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Research

Measurements of salivary alpha-amylase in horse: Comparison of 2 different assays

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ABSTRACT

The aim of this study was to develop and validate 2 different methods (enzymatic vs. time-resolved immunofluorometric assay [TR-IFMA]) for the detection and measurement of salivary α-amylase (s-α) in horses. The analytical validation of the enzymatic assay showed intra-assay and interassay coefficients of variation of 2.5% and 7.2%, respectively, and a limit of detection of 0.045 IU/L. On the other hand, the analytical validation of the TR-IFMA showed intra-assay and interassay coefficients of variation of 5.4% and 9.9%, respectively, and a limit of detection of 0.097 ng/mL. Both assays also demonstrated a high level of accuracy as determined by linearity under dilution. To check the presence of α-amylase in saliva of horses, 3 different models of physical exercises (exercise in a horse walker and 2 different types of dressage) were made in a total of 27 animals. Saliva samples were obtained before, immediately after, and 30 minutes after the physical exercise. Samples were measured by enzymatic assay and TR-IFMA, and both methods were able to detect the enzyme. Only the results of TR-IFMA showed a significant increase ($P < 0.05$) in s-α concentration in saliva after the performance of one type of physical exercise and a significant decrease ($P < 0.05$) in s-α concentration in saliva after the performance of other type of physical exercise, but these significances did not exist in the case of enzymatic measures in both types of exercises. Moreover, TR-IFMA had a lower interindividual variability than the enzymatic assay. Both assays validated in this study could measure s-α in saliva of horses in a reliable and robust way, showing the TR-IFMA advantages that could make this test a suitable alternative to enzymatic measures.

Introduction

In the last decade, there has been a growing concern in society about the importance of welfare in farm animals (Vanhonacker et al., 2008). The response against stress situations is especially interesting in horses because of the close relationship between this species and humans.

Several analytes can be measured from blood and plasma to evaluate the response of the animals against several situations. These analytes can be catecholamine (epinephrine and norepinephrine) as markers of the sympathetic adrenal medullary (SAM) system or cortisol as a marker of activation of hypothalamic-pituitary-adrenocortical axis (Jimenez et al., 1998). The use of salivary samples to measure stress markers has several advantages compared to blood samples. For example, saliva can be obtained by noninvasive procedures, does not produce additional stress in animals, is more economic, and so forth. Therefore, salivary cortisol is widely used for stress analysis in horses (Von Lewinski et al., 2013; Christensen et al., 2014).

α-Amylase is a protein that has been used as a biomarker of the sympathetic adrenal medullary (Bosch et al., 2002; Nater and Rohleder, 2009) in humans (Furlan et al., 2012; Rashkova et al., 2012) and in other animal species such as pigs (Fuentes et al., 2011; Muneta et al., 2010) although no references have been found in relation to horses. In humans, it has been shown that the secretion of salivary α-amylase (s-α) is an indicator of autonomic activity because it occurs in response to neurotransmitter stimulation, and salivary glands are innervated by both sympathetic and

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parasympathetic nerves (Garrett, 1999). Moreover, it is considered that sympathetic stimulation (via norepinephrine) leads to high levels of protein concentrations such as α-amylase (Baum, 1993). However, studies on animals suggest that both parasympathetic and sympathetic activation lead to an increase in α-amylase levels, and the 2 branches of the autonomic nervous system do not act independently (Nater et al., 2005). On the other hand, the literature shows that amylase secretion is primarily mediated by activation of beta-1 adrenoceptors (Schneyer and Hall, 1991).

The main purpose of this study was to determine the presence of salivary amylase (s-α) in saliva of horses and its measurement using 2 different analytical methods (enzymatic and immunofluorometric) under conditions of physical exercise and to open the path to possible future research on the role of this protein as a biomarker of stress in the equine species.

Material and methods

Animals, experimental procedures, and sampling

Animals were divided into 3 different groups that were subjected to different physical exercises. Taking into account the diversity of the exercises and the age of the animals, the measures obtained after the physical exercise were compared with a baseline sample (TB) of saliva for each animal in a repeated measures design.

Group I animals (n = 10) belonged to the breeding of Yeguada de la Cartuja (Jerez de la Frontera, Cádiz, Spain). All horses were healthy stallions and were aged 11.2 ± 4.2 years. The stallions were housed in boxes of 8 square meters with free access to water and straw and controlled feeding to prevent overweight. The animals were used for breeding and exhibitions of dressage (highest expression of horse training). The procedure to which they were subjected consisted of a training session in a horse walker for 30 minutes at different velocities (walking and trotting).

Group II animals (n = 7) belonged also to the breeding of Yeguada de la Cartuja. All animals were healthy stallions, were aged 3.2 ± 0.48 years, and were housed in conditions similar to those of group I. The animals were used for breeding and exhibitions of dressage. The procedure to which they were subjected consisted of a training session of dressage exercises for 10 minutes on a riding arena, performed regularly for 2 or 3 days a week, depending on whether there was a display of dressage in the stud during the week.

Group III animals (n = 10) belonged to the breeding of Pablo Hermoso de Mendoza (Estella, Navarra, Spain) and they were males of the Lusitano breed. All animals were healthy stallions and were aged 7.1 ± 2.28 years. The stallions were housed in boxes of 8 square meters with free access to water and straw and controlled feeding to prevent overweight. The animals were used for dressage exercises with bulls in the presence of public. The procedure to which they were subjected consisted of a dressage exercise with bulls in front of an audience during a variable period of time, not exceeding 5 minutes per animal. Usually horses performed dressage exercises daily, with sporadic workouts with bulls but with no public.

In all groups, saliva samples were collected as described in other species (Fuentes et al., 2011) by introducing a small sponge in the horse's mouth for at least 1 minute. The space between the incisors and molars was used for easy access to the animal's mouth safely. All horses were sufficiently accustomed to sampling and tolerated this procedure without resistance. The sponges were placed in collection devices (Salivette, Sarstedt AG & Co., Nümbrecht, Germany) and were centrifuged at 4,000 relative centrifugal force (rcf) for 8 minutes. Saliva samples were kept at −80°C until analysis.

Sampling was identical in all groups. Three salivary samples were taken from each animal. The first sample was taken 10 minutes before the task (TB), and the remaining samples were taken just at the end of the exercise and 30 minutes later (T0 and T30, respectively). Samples were brought to the laboratory, and s-α activity (enzymatic assay) and concentration (immunofluorometric assay) were analyzed as described later. The research protocol used in the present study was approved by the Bioethical Commission of the University of Murcia according to the European Council Directives regarding the protection of animals used for experimental purposes (license number CEEA 71/2014).

Salivary α-amylase analysis

Two different types of assays were used to measure α-amylase in the saliva of horses: an enzymatic assay and a time-resolved immunofluorometric assay.

Enzymatic assay

α-Amylase was determined by a commercial kit (α-Amylase, Beckman Coulter Inc., Fullerton, CA.) using the International Federation of Clinical Chemistry and Laboratory Medicine method (International Federation of Clinical Chemistry, Scientific Division, Working Group on Enzymes). This is a kinetic spectrophotometric assay that uses 4,6-ethylidene(G7)-p-nitrophenol(G1)-alpha-D-maltoheptaoside as a substrate of the enzyme. The intermediate product of substrate hydrolysis reacts with α-glucosidase, giving p-nitrophenol as the final product of the reaction. The rate of p-nitrophenol formation is directly proportional to the α-amylase activity of the sample and can be determined by measuring the absorbance at 405 nm. Volume sample was modified to optimize the method to horse saliva. Reagent volumes were adjusted following the manufacturer's indications. The assay was adapted to an automatic analyzer (Cobas Mira Plus; ABX Diagnostica, Montpellier, France).

Time-resolved immunofluorometric assay

Streptavidin-coated plates (Streptavidin Microtitration Strips, DELFIA, PerkinElmer, Turku, Finland) were used for the development of this assay. The assay was constructed as a noncompetitive indirect sandwich method based on anti−α-amylase polyclonal antibody biotin-labeled as capture reagent and the anti−α amylase polyclonal antibody Eu3+-chelates labeled as detector. The procedure was as follows.

In the first step, wells of streptavidin-coated plates (DELFIA) were coated for 45 minutes at room temperature with anti−α-amylase polyclonal antibody biotin-labeled in assay buffer (DELFIA assay buffer, PerkinElmer) per well with gentle shaking. After coating, wells were washed 4 times with wash solution (DELFIA Wash Concentrate, PerkinElmer) by using a Wallac Delfia 1296-024 Microplate Washer (PerkinElmer).

In the second step, wells were incubated for 45 minutes with saliva samples diluted (1:2) in assay buffer (DELFIA) per well with gentle shaking. After incubation, wells were washed 4 times with wash solution (DELFIA) by using a Wallac Delfia 1296-024 Microplate Washer (PerkinElmer).

In the third step, wells were incubated for 45 minutes with anti−α-amylase polyclonal antibody Eu3+-chelates labeled in assay buffer (DELFIA) per well with gentle shaking. After incubation, wells were washed 4 times with wash solution (DELFIA) by using a Wallac Delfia 1296-024 Microplate Washer (PerkinElmer).

Finally, enhancement solution (DELFIA Enhancement Solution, PerkinElmer) was added per well and incubated for 5 minutes with gentle shaking to allow Eu3+ to form fluorescent chelates. The enhanced fluorescence, proportional to the quantity of α-amylase in