



Association between the levels of serum cortisol and the presence of gastric ulcers in working mules

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ABSTRACT

The relationship between stress and gastric ulceration has been described in laboratory animals and human peptic ulcer disease. In horses, high levels of cortisol have been associated with ulcerations in the glandular mucosa but not in the squamous mucosa. However, currently, there are no data on gastric ulceration and cortisol concentrations in working mules. The objective of this study was to assess the association between serum cortisol levels and the presence of ulcers in both types of gastric mucosa. Blood samples of 97 clinically healthy mules of both sexes, with an average age of 8.7 ± 4.4 years, mean body weight (BW) of 290.5 ± 37.6 kg, and a body condition score (BCS) of 5 ± 0.8 , were subjected to a gastroscopic study to evaluate and classify the lesions found in both gastric mucosa. In addition, blood samples were taken to measure the concentration of serum cortisol through commercial sandwich ELISA kit (AccuBind®, Monobind Inc., CA, USA). Mule gastric ulcerative syndrome (MGUS) was identified in 44.6%, 27.8% and 19.6% for mule squamous gastric disease (MSGD) and mule glandular gastric disease (MGGD), respectively. The mean cortisol concentration was 10.59 ± 4.06 µg/dL, with very similar concentrations between the ulcerated and non-ulcerated mule groups. The association analysis did not show statistical significance between the serum cortisol and the degree of ulceration in either type of gastric mucosa, differing from what has been reported in horses, possibly due to adaptive differences that these hybrids may have when compared to other equids.

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Introduction

The presence of Mule Gastric Ulcerative Syndrome (MGUS) was recently found in 44.6% of the population that carries out agricultural work (Calixto and Martínez-Aranzaes, 2024). Being an initial figure for the species, which, in the absence of information on MGUS, were extrapolated from the reports of equines and donkeys, which, unlike mules, have been widely studied in different breeds around the world.

The importance of studying this species is due to the multiple activities to which they are subjected in many countries. The characteristics of resistance, rusticity, and stoicism anecdotally attributed to mules differentiate them and make them preferential for some work in relation to other equids. The clinical characterization and predisposing factors in each of the gastric mucosa in equids

experiencing stressors should motivate the proper and current use of welfare concepts (Sykes et al., 2015).

Equine gastric ulcer syndrome (EGUS) is considered multifactorial, where factors both inherent to equines and exploitation and management systems have been identified according to the activities they carry out (Lorenzo-Figueras and Merritt, 2002; Bell et al., 2007; Orsini et al., 2009; Freymond et al., 2015). Factors, such as prolonged fasting, stress, exercise, housing, type and frequency of feeding, medications, and microbiome, among others, have been identified as inducers of this syndrome (Martínez and Silveira, 2014; Pedersen et al., 2018; Padalino et al., 2020). With respect to this research and their work purpose, mules are subjected to long hours and prolonged periods of fasting, taking advantage of the resistance and abilities of the species, these being the main generators of stress that could possibly be involved in the pathophysiology of MGUS. Follow-up studies have been carried out in other equids but not mules.

Stress induced by various activities derived from the management systems and intensity of work or sports training has been related to the presence of ulcers in both the glandular gastric mucosa and squamous mucosa of horses (Sykes and Jokisalo, 2014; Sykes

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et al., 2019). Such stress can generate neuroendocrine imbalances, such as increased production of gastrin and histamine, which translates into greater production of hydrochloric acid, having a caustic effect on the squamous mucosa, while high levels of cortisol, could alter the protection mechanisms of the gastric glandular mucosa. However, there is a lack of clarity on the effect of the stress factor in the dynamics of injury to the gastric mucosa of mules, which are extremely exposed to ulcerogenic aspects identified for equines and donkeys.

Cortisol is frequently used as a marker of stress, absence of good welfare, predictor of disease, and indicator of athletic conditioning in horses for sport (Broom, 2006; Freymond et al., 2015; Zulúaga and Martínez, 2017). It has been measured in various substrates such as blood, saliva, sweat, tears, feces, and hair (Nagel et al., 2012; Bohák et al., 2013; Monk et al., 2014; Nuñez et al., 2014; Mönki et al., 2016; Bonelli et al., 2019).

Recently, interest has been shown in understanding the role of the cortisol level in the pathogenesis of ulcerative lesions in equine gastric glandular disease (EGGD) and equine gastric squamous disease (ESGD), within EGUS (Sykes et al., 2019). Therefore, this work is aimed to assessing the association between serum cortisol levels and the presence of ulcers in both gastric mucous of mules dedicated for agricultural work.

Materials and Methods

Mules of study

A population of 97 clinically healthy mules, of both sexes, 93 with an average age of 8.7 ± 4.4 years, BW of 290.5 ± 37.6 kg, and a BCS of 5 ± 0.8 (Henneke et al., 1983), used in agricultural work (riding, pack, and ranch mules) for long hours per day (h)/week (3–11 h/3–7 d), managed in pastures, and were fed based on by-products of sugarcane, forage, and ad-libitum access to water, which were supplied at the end of each work activity.

Blood samples were collected and an endoscopic examination was completed. The mules who had received treatments based on antibiotics and steroidal and non-steroidal anti-inflammatory drugs or for gastric ulcers, in the last 6 months, were excluded.

Sampling and determination of cortisol concentration

Strict cleaning and antisepsis measures at the jugular vein venipuncture site preceded the collection of blood samples in a vacuum tube without additives in the morning period (7:00 am–11:00 am). The samples were centrifuged at 1500 rpm for 10 minutes to obtain the serum fraction and subsequently separated in Eppendorf tubes and frozen at -20°C until analysis.

The analysis of the serum cortisol concentration (SCC) was obtained through the use of the commercial sandwich ELISA kit (AccuBind®, Monobind Inc., Lake Forest, CA, USA) (Calixto and Martínez, 2021). ELISA plates were analyzed on a conventional wavelength reader at 450–630 nm (Stat Fax 303® Plus Microstrip Reader, Awareness Technology Inc., Palm City, FL, USA). Validation of the kit was performed using six kit calibrators and three commercial cortisol controls: Multi-ligand control A, 7.7 ng/ml; Multi-ligand control B, 97.4 ng/ml; and Multi-ligand control C, 193.2 ng/ml (QSure® Multi-Ligand Control Tri-Level, Monobind Inc.). Concentrations were expressed in ng/ml, and the cortisol AccuBind® ELISA Test System has a sensitivity of 3.7 ng/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainly) statistic to calculate the minimum dose. This range was validated with known concentrations. This test was compared with a coated-tube radioimmunoassay method. Biological specimens from low, normal, and high cortisol level populations

were used. The values range from 4 to 950 ng/ml according to information from the manufacturer.

Gastroscopic examination

After a fasting period of 12 and 4 hours for solid and 117 liquid food, respectively, the mules were sedated with 10% xylazine (1.3 mg/kg/IV). A flexible video endoscope (PortaScope®, 1800PVS, Bradenton, FL, USA) 300 cm long and 12 mm in external diameter was inserted via the nasal route; once located in the stomach, this organ was inflated with air and flushed with jets of water through the working channel to improve the visual field and to be able to properly evaluate the entire gastric mucosa. The endoscopic evaluation included inspection of the cardia, greater and lesser curvature, *margo plicatus*, pyloric antrum, pylorus, and anterior segment of the duodenum.

The findings in each mucosa were recorded and classified according to Andrews and Nadeau (1999) and the European Consensus for EGUS (Sykes et al., 2015), separately for ESGD and EGGD, with grades ranging from 0 to 4.

Statistical analysis

This was a descriptive cross-sectional study. The gastroscopic findings data were evaluated using the Kolmogorov-Smirnov test using the SAS® statistical program and Microsoft Excel® (Microsoft Office 2013®, Microsoft Corporation, WA, USA). Parametric data were reported as mean \pm standard deviation. Pearson's chi-square test and odds ratio were used to evaluate the level of association between cortisol concentration and MGUS. A significance level of $P \leq 0.05$ was considered.

Results

A total of 65 male mules (67%) and 32 female mules (33%) were studied, and all were clinically healthy at the general examination and clinical history of the last 6 months, as described by the owners. For mules without ulcers, 44.6% (46/97) of the animals presented MGUS, with 27.8% and 19.6% affected by MSGD and MGGD lesions, respectively. The SCC of these mules was 10.59 ± 4.06 $\mu\text{g/dL}$ (range: 144, 0.68–24.82 $\mu\text{g/dL}$), with similar concentrations between the ulcerated and non-ulcerated mules groups (Figure 1).

The association analysis between the variables, presence of ulcers, and SCC, for both gastric mucosa is shown in Table. Finally, the cortisol concentration is compared in each of the severity grades of the ulcers in MGGD and MSGD, respectively (Figures 2 and 3).

Discussion

The mules that were objects of study engaged in strenuous agricultural work days, as is usual in many countries. There is an absence of information on epidemiological aspects and diseases of this species. The frequency of MGUS presentation was lower than that reported in horses, despite the presence of predisposing factors identified for the other equids. This result indicates the possibility of differences or additional aspects related to management systems or racial particularities relevant to the mules' response that are still to be studied.

Among the predisposing factors for gastric ulcers in horses and donkeys are the intensity of exercise, prolonged fasting, concentrate-based diets, social isolation, anti-inflammatories, and bacterial flora, among others (Sykes et al., 2015; Scheidegger et al., 2017). Likewise, ulcer protective factors include pasture management and conditions close to the natural life of these animals (Nadeau et al., 2000). In this context, the mules worked shifts of up to 11 h/d/wk, considered high intensity, together with long periods of fasting from solid and liquid

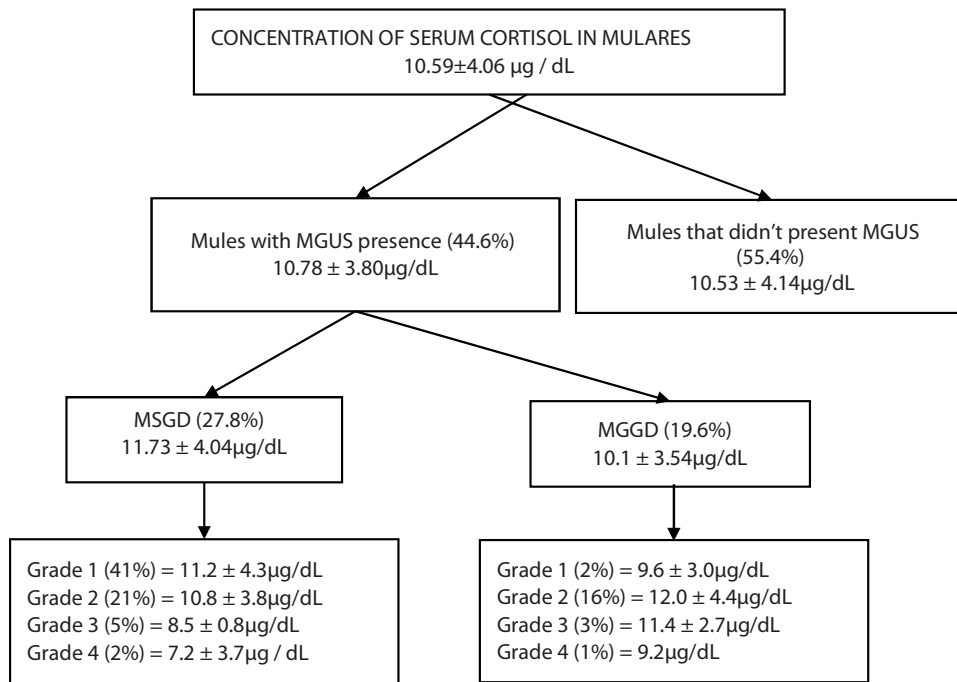


Figure 1. Percentage distribution of gastroscopic findings in both gastric mucosa of mules (mean ± standard deviation) (MSGD, MGGD), with their respective concentration of serum cortisol. MGGD, mule glandular gastric disease; MSGD, mule squamous gastric disease.

Table

Association analysis between serum cortisol concentration and MGGD and MSGD, of the mule population dedicated to agricultural work.

	Chi square	P-value	Odds ratio	P-value
MGGD	90.6	0.431 ^a	90.4	0.438 ^a
MSGD	89	0.464 ^a	106.4	0.101 ^a

MGGD, mule glandular gastric disease; MSGD, mule squamous gastric disease.

^a No statistical difference (*P* > 0.05).

foods, which were classified as the main ulcerogenic factors for this population. Gastroprotective factors were also observed, such as group management in paddocks, a fiber-based diet (by-products of sugarcane), and the absence of the use of medications before gastroscopic evaluation.

Due to the variety of predisposing factors, differences in the defense mechanisms of both gastric mucosa and discrepancies in presentation, ESGD and EGGD were considered separately within

EGUS (Sykes et al., 2015). The inducing factors and pathophysiological pathways for ESGD are well characterized (Sykes and Jokisalo, 2015a), in contrast to EGGD (Sykes and Jokisalo, 2015b; Mönki et al., 2016; Banse and Andrews, 2019). However, some factors remain controversial within the genesis of gastric ulcers, as is the case of stress generated by activities opposed to welfare or derived from physical activities (Lorenzo-Figueras and Merritt, 2002), such as work routines in difficult fields without access to food and water for long hours, which directly and indirectly can lead to the fragility and disruption of gastric surfaces and eventual formation of ulcers depending on the intensity and time of activity (Tamzali et al., 2011; Malmkvist et al., 2012). In mules, despite identifying the presence of stress, their involvement in MGUS is unknown.

The relationship between stress and gastric ulceration has been described in laboratory animals and human peptic ulcer disease (Malfertheiner et al., 2009). Likewise, high sensitivity of adrenocorticotropic hormone (ACTH) has been determined in pigs subjected to chronic stress as well as in horses with EGGD, which have



Figure 2. Relationship between serum cortisol concentration and degrees of ulceration in MGGD.

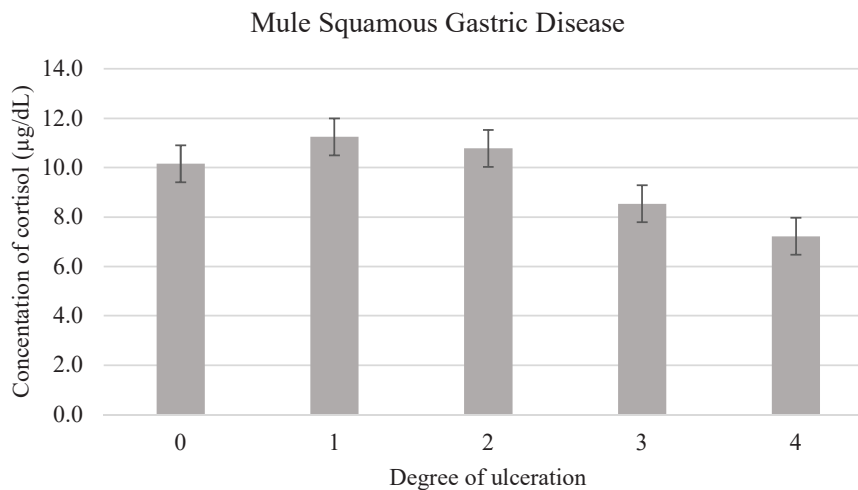


Figure 3. Relationship between serum cortisol concentration and degrees of ulceration in MSGD.

shown high levels of cortisol in feces and saliva (Malmkvist et al., 2012; Scheidegger et al., 2017; Bonelli et al., 2019), indicating that stress is an ulcerogenic factor of the glandular mucosa, which is contradictory in ESGD when determining a negative correlation with the concentration of cortisol in hair (Prinsloo et al., 2019). The foregoing could not be statistically verified in this study, but a behavior similar to that reported by Prinsloo et al. (2019) may have been manifested (Figure 3).

Unlike the study by Prinsloo et al. (2019), this work used blood cortisol, since hair cortisol concentrations indicated long-term stress (Duran et al., 2017). However, cortisol concentrations were similar across conditions, whereas the mules with a higher degree of MSGD (Figure 1) only 7% had a lower concentration, possibly indicating chronic stress, which can cause inhibition of cortisol production (Alexander and Irvine, 1998). In addition, this population of mules carried out strenuous work that generated various stressors. More studies are required to validate this negative association between these two variables, because the sample size in this study of the number of individuals with higher degrees of MSGD was relatively small.

Regarding this, there is evidence of the high correspondence between cortisol levels and ulcers in the glandular mucosa in horses (Malmkvist et al., 2012; Scheidegger et al., 2017). However, this association was not found in mules with MGGD. This result could have been due to the low number of animals with MGGD in high degrees of severity and to the particularities of the species. However, variability in cortisol concentration has also been reported due to various factors, depending on the test used. There has been reported overlap in the ACTH sensitivity test between healthy and ulcerated patients (Scheidegger et al., 2017).

In contrast to the association of the level of acute and chronic stress with the degrees of ulcers in both gastric mucosa of the equines, previous studies have shown a low presence of gastric ulcerative lesions in mules subjected to risk factors, classified as ulcerogenic in other equids (Alexander and Irvine, 1998; Tamzali et al., 2011; Sykes et al., 2015), due to the high level of stress they generate.

Different equids may have different adaptive responses. Studies should consider the effects of the circadian cycle on cortisol measures, since the response of this analyte depends on the time of exposure to stress factors, potentially differentially affecting the gastric mucosa.

Conclusions

Under the conditions of this study, no association was determined between serum cortisol levels and the degrees of ulceration of both gastric mucosa, in contrast to what was reported in

horses. These data suggest that the adaptation response to stress factors is different, due to the low frequency of MGUS presentation, in the face of exposure to stress factors derived from extreme working conditions.

Authors' contribution

The idea for the study was conceived by José Martínez-Aranzales. The data collection and analysis were designed by Lady Calixto-Vega and José Martínez-Aranzales. The article was written by Lady Calixto-Vega, with contributions from Jose Martínez-Aranzales.

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Ethical Considerations

This study was carried out with authorization and completion of an informed consent by the mule owners, and approved by the Ethics Committee for Animal Experimentation of Universidad de Antioquia (protocol No. 1222019).

Conflict of Interest

The authors declare no conflict of interest.

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