad libitum access to a nutritionally balanced diet. Water was continuously available.

Selected pigs were sampled on two consecutive days, to discriminate between the cyclic and random sources of variation, at 0700, 1100, 1500 and 1900 h. This sampling regime had been previously used for the study of circadian cortisol levels in piglets (Gallagher et al., 2002) and the circadian pattern of acute phase proteins in growing pigs (Gutiérrez et al., 2013). The temperature of the pens at these sampling time-points varied from 18 ºC in the morning to 30 ºC at the midday in the month of April.

2.4. Models of induction stress

Two models of stress induction were used to evaluate the effect of stress on salivary testosterone concentrations with two different groups of animals. One was immobilization with nose-snare,