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Oxytocin in horse saliva: validation of a highly sensitive assay and a pilot report about changes in equine gastric ulcer syndrome

María Botía[1], Alberto Muñoz-Prieto[1*], Silvia Martínez-Subiela[1], María Martín-Cuervo[2], Sanni Hansen[3], Xavier Manteca[4], José Joaquín Cerón[1] and Marina López-Arjona[4]

Abstract

Background Equine gastric ulcer syndrome (EGUS) is a frequent disease in horses that comprises two different entities: equine squamous gastric disease (ESGD) and equine glandular gastric disease (EGGD). This disease considerably reduces the quality of life of affected horses and can negatively affect performance. Saliva contains biomarkers, such as oxytocin, that have been used as a welfare indicator and can develop a function as a protective factor against stress-induced changes in gastric function due to its gastric antisecretory and antiulcer effects. The objective of this work was to evaluate changes in salivary oxytocin concentrations in healthy and EGUS horses. For this purpose, an immunoassay based on AlphaLISA technology was validated for the quantification of salivary oxytocin and applied in a total of 102 horses divided into 5 groups: 25 with both EGUS, 23 with only EGGD, 21 with only ESGD, 19 horses with other diseases, and 14 healthy horses.

Results The analytical validation of the method showed good precision and linearity under dilution. Salivary oxytocin concentrations in healthy horses were higher compared to horses with both ESGD and EGGD and only EGGD. Salivary oxytocin concentrations in horses with only ESGD were higher compared to horses with both ESGD and EGGD and horses with only EGGD. In addition, salivary oxytocin concentrations in horses with other diseases different from ESGD were significantly increased compared to horses with both ESGD and EGGD and horses with only EGGD.

Conclusions This report validates a new assay that can measure oxytocin in saliva in horses in a precise and accurate way. The lower oxytocin values in horses with EGGD and both EGGD and ESGD than in horses with ESGD, horses with other diseases, and healthy horses could indicate a possible relation of oxytocin with this disease.

Keywords Biomarker, EGUS, Horse

*Correspondence:
Alberto Muñoz-Prieto
alberto.munoz@um.es
[1] Interdisciplinary Laboratory of Clinical Analysis (INTERLAB-UMU), Department of Animal Medicine and Surgery, Veterinary School, Regional Campus of International Excellence Mare Nostrum, University of Murcia, 30100 Murcia, Spain
[2] Department of Animal Medicine, Faculty of Veterinary Medicine of Cáceres, University of Extremadura, Av. de La Universidad S-N, Cáceres 10005, Spain
[3] Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[4] Department of Animal and Food Science, Autonomous University of Barcelona, Cerdanyola del Vallès, Barcelona 08193, Spain

Introduction

Equine gastric ulcer syndrome (EGUS) comprises mucosal diseases of the equine stomach, and specific nomenclature has been used to differentiate diseases affecting the squamous and glandular mucosa: Equine Squamous Gastric Disease (ESGD) and Equine Glandular Gastric Disease (EGGD) [1]. Equine Squamous Gastric Disease involves lesions affecting the squamous mucosa, while EGGD refers to lesions of the glandular mucosa [1]. The highest prevalence of these diseases is observed in performance horses, and the risk of both ESGD and EGGD increased with exercise intensity and duration [2]. The clinical signs of this disease depend on the kind of EGUS (ESGD or EGGD), but it can include colic, weight loss, poor body condition, poor coat condition, reduced appetite, diarrhea, bruxism, behavioral changes and poor performance [1, 3].

The use of saliva as a biological sample to evaluate changes in different analytes related to different organic processes has many advantages, including the fact that it can be obtained non-invasively and with minimal pain or stress to the animal [4]. In horses, saliva samples have been used to evaluate changes in saliva analytes by gel proteomics in healthy and horses treated for EGUS [5], changes in salivary cortisol in horses with abdominal pain [6], changes in salivary calprotectin and aldolase in horses with EGUS and other pathologies [7], and oxidative status biomarkers in the saliva of horses with EGUS and colic of intestinal etiology [8].

In recent years, measurement of oxytocin has been used for stress and welfare evaluation in different species [9, 10]. In experimental studies, it has been described the relationship between oxytocin and gastric function since oxytocin can function as a protective factor against stress-induced changes, e.g. protecting against the negative impacts of immersion of the xiphoid process in cold water on gastric function in rats [11]. Systemically administrated oxytocin possesses gastric antisecretory and antiulcer effects in certain experimentally induced gastric and duodenal ulcers, such as acetic acid-induced chronic gastric ulcer models, in rats and guinea pigs. These effects can be attributed principally to the antisecretory activity of oxytocin [12].

In horses, a few studies have demonstrated changes in oxytocin concentrations in different situations, e.g. salivary oxytocin as a non-invasive indicator of emotional responses in young horses [13] and increased plasma oxytocin levels when a participant gently rubbed the neck and withers of the horse compared to standing beside it without touching it [14]. These reports have used commercial ELISA assays to measure oxytocin in saliva and plasma. AlphaLISA assays, an alternative technology, are immunoassays that use luminescent oxygen-channeling chemistry to amplify the assay signals [15]. These assays have some advantages over commercial ELISA kits, such as a low sample volume and a reduction in washing steps. AlphaLISA assays for the measurement of oxytocin in saliva has been validated in animal species such as pigs, cows, and dogs [16–18].

This study aimed to (1) develop and validate a new immunoassay based on AlphaLISA technology for oxytocin measurement in the saliva of horses and (2) evaluate possible changes in oxytocin concentrations in saliva from horses with EGUS. For this purpose, oxytocin concentrations in saliva of healthy horses and horses with both EGUS forms and only EGGD, only ESGD and horses with other diseases different than EGUS were measured.

Methods

Optimization and validation of AlphaLISA method

For immunoassay development, a monoclonal antibody against oxytocin previously described [17] was used. This method is a direct competition assay that uses AlphaLISA technology (PerkinElmer, (MA, USA)) where acceptor beads coated to the monoclonal anti-oxytocin antibody were used. The assay and standard curve optimization were developed as in previous reports [16, 18].

The inter- and intra-assay coefficients of variations (CVs) were calculated for analytical validation of the assay. To determine intra-assay precision, five replicates of each pooled sample with high, medium and low oxytocin concentrations were analyzed simultaneously. For inter-assay precision, five aliquots of each pool were stored in plastic vials at -80ºC until analysis.

Accuracy was assessed through linearity under dilution and recovery experiments. For the linearity evaluation, two samples were serially diluted from 1:2 to 1:128 with AlphaLISA HiBlock buffer. For the recovery test, varying amounts of commercially available oxytocin-BSA were added to saliva samples with known oxytocin concentrations, and the percentages of the measured versus expected oxytocin concentrations were calculated.

The lower limit of detection (LLD) was determined as the mean plus two standard deviations (SD) of 12 replicated measurements of the AlphaLISA HiBlock buffer. For the lower limit of quantification (LLQ), the lowest oxytocin concentration measurable with less than 20% imprecision was calculated. To achieve this, saliva samples with a known oxytocin concentration were serially diluted from 1:2 to 1:128 with AlphaLISA HiBlock buffer and analyzed five times in a single run for each dilution.

Animals

For the oxytocin measurements, 102 saliva samples were collected from horses diagnosed with EGUS, healthy horses, and horses with other diseases. This included 25

horses with both types of EGUS, 23 with only EGGD, 21 with only ESGG, 14 healthy horses, and 19 horses with other diseases.

All diseased horses were admitted to the Large Animal Teaching Hospital at the University of Copenhagen between February 2022 and March 2023. Horses with EGUS were privately owned and referred for gastroscopy due to the owner's suspicion of EGUS, which was based on behavioral issues (e.g., aggressive behavior), colic, riding issues (e.g., reluctant to move forward, bucking, poor performance), or weight loss. Horses with diseases different from EGUS presented clinical signs compatible with EGUS but were negative on gastroscopy. These horses were further diagnosed with another disease, such as colic (7), enteritis (5), gastric impaction (5), or no specific diagnosis (2) was found.

Healthy horses that were submitted for castration or OPU (Ovum Pick Up) procedures and had gastroscopy as part of their routine pre-surgical protocol were sampled at the Veterinary Teaching Hospital of the University of Extremadura during the same period. These horses were privately owned and considered healthy based on the absence of significant clinical findings in their medical history, clinical examination (heart and respiratory rates, rectal temperature, mucous membrane color, capillary refill time, borborygmi), complete blood count (CBC), and serum biochemistry profile. Healthy horses had an EGUS grading of 0 or 1, indicating intact epithelium without hyperkeratotic areas or glandular lesions.

Gastroscopy

Horses were admitted to the hospital the day before the gastroscopy and were starved for 16 h before the gastroscopy. Gastroscopy images were interpreted by a single experienced veterinarian at each hospital, both with over ten years of experience, according to the guidelines provided by the European College of Equine Internal Medicine (ECEIM) in its Consensus Statement [19]. The evaluators were blinded, and the images were graded in real-time or retrospectively from images stored in a video.

Saliva collection

Horses did not show evident signs of stress before saliva sample collection and did not later overreact to intravenous injections or the restraint. In addition, no sedatives were administered in the stall. The horses were in the stall at least 12 h after transportation.

Saliva samples were obtained from all horses prior to intravenous sedation and gastroscopy, immediately after the horses were positioned in the examination stand [7]. A sponge was used to collect saliva, which was then deposited into a Salivette ® tube. The tubes were kept at 4ºC until transported to the laboratory within a 10-min from the collection. Upon arrival, they were centrifuged at 3000 g for 10 min and then stored at – 80ºC in the period between collection and analysis.

Statistical analysis

The D'Agostino-Pearson normality test was performed to evaluate the distribution's normality, resulting in a non-parametric distribution. Then, the data were log-transformed, and the D'Agostino-Pearson normality test was run again to confirm a parametric distribution. Then, an ordinary one-way ANOVA followed by Tukey's multiple comparisons test was performed to compare oxytocin values obtained in different groups (healthy, both EGUS, EGGD, ESGD, and horses with other diseases). The Spearman correlation was used to correlate salivary oxytocin concentrations with body weight and BCS. A multiple linear regression (least squares) was used to assess whether age, breed, or sex influenced the oxytocin concentrations. In addition, a Spearman correlation was done between EGGD graduation of the lesions at endoscopy and the oxytocin levels, and ESGD graduation of the lesions at endoscopy and the oxytocin levels.

The statistical power of the results $(1-\beta)$ obtained from the previous statistical analysis was calculated by posthoc analysis by the G-Power program to evaluate the risk of a type I error incurred with the number of horses evaluated. The oxytocin concentrations were expressed as mean and standard deviation (SD). The analysis indicated that it is was required a minimum of 32 horses per group to achieve a 0.8 statistical power (1 – ß) using a type I error of $\alpha = 0.05$ in the different group comparatives.

Statistical analysis was performed using GraphPad's commercial statistics package (GraphPad Prism 9 for macOS). Values of $p < 0.05$ were selected to indicate significance for all analyses.

Results

Validation of AlphaLISA method

The assay procedure that resulted after optimization for AlphaLISA method with monoclonal antibody against oxytocin is shown in Fig. 1.

The method showed intra-assay CVs of 11.9–12.9% and inter-assay CVs of 7.8–15.0%. Dilution of saliva samples resulted in linear regression equations with a correlation coefficient between 0.97 and 0.98. The results of the recovery obtained were between 86 and 120%. The assay LLD and LLQ were 21.5 and 31.7 pg/mL, respectively.

Oxytocin concentrations in healthy, both EGUS, only EGGD, and only ESGD horses

Horses with EGUS had a mean age of 11.81 (± 4.95) years and consisted of 29 mares and 40 geldings, representing

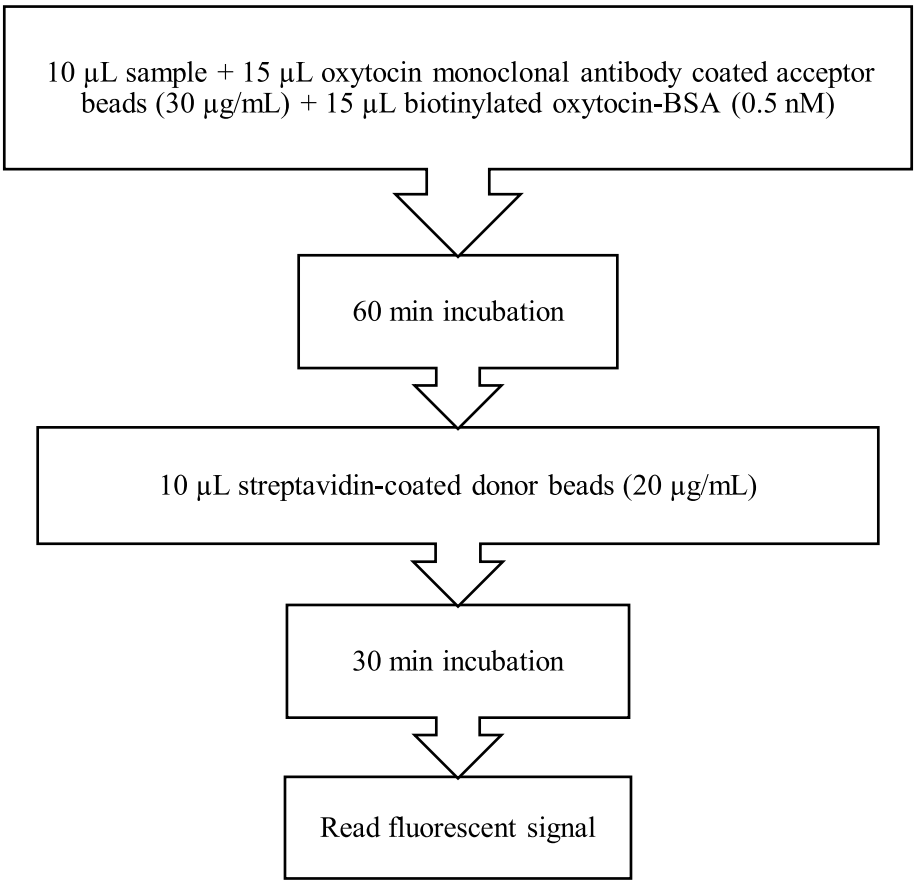

Fig. 1 AlphaLISA protocol for oxytocin measurement in horse saliva

various breeds, primarily Warmblood. Horses of the group with diseases different from EGUS had a mean age of 11 ± 5.64 years and consisted of 11 mares and 8 geldings, composed of different breeds being Warmblood the most represented. Horses of the healthy group had a mean age of 10.45 (± 4.20) years and consisted of 6 mares and 8 geldings, representing various breeds.

Horses with EGUS had a mean body weight of 521.41 ± 93.2 kgs, and a mean BCS, based on a previously reported scale [20], was 5.67 ± 1.19. Horses with diseases different than EGUS had a mean body weight of 478 ± 113 kgs, and a mean BCS of 5.2 ± 0.78. In the healthy group, the mean body weight of the horses was 518.1 ± 87.5 kgs, and the mean BCS [20] was 5.85 ± 1.27.

Salivary oxytocin concentrations in healthy horses (mean: 161.0 ± SD 70.1 pg/mL) were higher compared to horses with both ESGD and EGGD (mean: 100.3 ± SD 94.2 pg/mL, $P = 0.0046$) and to horses with EGGD (mean: 105.6 pg/mL, ± SD 62.8, $P = 0.0398$). Salivary oxytocin concentrations in horses with only ESGD (mean: 164.5 ± SD88.3 pg/mL) were higher compared to horses with both ESGD and EGGD (mean: 100.3 ± SD 94.2 pg/mL, $P = 0.0007$) and horses with EGGD (mean: 105.6, ± SD 62.8 pg/mL, $P = 0.0106$) (Fig. 2). Salivary oxytocin concentrations in horses with other diseases (mean: 140.2 ± SD 16.7 pg/mL) were higher compared to horses with both ESGD and EGGD (mean: 100.3 ± SD 94.2 pg/mL, $P = 0.0039$) and horses with EGGD (mean: 105.6 ± SD 62.8 pg/mL, $P = 0.0420$) (Fig. 2).

No difference was found in saliva oxytocin concentration regarding age ($p = 0.281$), breed ($p = 0.932$) and sex ($p = 0.874$). Further, no correlation between oxytocin concentration and body weight ($p = 0.291$) or body condition score ($p = 0.632$) was found.

No correlation was found between oxytocin concentrations (pg/ml) and ESGD grading in the group of horses with only ESGD ($r = 0{,}271$, $p = 0.232$, $n = 21$). In addition, no correlation was found between oxytocin concentrations and EGGD grading in the group of horses with only EGGD ($r = 0.124$, $p = 0.570$, $n = 23$).

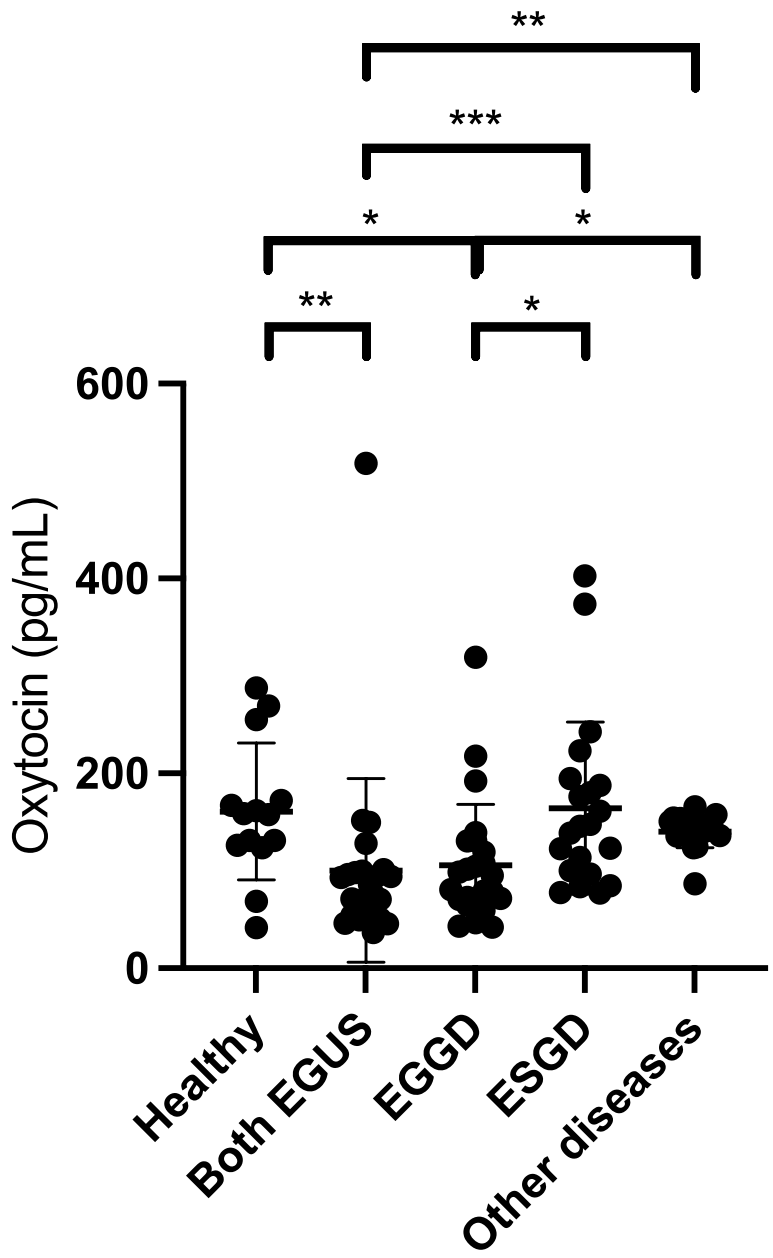

Fig. 2 Saliva oxytocin concentration in healthy control horses, horses diagnosed with both ESGD and EGGD, horses diagnosed with either of the two syndromes, ESGD or EGGD, and horses with other diseases. The groups comprising the EGGD syndrome had a lower saliva oxytocin concentration than control or ESGD horses. Dots represent the individual values and lines represent the mean and SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Discussion

The development and validation of a new method for the detection of oxytocin in equine saliva based on AlphaLISA technology was presented in the present study. The LLD (31.7 pg/mL) and LLQ (50.5 pg/mL) obtained by this method were indicative of a high sensitivity, allowing detection of low concentrations of oxytocin in saliva. This is in accordance with results in previous studies in which salivary oxytocin was measured with a similar method in the porcine, canine and bovine species [16–18]. Two studies in which oxytocin has been measured in the saliva of horses using a commercial ELISA kit previously validated for use in horses reported values in unextracted samples between 50–175 pg/mL [13, 21]. This is in line with the results of the present study. In addition, many of the values of the healthy horses in the present study are

inside the range (117.33 ± 57 pg/mL) of a previous report [17]. The principal advantages of the AlphaLISA method presented in this study are the lack of washing steps and low sample volume (10 µL vs 100 µL of sample requirement with the commercial ELISA kit indicated before), making it an advantage in clinical settings. In addition, the intra- and inter-assay CVs for the AlphaLISA were both below the recommended 20% [22], similar to those previously obtained with pig saliva by AlphaLISA [17]. Furthermore, the linearity under serial sample dilution indicated that the assay can detect oxytocin in an accurate way.

Horses with diseases different from EGUS and horses with ESGD showed higher oxytocin values than the group with only EGGD and both ESGD and EGGD. While the pathogenesis of ESGD is well understood, that of EGGD has not been fully elucidated [1]. However, a higher prevalence of EGGD in horses exposed to potential stressors (e.g. more training days, more caretakers, and not being turned out on pasture) has been found [3, 23, 24]. Based on these studies, stress in different forms has been suggested to play a role in EGGD pathogenesis. One possible mechanism could be that a higher endogenous cortisol release (provoked by stress response) decreases local prostaglandin E production, resulting in decreased mucosal blood flow, decreased mucus production, and increased HCl secretion [25]. The compromised protective factors and regenerative capacity of the glandular mucosa could make the gastric mucosa susceptible to ulceration. Overall, there is a relation between high levels of oxytocin and wellbeing [26] and low levels of oxytocin measured in both saliva and plasma has been related to emotional stress in the horse [13, 27]. Horses showing behavioral signs of discomfort during training had a lower oxytocin response than horses with no such signs [13]. Also, in humans, low oxytocin levels can favor the appearance of stress since oxytocin release can have an anti-stress effect [28] and be a protective factor against stress-induced changes to gastric function [11].

Oxytocin has also direct antiulcer effects, possibly related to an antihistaminergic mechanism [29] or its protective role in case of gastric ischemia [30]. In this line, oxytocin has vasodilator effects and, therefore, could protect the glandular mucosae from ulcers in cases of inhibition of vasodilator prostaglandins; mechanism involved in the production of gastric ulcers due to NSAIDs administration [31, 32]. It is interesting to note the high values of oxytocin in saliva that horses with diseases different from EGUS had compared to horses with EGGD and both EGGD and ESGD. This could reinforce the possible involvement of the decrease of oxytocin in saliva in the pathogenesis of EGGD. Although it could be postulated that oxytocin would decrease associated with the disease, in other animal species, such as pigs, in some situations of pain or infection, increases in oxytocin have been described as a probable compensatory mechanism [33]. As such the higher oxytocin observed in the group with diseases different from EGUS might be indicative of a direct protective effect independent of stress.

In addition to the hypothesis of the role of oxytocin in the development of EGUS, the idea that EGUS results in a stress response that might cause changes in oxytocin should also be considered as a hypothesis. Horses with severe grades of EGGD have an exaggerated response to ACTH, measured by saliva cortisol [34, 35]. Further, a previous report [36] indicated that horses with EGGD were more stress-sensitive to a novel object test than horses without EGGD. This hypothesis could be supported by the fact that ESGD, which is caused mainly by management factors independent of stress, can lead to changes in hair cortisol [37].

In the present study, no difference regarding age, sex or breed for saliva oxytocin measurement was found. This is in accordance with a previous study, which similarly found no difference in plasma oxytocin concerning age, sex or breed [38].

The main limitation of this report is the population differences between diseased and healthy horses since they are from two different countries with probably different management systems. The lack of equivalence between populations is a major weakness of the study design, and this design flaw might have influenced results since the differences observed in oxytocin between healthy and horses with EGUS could be related to a confounding factor at a population level. In addition, the minimum required sample size per group was set at 32 individuals to reach a 0.8 statistical power, and since in our study we did not reach this number, the interpretation of the results could be affected by it. Therefore, this should be considered as a pilot study, and further trials with a homogenous population and a large number of animals should be conducted to confirm the reported findings. In addition, it would be of interest to evaluate the potential of this marker to monitor the EGUS evaluation and efficiency of treatments.

Conclusions

The present study validated a new assay that can measure oxytocin in saliva in horses in a precise and accurate way. In addition, although there was a large degree of cross-over between the data, horses with EGGD and mixed EGGD and ESGD had lower oxytocin concentration measured in saliva than horses with ESGD, healthy horses and horses with other diseases. The heterogeneity of the population between the diseased and healthy

horses and the relatively low number of individuals used indicated the need for additional research to elucidate and clarify the role of oxytocin in EGUS.

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Authors' contributions
M.L.-A., J.J.C. and X.M. were working on funding, preparation of the manuscript and conception, M.B., A.M., S.M.-S., M.M.-C. and S.H. were working the investigation, M.L.-A. and A.M. were working on the preparation of the manuscript and prepared the figures and tables. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Murcia University Ethics Committee (protocol code CEEA 288/2017, approval date: 13 January 2016) and by the local committee of the Large Animal Teaching Hospital of the University of Copenhagen (protocol code #2020–020, approval date: 1 January 2020 and # 2024–001, approval date: 1 January 2024). Informed consent from owners was obtained to use the horses in the current study.

Consent for publication
All authors have approved the manuscript and agree with its publication.

Competing interests
The authors declare no competing interests.

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