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Expression of Cyclooxygenase Isoforms in Equine Gastric Ulcers

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EXPRESSION OF CYCLOOXYGENASE ISOFORMS IN EQUINE GASTRIC ULCERS

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RÉSUMÉ

Le syndrome d'ulcère gastrique équin est une maladie complexe qui affecte des chevaux adultes. Sa prévalence peut atteindre 90 % chez les chevaux de course. Bien que plusieurs théories aient été proposées, la pathophysiologie de ce syndrome demeure inconnue. Le rôle de la cyclooxygénase dans la protection de la muqueuse gastrique a été étudié chez plusieurs espèces, mais pas chez le cheval. L'objectif de cette étude était de caractériser l'expression de la COX-1 et de la COX-2 dans les ulcères gastriques équins. Dix échantillons de muqueuse gastrique non glandulaire équine normale et 38 échantillons de muqueuse ulcérée ont été prélevés post-mortem. Un spécimen de chaque échantillon a été analysé par immunohistochimie et un autre par immunobuvardage. Les anticorps MF241 et MF243 dirigés contre la COX-1 et la COX-2, respectivement, ont été utilisés. L'immunoréactivité a été évaluée avec un système de notation de 0 à 3. L'expression des isoformes de la cyclooxygénase a été confirmée par immunobuvardage. Tous les échantillons de muqueuse normale exprimaient fortement la COX-1, alors que 80 % n'exprimaient pas la COX-2. L'expression de COX-1 et COX-2 était variable dans les échantillons de muqueuses ulcérées. L'expression de la COX-1 était significativement inférieure tandis que l'expression de la COX-2 était significativement supérieure dans la muqueuse gastrique ulcérée comparativement à la muqueuse normale ($p < 0.0001$). Une expression plus élevée de COX-2 dans les ulcères gastriques du cheval suggère un rôle possible pour cette enzyme dans leur guérison. L'utilisation des inhibiteurs COX-2 spécifiques en guise d'agents anti-inflammatoires chez les chevaux devrait donc être faite avec prudence.

Mots clés : Ulcères gastriques, estomac, cyclooxygénase-1, cyclooxygénase-2, cheval.

SUMMARY

The equine gastric ulcer syndrome is a complex disease which affects adult horses. Its prevalence can reach up to 90% in racehorses. Although several theories have been proposed, the pathophysiology of this syndrome remains unknown. The role of cyclooxygenase in the protection of the gastric mucosa has been studied in several species, but not in the horse. The objective of this study was to characterize the expression of COX-1 and COX-2 in equine gastric ulcers. Ten samples of normal equine nonglandular gastric mucosa and 38 samples of ulcerated mucosa were obtained post-mortem. One specimen of each sample was analyzed by immunohistochemistry and another one by immunoblot. Antibodies MF241 and MF243 directed against COX-1 and COX-2, respectively, were used. The immunoreactivity was evaluated by a scoring system from 0 to 3. Expression of the cyclooxygenase isoforms was confirmed by immunoblot. All normal mucosal samples strongly expressed COX-1, whereas 80% did not express COX-2. The expression of COX-1 and COX-2 varied considerably in the ulcerated mucosal samples. COX-1 expression was significantly lower while COX-2 expression was significantly higher in the ulcerated gastric mucosa than in the normal mucosa ($p<0.0001$). A greater expression of COX-2 in equine gastric ulcers suggests a possible role for this enzyme in their healing. The use of specific COX-2 inhibitors as anti-inflammatory drugs in the horse should therefore be made cautiously.

Key words: Gastric ulcers, stomach, cyclooxygenase-1, cyclooxygenase-2, horse.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
bFGF	Basic fibroblast growth factor
C	Cardia
COX	Cyclooxygenase
COXIBs	Selective COX-2 inhibitors
COX-1	Cyclooxygenase-1
COX-1b	Cyclooxygenase-1b
COX-1v	Cyclooxygenase-1 variant
COX-1$^{-/-}$	Cyclooxygenase-1 knockout
COX-2	Cyclooxygenase-2
COX-2$^{-/-}$	Cyclooxygenase-2 knockout
COX-3	Cyclooxygenase-3
DAB	Diaminobenzidine tetrahydrochloride
DFU	5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furan one
DNA	Deoxyribonucleic acid
ECL	Enterochromaffin-like
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factors
EGUS	Equine gastric ulcer syndrome
F	Fundus
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
GIT	Gastrointestinal tract
GL	Glandular mucosa
HCl	Hydrocloric acid
HGF	Hepatocyte growth factor
LPS	Lipopolysaccarides
LT	Leukotriene
MP	*Margo plicatus*
mRNA	Messenger ribonucleic acid

NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
O_2	Oxygen
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PGA_2	Prostaglandin A_2
PGD_2	Prostaglandin D_2
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
RT	Room temperature
TGF-α	Transforming growth factor
TX	Tromboxane

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INTRODUCTION

Gastric ulceration is a frequent condition in horses; it has been associated with feeding practices, stress and intensive training or exercise. The exact impact of this disease on athletic performance has not been assessed; however it is believed that gastric ulceration results in performance decline. Along with medical treatment costs, this loss of performance could lead to important economical losses to racehorse owners.

Understanding the pathogenesis of equine gastric ulcer syndrome (EGUS), as well as the major players involved in its healing process, could lead to a solution to prevent or control this disorder.

Prostaglandins protect the gastric mucosa against injury from noxious agents; they are involved with the gastric mucosal defence barrier in numerous ways. Cyclooxygenase (COX) is the core enzyme responsible for prostaglandin synthesis, and its role in gastric ulceration has been studied in several species. It was suggested that cyclooxygenase-2 (COX-2), one of the cyclooxygenase isoforms, had an important role to play in ulcer healing. In order to examine the potential role of COX-2 in EGUS, it is necessary to identify the expression of this enzyme in the gastric mucosa of horses under physiologic and ulcerated conditions. Developing a transendoscopic technique for biopsying the equine stomach multiple times, *in vivo*, without danger to the study subject would be a wonderful tool for achieving this goal.

LITERATURE REVIEW

Chapter 1 – The Equine Stomach

1.1 Anatomy and Histology of the Equine Stomach

In contrast with the rest of the equine gastro-intestinal system, the stomach, which has a volume of 8 to 15 liters, is very small. The entry of the stomach is the cardia; the exit is the pylorus (Picavet 2002). Unlike most domestic species, the horse's stomach is lined by two different mucosal types, separated by the *margo plicatus*. The nonglandular or squamous mucosa, where ulcers typically form in adult horses, is a stratified epithelium that consists of four histological layers and is located in the upper compartment of the stomach. The glandular mucosa is found in the stomach's lower compartment and is composed of gastric glands and mucus-secreting cells. The first is considered an extension of the esophagus and has no glandular structures, while the latter secretes mucus, hydrochloric acid (HCl) and pepsinogen (The Equine Gastric Ulcer Council 1999).

The nonglandular mucosa's histological layers are the stratum corneum, which is formed by several levels of cornified pyknotic cells; the stratum transitionale, with its large round nucleated cells; the stratum spinosum, with smaller oblong cells; and the stratum basale, which is composed of cells of cuboidal shape (Argenzio 1999).

The gastric glands of the glandular portion of the equine stomach are composed of six cell types, each one releasing a different substance. Parietal cells release HCl; zymogen chief cells release pepsinogen; D-cells release somatostatin; mast cells release histamine; surface mucus cells secrete mucus and enterochromaffin-like (ECL) cells release histamine as well as serotonin (Garner, Flemstrom et al. 1984; The Equine Gastric Ulcer Council 1999).

1.2 Physiology of the Equine Stomach

Mature horses' stomachs can secrete 1.5 liters of gastric juice per hour with an acid output range between 4 to 60 mmol of HCl hourly (Campbell-Thompson 1989). The gastric pH ranges from 1.5 to 7.0, depending on the region where it is measured. The dorsal portion of the oesophageal region can reach a neutral pH, while regions close to the *margo plicatus* present a pH between 3.0 and 6.0 and the region near the pylorus on the glandular mucosa has a pH ranging from 1.5 to 4.0 (Murray and Grodinsky 1989) (Figure 1).

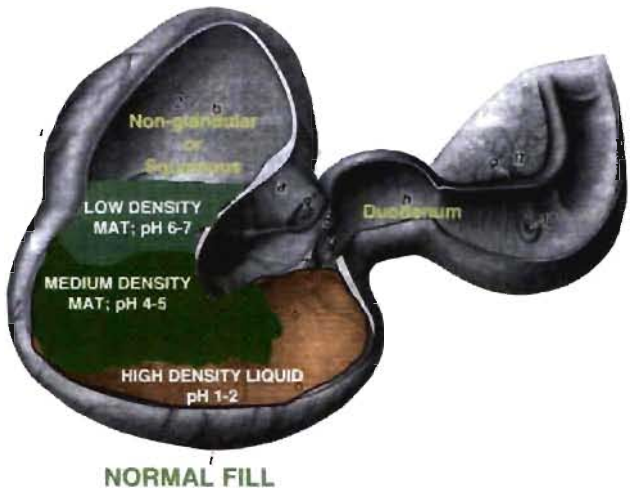

Figure 1: Illustration of the Equine Stomach Fill and Contents pH

(Merritt 2003).

Contrary to many species that can adjust hydrochloric acid secretion based on food ingestion, acid is continuously secreted in the equine stomach independently of food intake (Campbell-Thompson and Merritt 1987; Murray 1997; Merritt 1999; Herdt 2007).

This is likely due to the fact that horses are constant grazers and would eat uninterruptedly if allowed access to feed 24 hours per day (Herdt 2007). Nowadays, however, this aspect of equine physiology can play a role in gastric injury, since horses are fed only twice a day, in most regimens, making the squamous lining of their stomach prone to acid grievance.

Through the buffering effects of increased saliva production, feeding helps to increase gastric pH (Murray and Schusser 1993). Gastric emptying of a hay meal occurs in 24 hours, whereas the same process for a liquid meal can be as fast as half an hour (Healy, Lawrence et al. 1993). This may be important since roughage meals are considered buffers for gastric acidity. This particular aspect of the horse's gastric physiology explains why, nowadays, horses that are fed only twice a day can develop gastric injuries, especially to the squamous portion of their stomach, due to constant acid exposure. In order to avoid injuries or digestion of the gastric mucosa by acidic gastric juices, different mechanisms of mucosal defence have evolved between the glandular and nonglandular mucosa.

1.3 Mechanisms of Glandular Mucosal Defence

Although high concentrations of HCl and pepsin are present in the stomach, injury to the glandular gastric mucosa does not often occur due to mucosal barrier protection mechanisms (Sjaastad, Hove et al. 2003). Prostaglandin E_2 plays an important role in the glandular gastric defence system, promoting bicarbonate and mucus secretion, suppression of HCl secretion, maintenance of intercellular tight gap junctions and adequate mucosal blood flow (Miller 1983; The Equine Gastric Ulcer Council 1999). Gastric mucosal cells will secrete bicarbonate in response to an increase in acid concentration, mechanical irritation and production of endogenous prostaglandin. Regardless of the acidic pH of the luminal surface, the adherence of bicarbonate generates a neutral pH at the mucosal surface (Murray 1999). Viscous hydrophobic mucus secreted by surface mucous cells also adheres to the mucosal surface to prevent

damage from contact with acid and pepsin. Mechanical damage is minimized since the mucus acts as a lubricant as well (The Equine Gastric Ulcer Council 1999).

The tight junctions between gastric epithelial cells also prevent hydrogen ions from diffusing amid them (Sjaastad, Hove et al. 2003). Also, adequate mucosal blood flow is imperative in order to remove hydrogen ions that have back diffused into the mucosa from the lumen and to supply oxygen and nutrients to sustain the high cellular metabolic and regenerative activity (Sorbye and Svanes 1994; Wallace 2001; Crawford and Kumar 2003).

Epidermal growth factors (EGFs) are also major players of the glandular mucosal defence system. They are secreted by the salivary gland and modulate the rapid cell turnover within the stomach by the means of deoxyribonucleic acid (DNA) synthesis promotion and proliferation of gastric mucosal cells (Jeffrey, Murray et al. 2001). EGFs ensure that glandular gastric cells have a life-span as short as 2 to 3 days, allowing damaged surface cells to be replaced by new ones before any further harm can be done to the mucosa (Sjaastad, Hove et al. 2003). EGFs' role is not restricted to epithelial cell restitution, since they also inhibit the secretion of HCl by parietal cells and are involved in prostaglandin synthesis (The Equine Gastric Ulcer Council 1999). All of the above mentioned mechanisms work together in order to protect the glandular gastric mucosa from pepsin and acid assault.

1.4 Mechanisms of Nonglandular Mucosal Defence

The nonglandular or squamous mucosa is known to have restricted mechanisms of defence. Acid repulsion and intracellular buffering are the most important ones (Murray 1999; The Equine Gastric Ulcer Council 1999). The squamous epithelium has no mucus-secreting glands (Herdt 2007), and the lack of alkaline mucus coating on the nonglandular epithelium surface is thought to partially explain why this tissue is prone to ulcer development. However, recent studies have raised the possibility of the presence of

surface mucus on this epithelium (Murray and Mahaffey 1993; Bullimore, Corfield et al. 2001), although its role or physical properties have not been elucidated to date.

Another study has proposed that surfactant may exist on the equine nonglandular mucosa in the form of osmiophilic phospholipid material and could be an additional physical barrier to back-diffusion of acid where other defence mechanisms are absent (Ethell, Hodgson et al. 2000). Because of the paucity of defence mechanisms, the best protection of the equine nonglandular mucosa from peptic injury remains its limited exposure to acidic gastric juices.

Chapter 2 – Gastric Ulcers

2.1 Equine Gastric Ulcer Syndrome

Equine Gastric Ulcer Syndrome or EGUS is a widespread disease complex that affects adult horses (Pagan 1997; Picavet 2002). The high prevalence illustrated by different authors in active horses ranges between 40%, in western performance horses (Bertone 2000), to greater than 90%, in Standardbreds in active race training (Ferrucci, Zucca et al. 2003; Roy, Vrins et al. 2005). In adult horses, ulcers are typically formed in the squamous mucosa alongside the *margo plicatus*, nevertheless they can also be found in the glandular and pyloric areas (Andrews and Nadeau 1999; Dionne, Vrins et al. 2003; Bezdekova, Jahn et al. 2007). Most affected horses are asymptomatic, although some symptoms may be decreased appetite, weight loss, rough hair coat, loose faeces, diminished performance and slight or recurrent colic (Murray 1999; The Equine Gastric Ulcer Council 1999). The pathogenesis of this syndrome has yet to be elucidated and many theories have been proposed which will be discussed further in this chapter.

In equine practice, the diagnosis of EGUS is routinely made by assessing treatment response. Although some have been proposed (O'Conner, Steiner et al. 2004; Hewetson, Cohen et al. 2006; Taharaguchi, Nagano et al. 2007), there are no validated laboratory

tests available to confirm the diagnosis of equine gastric ulcers to date. The only trustworthy method for diagnosing EGUS is therefore gastroscopy.

The ulceration process of the squamous gastric mucosa follows a complex sequence of events. Reddening of the epithelium is the first change observed endoscopically. Histologically this change appears as congested capillaries in the *lamina propria* and epithelium. Prolonged contact of the nonglandular mucosa to HCl then leads to denudation of epithelial superficial layers. When this injury extends to the basal epithelial cells, erosion occurs. If the erosion deepens into the *lamina propria*, the lesion becomes an ulcer. Most of the time ulcers do not extend to the muscularis mucosa (Murray 1994; Murray and Eichorn 1996).

Several grading systems have been used with the intention of evaluating gastric lesion severity (MacAllister, Andrews et al. 1997; Andrews, Reinemeyer et al. 2002). Among these, the most accepted and used nowadays is the one proposed by the Equine Gastric Ulcer Council, which assigns scores from 0 to 4, where 0 corresponds to intact mucosa and 4 to broad, deep lesions (The Equine Gastric Ulcer Council 1999; Bell, Kingston et al. 2007).

The purpose of EGUS treatment is to lower stomach acidity in order to provide an environment where ulcers can heal (Buchanan and Andrews 2003). Although many therapeutic regimens have been employed to treat gastric ulcers in horses, it has been shown by many studies that the most efficient agent is the proton ion pump inhibitor, omeprazole (The Equine Gastric Ulcer Council 1999; Buchanan and Andrews 2003; Orsini, Haddock et al. 2003; Andrews, Frank et al. 2006).

2.2 Comparative Pathogenesis of Gastric Ulcers

Ulcers occurring in the equine gastric squamous mucosa are comparable to gastro-esophageal reflux disease (GERD) in humans and porcine gastro-esophageal ulcer

disease, since, as in the oesophagus, the gastric squamous mucosa lacks developed intrinsic mucosal protective factors (The Equine Gastric Ulcer Council 1999; Murray, Eichorn et al. 2001).

GERD is caused by excessive exposure of the esophageal epithelium to refluxed gastric contents, and it occurs mainly because of hiatal hernia or lower esophageal sphincter incompetence that permit free reflux to occur (Hunt 1999).

Porcine gastric ulcers occur primarily in the nonglandular portion of the stomach, called the *pars oesophagea*, and result from risk factors including dietary particle size, gastric fluidity, feed carbohydrate content, and existence of commensal gastric organisms responsible for dietary carbohydrate fermentation. The role of *Helicobacter sp.* in the development of porcine gastric ulcers has yet to be defined (Doster 2000).

2.3 Pathogenesis of Equine Gastric Ulcer Syndrome

EGUS results from an imbalance between mucosal aggressive and defensive mechanisms (Andrews and Nadeau 1999). Because mucosal protective factors are more developed in the glandular than in the squamous mucosa, the mechanisms which cause ulceration in each tissue differ.

Since naturally occurring gastric ulcers in adult horses is the primary topic of this study, the pathogenesis of ulcers in the nonglandular region of the stomach will be the main focus of this review while the pathogenesis of glandular ulcers will only be reviewed briefly.

In the glandular mucosa, ulcers are mainly attributable to blood flow disruption and decreased secretion of mucus and bicarbonate. Prostaglandin (PG) inhibition may be the leading factor to mucosal protective mechanism disorders, and is considered the primary cause of equine gastric glandular ulceration. In fact, the most common occurrence of PG

inhibition in horses leading to ulceration of the glandular mucosa is the excessive or long term administration of non-selective non-steroidal anti-inflammatory drugs such as phenylbutazone.

The pathogenesis of ulceration in the nonglandular region of the equine stomach is related to the fact that increased exposure to gastric juices quickly overcomes the limited defence mechanisms. Continuous exposure of the squamous mucosa to acid contents leads to loss of the superficial epithelial layers. The severity of the lesions is associated with the duration of exposure to acidity (Furr, Murray et al. 1992), hence the importance of identifying the risk factors associated with EGUS.

It was initially thought that horses confined to stalls have a higher risk of developing EGUS than those maintained at pasture because they are more prone to stress and less likely to feed constantly (Murray and Eichorn 1996; Orsini and Pipers 1997), however recent studies in active racehorses and broodmares that were kept at pasture showed similar EGUS prevalence to horses stabled full time (Bell, Kingston et al. 2007; le Jeune, Nieto et al. 2008). Therefore, it appears that pasture turnout alone is insufficient protection against gastric ulceration.

Despite the results reported in two papers (Vatistas, Snyder et al. 1999; Rabuffo, Orsini et al. 2002), most studies support that sex and age do not seem to influence EGUS prevalence (Murray, Schusser et al. 1996; Roy, Vrins et al. 2005; Bell, Kingston et al. 2007; Bezdekova, Jahn et al. 2007).

Prolonged fast has also been shown to cause severe ulceration of the gastric squamous epithelial mucosa, triggered by excess acidity (Murray and Eichorn 1996). Suppression of gastric acidity by ranitidine efficiently reduced the area of ulceration caused by feed deprivation (Murray and Eichorn 1996).

The type of feed may also influence the incidence of EGUS. Horses fed alfafa hay or alfafa hay and grain had significantly higher gastric juice pH and lower gastric ulcer

scores than horses fed bromegrass hay (Nadeau, Andrews et al. 1998; Nadeau, Andrews et al. 2000). Because gastric acid output significantly decreased when corn oil was administered to ponies (Cargile, Burrow et al. 2004), it has been hypothesized that dietary oils could prevent equine gastric ulceration. However Frank and colleagues assessed the antiulcerogenic properties of 3 dietary oils and could not confirm this hypothesis (Frank, Andrews et al. 2005). Understanding the difference between gastric acid output and pH is fundamental to interpret these findings, because the decrease of acid output will only affect the pH of gastric fluid when the output becomes minimal (The Equine Gastric Ulcer Council 1999).

A recent *in-vitro* study suggested that hydrogen ions cause an increase in outer barrier permeability of the nonglandular gastric mucosa, allowing diffusion into the deeper sodium-transporting cell layers, which become acidified causing a decrease in sodium transport and ultimately cell swelling (Andrews, Buchanan et al. 2008). The same study showed that lactated Ringer solution did not alter the bioelectric properties of equine nonglandular mucosa, contradicting previous reports where tissue sodium transport and resistance were decreased after exposure to volatile fatty acids (Nadeau, Andrews et al. 2003; Nadeau, Andrews et al. 2003; Andrews, Buchanan et al. 2006).

Helicobacter pylori infections are associated with most peptic ulcers in humans (Gisbert, Calvet et al. 2007; Wilschanski, Schlesinger et al. 2007; Caetano, Felix et al. 2008). Helicobacter-like DNA was detected in the healthy and ulcerated gastric mucosa of horses (Contreras, Morales et al. 2007), however the pathogenic potential of this finding has yet to be assessed.

Exercise has long been linked to gastric ulceration in several clinical prevalence studies (Murray, Schusser et al. 1996; Vatistas, Sifferman et al. 1999; Vatistas, Snyder et al. 1999; Dionne, Vrins et al. 2003; Roy, Vrins et al. 2005; Bell, Kingston et al. 2007), and more specifically exercise intensity was shown to be a significant risk factor in Standardbred horses (Roy, Vrins et al. 2005). Furthermore, it was found that horses exercised on a treadmill had increased intra gastric pressure and decreased gastric juice

pH as soon as gait passed from walk to trot and from trot to gallop (Lorenzo-Figueras and Merritt 2002). Based on these findings, it was hypothesized that when horses move in faster gaits abdominal muscles tense and consequently abdominal pressure increases. The increased pressure pushes gastric contents higher than their normal fill and places the nonglandular mucosa in contact with acidic content.

Conversely, a recent study demonstrated that there was no difference between pregnant and non-pregnant mares with regards to the presence, location and severity of gastric ulcers (le Jeune, Nieto et al. 2008). Abdominal pressure has not yet been measured in pregnant mares.

To date, most of the studies investigated general EGUS pathogenesis, however information is lacking concerning the molecular pathophysiology of this syndrome, and the roles played by different enzymes.

Chapter 3 – Cyclooxygenase

3.1 Cyclooxygenase Isoforms

Prostaglandins (PG) are derivatives of arachidonic acid (AA), a 20-carbon unsaturated fatty acid produced from membrane phospholipids (Smith 1989). The principal pathways of AA metabolism are 1) the lipoxygenase pathway that results in production of leukotrienes (LT), and 2) the cyclooxygenase (COX) pathway, which produces two short lived intermediates: prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) (Smith, Marnett et al. 1991). The latter is a substrate for two enzymatic pathways: one that results in production of several prostaglandins and another leading to the production of thromboxanes (TX). COX is composed of two active sites: a heme with peroxidase activity, responsible for the reduction of PGG_2 to PGH_2, and a cyclooxygenase site, where AA is converted into the hydroperoxy endoperoxide PGG_2 (van der Ouderaa, Buytenhek et al. 1979). The reaction acts through hydrogen atom abstraction from AA

by tyrosine radical generated by the peroxidase active site. Two oxygen (O_2) molecules then react with the AA, yielding PGG_2 (Figure 2).

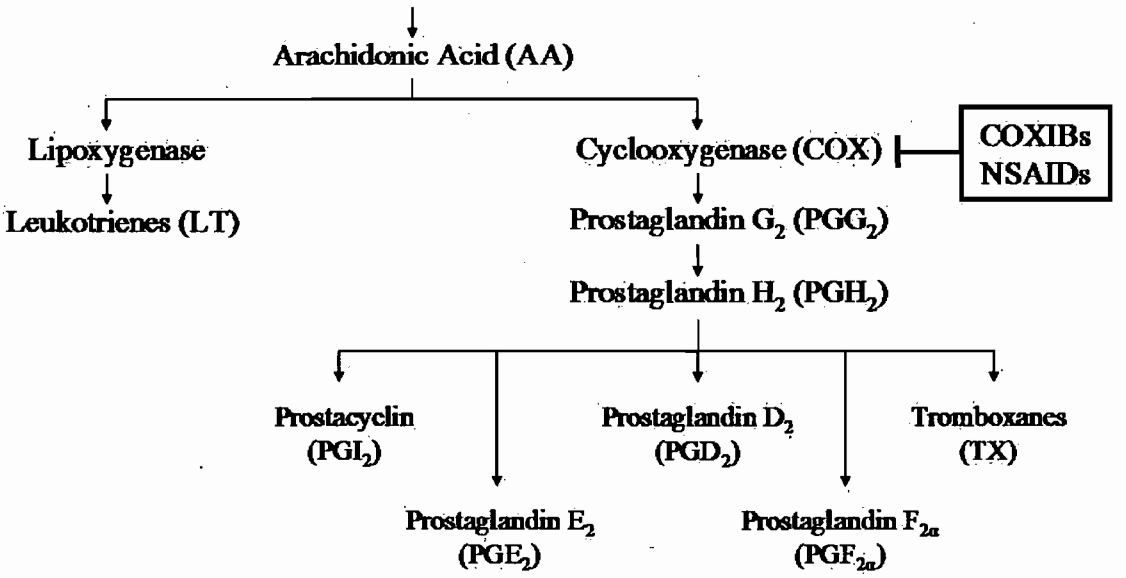

Figure 2: Representation of COX Activity

Currently three isoenzymes of cyclooxygenase have been identified: COX-1, COX-2, and COX-3 (DuBois, Radhika et al. 1996; Shaftel, Olschowka et al. 2003). COX-1 is constitutively expressed, found in most mammalian cells and is associated with maintenance of essential physiological functions such as gastric mucosal integrity, renal function and platelet homeostasis. COX-2 is responsible for production of PG that occurs during inflammatory processes and is upregulated during the inflammatory response and in various carcinomas, playing a role in carcinogenesis (Lee, Soyoola et al. 1992; Xie, Robertson et al. 1992; Meade, Smith et al. 1993; Mitchell, Akarasereenont et al. 1993; O'Sullivan, Huggins et al. 1993; Eberhart, Coffey et al. 1994; DuBois, Radhika et al. 1996). Under normal conditions, COX-2 is constitutively expressed to a lesser

extent in brain, kidney, female reproductive system and bones (Seibert, Zhang et al. 1997; Miller 2006). During an inflammatory response, inducible COX-2 becomes abundant in activated macrophages and other cells at the site of inflammation (Mitchell, Akarasereenont et al. 1993; Masferrer and Seibert 1994; Crofford 1997). Different tissues express varying levels of COX-1 and COX-2. Finally, the third form of COX, termed COX-3, is a splice variant of COX-1 that retains intron one and has a frameshift mutation; some authors refer to it as COX-1b or COX-1 variant (COX-1v) (Shaftel, Olschowka et al. 2003). Its exact functions have yet to be determined (Shaftel, Olschowka et al. 2003).

3.2 COX-1 and COX-2 Expression in the Gastrointestinal Tract

In the gastrointestinal tract (GIT), COX-1 is the predominant isoform and has been found in the gastric fundus, corpus antrum, and/or pylorus, duodenum, jejunum, ileum, cecum, and colon. COX-1 is normally expressed in rodents, dogs, humans, and primates whereas COX-2 is nearly absent in these species with low levels detected in the large intestine (Kargman, Charleson et al. 1996; Seibert, Zhang et al. 1997; Meyer-Kirchrath and Schror 2000; Koki, Khan et al. 2002).

COX-1 is also present in the mucosal epithelium, vascular endothelium, and in smooth muscle cells of the *tunica muscularis*. However, a wide intra-anatomical and interspecies variability of COX-1 expression levels is present in the GIT. In horses, COX-1 protein was expressed in ischemic-injured and non-ischemic tissues (Tomlinson, Wilder et al. 2004). In dogs, protein levels of COX-1 are 10-fold higher in the gastric antrum and/or pyloric region when compared to the small intestine (Seibert, Zhang et al. 1997). The primate small intestine contains five-fold more COX-1 protein than rodent or canine small intestinal tissues (Kargman, Charleson et al. 1996) and rodents express lower levels of COX-1 in the GIT than primates or humans. In humans, the highest area of COX-1 expression is in the small intestine and the lowest is in the gastric fundus/antrum (Kargman, Charleson et al. 1996). Another study demonstrated strong COX-1 and

COX-2 immunoreactivity in parietal cells of normal human gastric mucosa (Jackson, Wu et al. 2000).

Expression of COX-1 and COX-2 was observed in horses where both isoenzymes were expressed in non-ischemic- and ischemic-injured jejunal mucosal tissues. Ischemia was found to induce significant upregulation of both isoforms (Tomlinson, Wilder et al. 2004). Normally, COX-2 is absent from the intestinal tract in dogs, non-human primates, and humans, except in the colonic mucosa (Koki, Khan et al. 2002). Inflammation of GIT induces COX-2 expression and its inhibition by NSAIDs has been shown to delay recovery of gastrointestinal (GI) injury in rats (Kishimoto, Wada et al. 1998). In a rat model of ischemia-reperfusion-induced acute gastric mucosal injury, an increase in COX-2 expression was maximally observed at 24 hours (Kishimoto, Wada et al. 1998). The inflamed GI mucosa-mediated COX-2 expression may also affect the chloride and fluid flux, both GI secretory responses to infection by bacteria (Eckmann, Stenson et al. 1997). Greater immunostaining of COX-1 and COX-2 was found at the rim of ulcers and in *Helicobacter pylori* gastritis in humans, more specifically at the mid-glandular zone and *lamina propria* inflammatory cells (Jackson, Wu et al. 2000). In humans, increased COX-2 has also been found in various hyperplastic and neoplastic lesions of GIT including colon cancer, familial adenomatous polyposis, and sporadic adenomatous polyps in the colon (Soslow, Dannenberg et al. 2000; Khan, Masferrer et al. 2001; Koki, Khan et al. 2002).

In summary, COX-1 is considered to be the predominant isoform in the normal GIT while COX-2 does not appear to be present in normal GIT of most species studied to date. Significant interspecies differences in both the level of COX-1 expression and the ratio of COX-1 and COX-2 expression in the GIT have been observed. Dogs and rats have higher COX-1 and COX-1/COX-2 ratio expression than humans and non-human primates, which may in part explain the sensitivity of these species to sub-therapeutic doses of NSAIDs. As described further in this chapter, in horses, both COX-1 and

COX-2 proteins were expressed in ischemic-injured and non-ischemic tissues (Tomlinson, Wilder et al. 2004).

3.3 Role of COX-1 and COX-2 in Gastric Mucosal Defence System

As described previously, a complex protection system exists in the gastric mucosa to strengthen its resistance to injury. Prostaglandins play a crucial role in this system. In the GIT, maintenance of mucosal integrity was initially attributed exclusively to COX-1 isoform with no contribution of COX-2 (Vane and Botting 1995). Induction of COX-2 was associated with pathophysiological reactions such as inflammation. Recently, studies have shown that COX-2 may also have regulatory functions under physiological conditions (Robertson 1998; McAdam, Catella-Lawson et al. 1999). These observations indicate that both COX-1 and COX-2 either alone or in concert contribute to gastric mucosal integrity.

Specific inhibition of COX-1 in normal rat glandular gastric mucosa did not induce mucosal lesions (Wallace, McKnight et al. 2000; Gretzer, Maricic et al. 2001). Selective COX-2 inhibitors also did not injure the gastric mucosa when administered alone; however, combination with a COX-1 inhibitor resulted in severe gastric damage. Recent findings also showed an induction of gastric mucosal injury after combined treatment with SC-560 (a specific COX-1 inhibitor) and the COX-2 inhibitor celecoxib but not SC-560 alone (Wallace, McKnight et al. 2000). Also SC-560 but not celecoxib induced reduction in gastric mucosal blood flow and celecoxib but not SC-560 increased leukocyte adherence in mesenteric venules suggesting that the two COX isoforms differ in their biological activity. COX-1 deficient mice presented no gastric lesions and lower gastric PGE_2 levels (< 1%) compared to wild type mice (Langenbach, Morham et al. 1995). Lack of ulcerogenicity was also observed in COX-2 deficient mice (Morham, Langenbach et al. 1995). As previously described, selective pharmacological suppression of each COX isoform did not induce damage to the glandular gastric mucosa.

In contrast to normal glandular gastric mucosa that requires inhibition of both isoforms for ulcerogenic lesions to occur, in the acid-challenged rat stomach, inhibition of COX-1 alone resulted in injury which was further increased by addition of a COX-2 inhibitor or prevention of COX-2 up-regulation by dexamethasone (Gretzer, Maricic et al. 2001). However, specific inhibition of COX-2 with rofecoxib and DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furan one) in rat mucosa after acid challenge did not induce lesions (Gretzer, Maricic et al. 2001). In normal glandular gastric mucosa, COX-1 mRNA expression is high and COX-2 mRNA low but COX-2 mRNA was substantially up-regulated after intragastric acid challenge with no effect on COX-1 expression (Kargman, Charleson et al. 1996; Maricic, Ehrlich et al. 1999; Gretzer, Maricic et al. 2001). Acid-induced up-regulation of COX-2 mRNA was prevented by pretreatment with dexamethasone without alteration in COX-1 mRNA (Gretzer, Maricic et al. 2001).

Various mediators also act in concert to increase mucosal resistance against injury. Prostaglandins, nitric oxide (NO) and afferent nerves are some members of the glandular gastric defence system (Whittle, Lopez-Belmonte et al. 1990). As described before, inhibition of COX-2 alone did not induce gastric damage even in the presence of acid-challenge (Gretzer, Maricic et al. 2001). However, association of COX-2 inhibitors DFU or NS-398 with a NO inhibitor L-NAME resulted in severe and dose-dependent injury in acid-challenged mucosa (Maricic, Ehrlich et al. 1999). Combination of a COX-2 inhibitor with capsaicin, a neurotoxic drug, which blocks afferent neurons, caused gastric damage even without suppression of NO. These findings indicate that when NO formation or afferent nerves are suppressed, inhibition of COX-2 alone causes severe gastric damage in glandular mucosa.

Finally, in rats, ischemia-reperfusion damage resulted in increased mRNA levels of COX-2 in a time-dependent manner without an effect on COX-1 mRNA expression (Kishimoto, Wada et al. 1998; Maricic, Ehrlich et al. 1999). The up-regulation of COX-2 was attenuated after pre-treatment with dexamethasone during ischemia with

subsequent reperfusion but had no effect on COX-1 expression (Maricic, Ehrlich et al. 1999). In rats, occlusion of the gastric artery for 30 min followed by reperfusion for 60 min resulted in minor gastric injury. The injury was aggravated up to four-fold after treatment with COX-2 inhibitors or dexamethasone and prevented by concurrent administration of 16, 16-dimethyl-PGE_2 (Peskar, Maricic et al. 2001). The COX-1 inhibitor also resulted in gastric mucosal injury. These results suggest that both COX-1 and COX-2 act to reduce ischemia-reperfusion gastric damage and COX-2 may play a more important role in this condition in the glandular mucosa.

These findings demonstrate that the role of COX isoforms in glandular gastric mucosa protection differs in normal mucosa and in mucosa exposed to noxious agents. A combination of COX-1 and COX-2 inhibitors is necessary to induce injury in normal glandular mucosa while in the presence of noxious agents, isolated inhibition of COX-1 is sufficient to result in mucosal injury with increased expression of COX-2. It is unknown if these mechanisms of mucosal injury would be the same in nonglandular type mucosa. The implications of COX-1 and COX-2 in the nonglandular mucosal defence have yet to be elucidated. In naturally occurring nonglandular porcine gastric ulcers, COX-2 was strongly expressed (80%) (Lajoie, Sirois et al. 2002). In rats, COX-1 mRNA and protein levels were unaltered while COX-2 mRNA and protein levels were increased 2.5-fold and threefold, respectively, in esophageal ulceration when compared to normal nonglandular esophageal tissue (Baatar, Jones et al. 2002). The same study showed that treatment with celecoxib, a selective COX-2 inhibitor, significantly delays esophageal ulcer healing. This finding suggests that COX-2 may play a role in nonglandular ulcer healing.

3.4 Cyclooxygenase and Ulcer Healing

Ulcer healing is a multifarious process involving cell migration, proliferation, re-epithelialization, angiogenesis and matrix deposition (Figure 3). All of these events lead

to scar formation and are controlled by growth factors, transcription factors and cytokines (Tarnawski 1993; Tarnawski 2000; Wong, Playford et al. 2000; Vanwijck 2001).

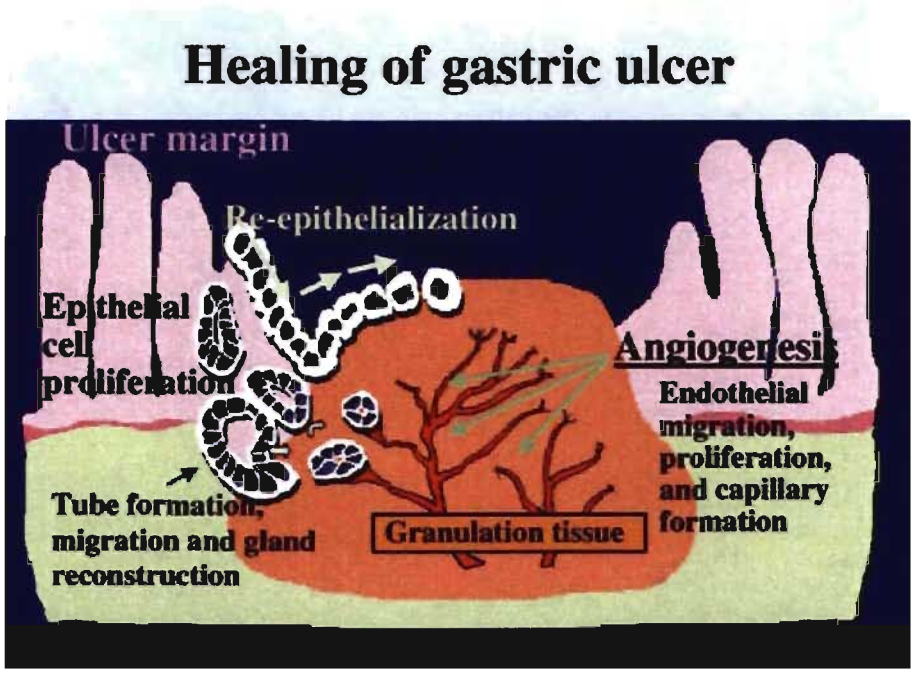

Figure 3: Illustration of the Ulcer Healing Complex Process

(Tarnawski 2005).

Cyclooxygenases have also been shown to be involved in ulcer healing. COX-2 mRNA and protein were increased in gastric ulcers induced by subserosal injection of acetic acid in mice (Mizuno, Sakamoto et al. 1997) while no effect on COX-1 mRNA expression was observed in ulcerated or non-ulcerated mucosa. However, a threefold increase in prostaglandins (PGA_2, PGE_2, $PGF_{2\alpha}$) was demonstrated in ulcerated tissue when compared to normal tissue and was inhibited by exposure to the COX-2 inhibitor NS-398 (Mizuno, Sakamoto et al. 1997). In rats with chronic ulcers, the immunoreactivity of COX-2 was low in normal gastric wall and increased during the initial phase of ulceration up to day 5, in the tissue of the ulcer base (Schmassmann, Peskar et al. 1998). Immunoreactivity of COX-1 was located mainly in the non-ulcerated

mucosa and was reduced after gastric ulceration in the mucosa adjacent to the ulcer crater. COX-1 immunoreactivity reappeared from day 5 onwards in the apical cytoplasm of the regenerative epithelial cells. These observations demonstrated that, in glandular chronic ulcers, COX-1 and COX-2 exhibit different spatial and temporal patterns of expression. COX-2 is up-regulated in chronic glandular gastric ulcers and COX-2 inhibitors prevent the healing of ulcers, indicating that COX-2 plays an important role in acceleration of ulcer healing. NSAIDs which inhibit both COX-1 and COX-2 are known to impair the ulcer healing process. Daily administration of indomethacin and diclofenac to rats with cryoulcers (ulcers experimentally induced by applying a stainless-steel probe cooled in liquid nitrogen on the surface of the gastric mucosa) for 8 or 15 days resulted in a dose-dependent delay in gastric healing (Schmassmann, Peskar et al. 1998). Delayed ulcer healing was also observed after daily administration of the COX-2 inhibitor L-745,337 in rats. Nonetheless, the role of COX-1 in gastric ulcer healing remains to be elucidated. Gastric ulcer healing is associated with an increase in blood flow (Hirose, Takeuchi et al. 1991). As previously described, inhibition of COX-1 caused a decrease in gastric mucosal blood flow (Wallace, McKnight et al. 2000), suggesting a possible mechanism by which selective COX-1 inhibitors impair the healing process.

The development of COX-1 and COX-2 knockout mice and selective COX-1 and COX-2 inhibitors allowed to evaluate the role of COX-1 and COX-2 in ulcer healing. Wild-type COX-1 knockout ($COX\text{-}1^{-/-}$) and COX-2 knockout ($COX\text{-}2^{-/-}$) mice with gastric ulcers were treated with selective COX-1 (SC-560), COX-2 (celecoxib, rofecoxib, and valdedoxib) and non-selective COX (piroxicam) inhibitors (Schmassmann, Zoidl et al. 2006). Healing was moderately impaired by COX-2 gene disruption and COX-2 inhibitors. Severe healing impairment was observed in dual (SC-560 + rofecoxib) and unselective (piroxicam) COX inhibition and combined COX impairment (in $COX\text{-}1^{-/-}$ mice with COX-2 inhibition and $COX\text{-}2^{-/-}$ mice with COX-1 inhibition). Inhibition of COX-1 or gene expression had no effect on ulcer healing. These data suggested that COX-2 is an important mediator in ulcer healing and that COX-1 becomes important when COX-2 is impaired.

Ulcer healing also entails local expression of growth factors in the ulcer area, for instance, EGF, transforming growth factor (TGFα), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF), as well as gastrin (Brzozowski, Konturek et al. 2001). These are expressed at the ulcer margin during ulcer healing (Konturek, Brzozowski et al. 1997), which concur with hypergastrinemia, the inhibition of gastric acid secretion and increase of mucosal blood flow (Konturek 1990). Gastrin administration increases gastric acid secretion and conversely accelerates ulcer healing, showing that healing and gastric acid secretion effects are unrelated with regards to this hormone. Moreover, gastrin receptors were found in the regenerative mucosal ulcer margin (Schmassmann and Reubi 2000), emphasizing the theory that gastrin contributes to mucosal cell proliferation at the ulcer margin (Li and Helander 1996). Local application of antibodies against growth factors or gastrin abrogates the acceleration of ulcer healing (Brzozowski, Konturek et al. 2001), showing the specificity of the healing promotion of those substances through stimulation of mucosal growth and angiogenesis at the ulcer margin. COX-2 gene expression was raised in the ulcerated mucosa after treatment with growth factors while COX-1 was unaffected (Brzozowski, Konturek et al. 2000). Since COX-1 and COX-2 inhibitors delay ulcer healing mediated by growth factors and upregulation of COX-2 is reflected at the ulcer margin, it can be concluded that PGs resulting from the COX-2 pathway mediate ulcer healing via diverse growth factors expressed at the ulcer margin.

3.5 The Role of Cyclooxygenases in Equine Gastrointestinal Tract

Few studies to date have investigated the role of COX in the equine GIT. One study demonstrated that experimentally-induced ischemia increased the passage of lipopolysaccarides (LPS) across the equine jejunal mucosa *in vitro* and that flunixin

caused a delay in mucosal recovery without an increase on LPS absorption (Tomlinson and Blikslager 2004). Another study showed that permeability of ischemic-injured equine jejunum was increased after treatment with flunixin and addition of misoprostol (a PGE_1 analogue) and that treatment with deracoxib (a selective COX-2 inhibitor) improved the negative gastrointestinal effects of NSAIDs on the recovery of equine intestinal mucosa (Tomlinson and Blikslager 2005). Similar results were found after treatment with flunixin or etodolac during ischemia-induced jejunal mucosal injury (Tomlinson, Wilder et al. 2004). Both drugs delayed recovery of intestinal barrier function after 18 hours of reperfusion and ischemia, and caused an increase in expression of COX-1 and COX-2 in equine jejunal mucosa. The effects of non-selective COX inhibitors (indomethacin and flunixin meglumine) and selective COX-1 (SC-560) or COX-2 (celecoxib, DUP-398 and NS-697) inhibitors on horse small bowel motility *in vitro* have been investigated (Menozzi, Pozzoli et al. 2008). Selective COX-2 inhibitors decreased both tonic contraction and spontaneous phasic contractions and non-selective COX inhibitors did not induce major effects on motility, except for an inhibition of tonic contraction shown by flunixin meglumine. This report indicates that the effects of COX inhibitors on horse small intestinal motility are not linked to PG depletion. The precise role of COX-1 and COX-2 in equine mucosal damage requires further elucidation and may provide valuable information for the development of novel therapeutic approaches to prevent mucosal injury and improve survival of horses with intestinal disease.

Only one report to date has described COX activity in equine nonglandular and glandular gastric mucosa (Morrissey, Bellenger et al. 2008). Authors observed that, in both mucosa types, COX-1 was the most important pathway to PGE_2 production under basal circumstances, and COX-2 was the primary pathway when it came to bradykinin-stimulated tissue *in vitro*. Neither the expression nor the role of the COX isoforms has been reported in equine ulcerated stomach.

3.6 Clinical Use of Coxibs in Veterinary Medicine

A variety of non-steroidal anti-inflammatory drugs (NSAIDs) target both COX-1 and -2 (DeWitt 1999). These classic COX inhibitors are not selective and the main adverse effects of their use in humans are peptic ulceration and dyspepsia. It is believed that this may be due to the "dual-insult" of NSAIDs by direct irritation of the gastric mucosa since many NSAIDs are acids, as well as by inhibition of PG synthesis by COX-1. Recently, selective COX-2 inhibitors (COXIBs) were developed to provide therapeutic benefits similar to NSAIDs without the attendant COX-1 mediated gastrointestinal toxicity (Radi and Khan 2006). Compared with traditional NSAIDs, COX-2 selective inhibitors demonstrate a reduced risk of gastric ulceration and decreased gastrointestinal side effects; however, this protection is lost if aspirin is coadministrated (Silverstein, Faich et al. 2000). However, traditional NSAIDs and selective COX-2 inhibitors reduced epithelial cell proliferation and delayed ulcer healing in rodents (Mizuno, Sakamoto et al. 1997; Schmassmann, Peskar et al. 1998), indicating that side effects could be anticipated from their use. Moreover, an increase in cardiovascular events has been reported in human patients treated with selective COX-2 inhibitors (Bresalier, Sandler et al. 2005).

Although several coxibs have been developed (rofecoxib, celecoxib, valdecoxib, parecoxib, etoricoxib, lumiracoxib), most of them have been withdrawn or were never approved in humans due to safety concerns. Increased risk of myocardial infarction, nephrotoxicity, hepatotoxicity and increased rate of serious and potentially life threatening skin reactions were the primary side-effects related to coxib use in humans (Schneeweiss, Solomon et al. 2006; Solomon, Avorn et al. 2006; Yan, Leung et al. 2006; Laine, White et al. 2008). Gastro-intestinal side effects were also common in patients who received coxibs (Laine, Connors et al. 2003; Schnitzer, Burmester et al. 2004). Co-administration with proton-pump inhibitors, such as omeprazole, was frequently recommended in cases of long term treatment (Bertin and Avouac 2003; Targownik, Metge et al. 2008).

In veterinary medicine, to date, only deracoxib and firocoxib have been approved for administration in dogs (Clark 2006), and only firocoxib has been approved for use in horses (Doucet, Bertone et al. 2008).

Deracoxib selectiveness for COX-2 was demonstrated in dogs through an *in vivo* study (Sessions, Reynolds et al. 2005), and its effectiveness in preventing pain and lameness associated with synovitis was shown in an experimentally-induced model (Millis, Weigel et al. 2002). In dogs with osteoarthritis, treatment with deracoxib did not alter platelet function; however hypercoagulability with increased clot strength and high coagulation index was reported (Brainard, Meredith et al. 2007). Three day treatment with deracoxib in clinically normal dogs did not alter the macroscopic appearance of pyloric or duodenal mucosae nor lead to histological evidence of ulceration in those tissues (Wooten, Blikslager et al. 2008). In the same study, deracoxib had no effect on mucosal COX-2 protein expression; however mild to moderate abnormalities were evident in pyloric antral and duodenal biopsy specimens. Deracoxib decreased gastric PGE_2, but not PGE_1 concentration after 3 days of treatment (Sessions, Reynolds et al. 2005). Deracoxib administered orally once a day at 4 mg/kg worsened the endoscopic score of gastric fundus, antrum and lesser curvature of five healthy dogs one day after administration (Dowers, Uhrig et al. 2006). Less than a year after deracoxib approval in the United States, the Novartis pharmacovigilance database had registered 29 cases of dogs with gastrointestinal tract perforation, however in 90% of the cases, deracoxib had been administered at higher-than-approved dosage or in close temporal association with another NSAID or corticosteroid (Lascelles, Blikslager et al. 2005).

In a clinical trial that enrolled more than 1000 client-owned dogs, another coxib tested in dogs, firocoxib, was considered effective at minimizing osteoarthritis pain and improved the quality of life of more than 87 % of the patients at Days 10 and 40 (Ryan, Moldave et al. 2006). In the same study minimal side effects were reported. When firocoxib was used at the recommended dosage for 28 days on healthy dogs, no side effects were registered (Steagall, Mantovani et al. 2007). In another trial, firocoxib-treated dogs had greater improvement in the clinical signs of osteoarthritis and fewer

abnormal treatment-related events than etodolac-treated dogs (Hanson, Brooks et al. 2006). On a mixed four-period crossover study design in dogs (Drag, Kunkle et al. 2007), firocoxib's capacity to prevent pain in a urate-induced lameness model was equivalent to the other NSAIDs being tested (i.e. carprofen, deracoxib and meloxicam). In a similar trial, firocoxib was considered better than carprofen to reduce acute pain and improve weight bearing (Hazewinkel, van den Brom et al. 2008). Firocoxib suppressed plasma and synovial fluid concentrations of PGE_2 in osteoarthritic dogs (Punke, Speas et al. 2008), and caused 4 of the 8 dogs enrolled in the study to vomit. One study reported that 20% of 110 dogs being treated with firocoxib for 30 days had at least one physical condition recorded by the owner (Pollmeier, Toulemonde et al. 2006), including anorexia, constipation, diarrhea, emesis and polydipsia. Firocoxib slowed wound healing in a canine gastric mucosal injury model (Goodman, Torres et al. 2009).

Finally, a novel coxib is now being tested for use in companion animals. Robenacoxib selectivity for COX-2 exceeded 450 for both IC50 and IC80 COX-1/COX-2 ratios in feline whole blood assays (Giraudel, Toutain et al. 2009). In a preclinical study in rats, robenacoxib produced less gastric ulceration and intestinal permeability than diclofenac and had no relevant effects on kidney function (King, Dawson et al. 2009). It was shown that robenacoxib has a good bioavailability in dogs after oral and subcutaneous administration (Jung, Lees et al. 2009).

To date, few studies have been performed concerning the use of coxibs in horses. The highly selective COX-2 inhibitor in dogs, deracoxib, was shown to reduce the recovery of intestinal mucosal transepithelial electrical resistance after ischaemia in horses (Tomlinson and Blikslager 2005). The same study showed that deracoxib reduced the levels of PGE_2 and 6-keto$PGF_{1\alpha}$ on ischaemic-injured equine jejunum.

In a controlled clinical trial, 123 horses with osteoarthritis received 0.1 mg/kg of firocoxib once a day (Doucet, Bertone et al. 2008), from those, 104 patients (84.6%)

were considered to show improvement of their clinical condition while no direct treatment-related adverse effects were perceived during the study.

Evidence suggests that most common side effects of coxibs in veterinary medicine are related to the GIT. The effects of COX-2 inhibition on the healing of EGUS lesions should therefore be investigated. Characterizing COX-2 expression in normal and ulcerated gastric mucosa is a first step towards that objective.

OBJECTIVE

The objective of the present study was to characterize the expression of COX-1 and COX-2 isoenzymes in normal equine nonglandular stomach and in spontaneous equine nonglandular gastric ulcers.

The departure hypothesis was that COX-1 would remain with the same expression levels in both mucosa types. And COX-2 would be up-regulated in the ulcerated mucosa.

ARTICLE

Expression of Cyclooxygenase Isoforms in Equine Nonglandular Gastric Ulcers

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Summary

Objective

Characterize the expression of the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoforms in naturally occurring equine nonglandular gastric ulcers.

Sample Population

Thirty-eight ulcerated and ten healthy equine stomachs.

Procedures

Two specimens of each sample were taken from the gastric squamous mucosa, one was fixed in 10% formalin for immunohistochemistry and another was frozen at -70 °C for immunoblotting analysis. Immunnoreactivity to two antibodies, MF241 (selective for COX-1) and MF243 (selective for COX-2) , was evaluated by a veterinary pathologist using a scoring system from 0 to 3, where 0 denoted the absence of COX expression and 3 corresponded to the maximum expression of either COX isoform. COX-1 and COX-2 characterizations were confirmed by immunoblotting analyses.

Results

All normal samples strongly expressed COX-1, while only 20% expressed COX-2. The expression of both isoforms varied greatly in the ulcerated mucosal samples. The expression of COX-1 was significantly lower while the expression of COX-2 was significantly higher in ulcerated versus normal mucosae ($p < 0.0001$).

Conclusions and Clinical Relevance

An increased expression of COX-2 in equine squamous gastric ulcers suggests a role for this enzyme in gastric ulcer healing. Further studies are necessary to establish the impact of COX-2 inhibitors utilization in horses with EGUS.

Abbreviations

COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DAB	Diaminobenzidine tetrahydrochloride
EGUS	Equine gastric ulcer syndrome
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate Buffered Saline
PMNs	Polymorphonuclear leukocytes
RT	Room temperature

Introduction

The nonglandular (or squamous) form of the equine gastric ulcer syndrome (EGUS) is a widespread disease complex that often affects horses [a]. The high prevalence illustrated by different authors ranges between 40%, in western performance horses [1], to 93,6%, in Standardbreds in active race training[2-4]. In adult horses, ulcers are typically formed in the squamous mucosa alongside the *margo plicatus*, nevertheless they can also be found in the glandular and pyloric areas [2,5,6]. While many theories related to the mechanical consequences of exercise and other potential risk factors have been proposed to explain the location and pathogenesis of gastric ulcers in the nonglandular portion of the equine stomach[7,8 5,9-12]; the molecular pathogeneses of ulcer formation and healing have yet to be elucidated.

In essence, the role of the two cyclooxygenase isoforms, COX-1 and COX-2, on gastric mucosal defence has been studied in several species, yet information is lacking concerning equine patients. In horses, ulceration of the glandular portion of the stomach resembles the situation in other species. However, the pathophysiology of nonglandular equine gastric ulcers is thought to be diverse due to different physiologic and defence mechanisms proper to this mucosa. In the glandular gastric mucosa, a complex protection system exists to strengthen its resistance against injury. Prostaglandins play a crucial role in this system. In the gastrointestinal tract (GIT), maintenance of mucosal integrity was initially attributed exclusively to the COX-1 isoform with no contribution of COX-2 [13]. Induction of COX-2 was associated with pathophysiological reactions

such as inflammation. Recently, studies have shown that COX-2 may also have regulatory functions under physiological conditions [14,15]. These observations indicate that both COX-1 and COX-2 either alone or in concert contribute to maintaining gastric mucosal integrity in species where glandular ulcers predominate. Only one report to date has described COX activity in equine nonglandular and glandular gastric mucosa [16]. Authors observed that, in both mucosa types, COX-1 was the most important pathway to PGE_2 production under basal circumstances, and COX-2 was the primary pathway when it came to bradykinin stimulated tissue *in vitro*.

Cyclooxygenase-1 and -2 have also been shown to play a role in ulcer healing in several species in which the stomach is composed primarily of glandular mucosa. For example, in rats with chronic ulcers, the immunoreactivity of COX-2 was low in normal gastric wall and increased in tissue located at the ulcer base [17]. Further evidence that COX-2 plays an important role in acceleration of ulcer healing has also been demonstrated in studies where the administration of selective COX-2 inhibitors prevented ulcer healing[17]. In fact, NSAIDs which inhibit both COX-1 and COX-2 have been shown to impair the ulcer healing process in species with predominantly glandular mucosae. Since the development of new COX-2 selective agents for use in horses, it became imperative to comprehend the role of this enzyme on the molecular pathogenesis of EGUS. The objective of this study was therefore to characterize the expression of both cyclooxygenase isoforms in naturally occurring equine gastric ulcers of the nonglandular (squamous mucosa), in order to establish whether or not the use of new selective NSAIDs would be dangerous to horses.

Materials and Methods

Tissue Sample Collection and Anti-COX Antibodies

Ulcerated and normal stomachs from horses slaughtered at an abattoir in St-Aimé, Québec, by use of the free bullet method, were used in this study. Care was taken to include ulcerated and peripheral mucosa for each sample representing the ulcerated stomachs. All samples were rinsed in saline solution to remove gastric contents before being fixed in 10% neutral buffered formalin or being transported on ice until they could be placed in a freezer at -70 °C for immunoblotting analysis. Normal and ulcerated samples were evaluated by examination of haematoxylin-eosin-saffron-stained sections by a veterinary pathologist[18]. Stomachs with mucosal erosions or normal stomachs with evidence of inflammation were excluded. Ten (10) normal and 38 ulcerated samples were included in this study. Two anti-COX antibodies (MF241 and MF243)[b] were used. MF241 was raised in rabbits against ovine placental COX-1 and its selectivity for COX-1 has been shown [19]. MF243 was raised in rabbits against ovine placental COX-2 and its selectivity for equine COX-2 has previously been characterized[20].

Immunohistochemistry

Immunohistochemical staining was performed based on a previously described method [21,22]. Briefly, formalin-fixed tissues were paraffin-embedded and 3-μm-thick sections

were prepared, deparaffinised in toluene, and hydrated through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with normal goat serum[c] (1:74 dilution) for 20 min at room temperature (RT). Primary antibodies diluted in phosphate buffered saline (PBS) were applied (MF241 at 1:4000 dilution and MF243 at 1:10000 dilution) and sections were incubated overnight at 4C. Control sections were incubated with nonimmune rabbit serum. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody[c] (1:222 dilution) was applied, and sections were incubated for 45 min at RT. Sections were washed in PBS for 10 min and incubated with the avidin DH–biotinylated horseradish peroxidase H reagents[b] for 45 min at RT. After a PBS wash for 10 min, the reaction was revealed using 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB)[d] in Tris buffer (pH 7.6) as the chromogen and 0.05% hydrogen peroxide as the substrate. Sections were counterstained with Gill's haematoxylin and mounted. Immunoreactivity was evaluated by an independent observer using the scoring system proposed by Lajoie and colleagues[22], where a score of 0 denoted no staining; 1, 0-10% of positive cells; 2, 11-30% of positive cells and 3, ≥31% of positive cells.

Solubilized Cell Extracts and Immunoblotting Analysis

The immunoblotting technique used in this study has been previously described for porcine stomachs[22]. Briefly, solubilised cell extracts were prepared as previously described [23]. The protein concentration in each extract was determined by the method of

Bradford (Bio-Rad Protein Assay). Proteins were resolved by onedimensional SDS-PAGE and electrophoretically transferred to Hybond polyvinylidene difluoride membranes. Blocking of membranes was done using 5% non-fat dry milk in 0.1% TTBS (0.1% Tween-20, 10 mM Tris-buffered saline, pH 7.5) for 1 hr at RT, then washed twice for 2 minutes at RT with 0.1% TTBS. After blocking, membranes were incubated with anti-COX antibodies (MF241 at 1:4000 dilution and MF243 at 1:7500 dilution) diluted in 0.05% TTBS (0.05% Tween-20, 10 mM Tris-buffered saline, pH 7.5) containing 2% non-fat dry milk for 2 hr at RT. Membranes were incubated with a horseradish peroxidase-labelled donkey anti-rabbit secondary antibody[e] (1:15,000 dilution) for 1 hr at RT. The membranes were washed and the bound secondary antibody was detected using the enhanced chemiluminescence's (ECL) detection kit[f]. The signal was visualized on Kodak Bio-Max X-ray film[f].

Statistical analysis

Statistical analyses were performed using computer software (SAS V.9.1)[g]. The Cochran-Mantel-Haenszel test assuming unequal distances between scores was used to establish the association of the gastric mucosa status and the COX immunoreactivity expression and the correlation between the expression of COX-1 and COX-2. A value of $P \leq 0.05$ was considered significant.

Results

Normal samples

All (100%) normal stomachs strongly expressed COX-1 but only 20% expressed COX-2 (Table 1). COX-1 staining in normal stomachs was generally seen in cells located in the chorion under the epithelial surface and identified as fibroblasts, in blood vessels located in the mucosa, submucosa and muscularis layers, and sometimes in smooth muscle cells (Figure 1). The surface epithelium was consistently negative for COX-1. When present, COX-2 staining was observed in small cells in the muscularis mucosa which were thought to probably be capillaries. COX-2 staining was negative in the surface epithelium, in the mucosa and submucosa (Figure 1).

Ulcerated samples

Both COX-1 and COX-2 were expressed in ulcerated samples although the intensity of staining varied considerably (Table 1). When present, COX-1 immunoreactivity in ulcerated samples was principally located in fibroblast-like cells under the ulcerated surface (Figure 2). When compared with COX-1 expression in normal samples, a significant lower COX-1 expression was observed in ulcerated tissues ($p<0.0001$). COX-2 immunostaining was predominantly located in the cytoplasm of elongated fibroblast-like cells in the granulation tissue proliferating under the ulcerated area. COX-2 immunoreactivity was also present in the cytoplasm of mucosal epithelial cells

bordering the ulcers (Figure 3). COX-2 expression was significantly higher in ulcerated tissues compared to the normal stomach ($p<0.0001$). There was no significant correlation between the expressions of COX-1 and COX-2 ($p=0.41$).

Immunoblotting

When a selective anti-COX-1 antibody was used, a 69,000 M_r band was detected in both normal and ulcerated stomachs. In several extracts of gastric ulcers, COX-1 protein levels were markedly reduced (Figure 4A), confirming the immunohistological observation of lower COX-1 expression in ulcerated tissues. When a selective anti-COX-2 antibody was used, no signal was detected in normal stomachs but a strong band was observed in the protein extracts from gastric ulcers (Figure 4). Equine COX-2 appeared as a 72,000– 74,000 M_r doublet and a small 62,000 M_r band believed to correspond to a proteolytic fragment, as previously observed in other species [22,24,25].

Discussion

This is the first study looking at the expression of COX-1 and COX-2 in ulcers of the nonglandular portion of the equine stomach. Our results demonstrate that normal nonglandular equine gastric mucosa expresses COX-1, and that most stomachs do not express COX-2. This observation is in agreement with previous studies performed on human squamous oesophagus mucosa and on nonglandular gastric mucosa in pigs [22,26,27]. They are also in line with common knowledge that COX-1 is the constitutive and COX-2 the inducible isoform of cyclooxygenase in most tissues. Unexpectedly, however, COX-1 expression was found to be significantly decreased in nonglandular ulcerated tissues. In fact, while COX-1 is known to be involved in the complex process of mucosal protection in glandular mucosa and has been shown to increase in rats after experimentally-induced glandular gastric ulceration [17], the role of this COX isoform in nonglandular mucosal homeostasis is unclear. Yet, a similar decrease in COX-1 was also observed in porcine naturally-occurring nonglandular gastric ulcers[22] As in horses, porcine ulcers are located in the nonglandular portion of the stomach. A possible explanation for the decrease in COX-1 expression in both equine and porcine nonglandular mucosal ulcers could be the loss of normal submucosa with its COX-1 positive cells and its replacement by granulation tissue containing COX-2 expressing fibroblasts. Alternatively, it could result from a negative feedback following the increase in COX-2, although no correlation was found between COX-1 and COX-2 expression in ulcerated gastric mucosal samples in this study.

COX-2 expression was significantly induced in the ulcerated squamous mucosa of adult horses. The same response was reported in pigs [22]. Indeed, in naturally occurring porcine ulcers of the nonglandular portion of the stomach, COX-2 was also strongly expressed (80%) compared to normal mucosa where COX-2 expression was absent or very low [22]. In the present study, COX-2 expressing cells were predominantly fibroblasts present in the granulation tissue under the ulcer bed. The localization of COX-2 expression in the granulation tissue in equine ulcers suggests that the enzyme could be involved in the repair process of equine gastric ulcers.

The role of COX-1 and COX-2 in glandular ulcer healing has been elucidated largely due to the development of COX-1 and COX-2 knockout mice and selective COX-1 and COX-2 inhibitors. Wild-type, COX-$1^{-/-}$ and COX-$2^{-/-}$ mice with gastric ulcers were treated with selective COX-1 (SC-560), COX-2 (celecoxib, rofecoxib, and valdedoxib) and nonselective COX (piroxicam) inhibitors [28]. Healing was moderately impaired by COX-2 gene disruption and COX-2 inhibitors. Severe healing impairment was observed in dual (SC-560 + rofecoxib) and unselective (piroxicam) COX inhibition and combined COX impairment (in COX-$1^{-/-}$ mice with COX-2 inhibition and COX-$2^{-/-}$ mice with COX-1 inhibition). Inhibition of COX-1 or gene expression had no effect on ulcer healing. It is not known if the same response is seen on nonglandular ulcer healing although delayed ulcer healing caused by selective COX-2 inhibition has been shown to be due to a reduction in epithelial cell proliferation in rat esophageal ulcers[29].

Cyclooxygenase-2 stimulus has been shown to be the main pathway leading to endogenous prostaglandins' contribution to ulcer healing in the glandular gastric mucosa [17,30,31]. Prostaglandins are believed to contribute to glandular ulcer healing through the induction of hepatocyte growth factor[32], reduction of gastric acid secretion[33-35], angiogenesis[36] and stimulation of mucus and bicarbonate secretion[37,38]. Cyclooxygenases have also been shown to be involved in ulcer healing as COX-2 mRNA and protein were increased in gastric ulcers induced by subserosal injection of acetic acid in mice [31] while no effect on COX-1 mRNA expression was observed in ulcerated or non-ulcerated mucosae. A threefold increase in prostaglandin (PGA_2, PGE_2, $PGF_{2\alpha}$) levels was demonstrated in ulcerated tissue when compared to normal tissue and was inhibited by exposure to the COX-2 inhibitor NS-398 [31] indicating that those prostaglandins were synthesised principally via the COX-2 pathway. These data suggested that, at least in glandular-type mucosae, COX-2 is an important mediator in promoting ulcer healing and COX-1 becomes important when COX-2 is impaired. It is not clear whether the same mechanisms can be inferred to nonglandular type mucosa such as in horses

COX-2 immunoreactivity was primarily located in fibroblasts at the ulcer base in this study. Similarly, in rats with chronic ulcers, the immunoreactivity of COX-2 was low in normal gastric wall and strongly increased in the tissue of the ulcer base [17] where it was identified in the cytoplasm of different cell types situated in regions of maximal repair activity. Immunoreactivity of COX-1 was located mainly in the non-ulcerated mucosa and was reduced after gastric ulceration in the mucosa adjacent to the ulcer crater. In the

same rat study, COX-1 immunoreactivity reappeared from day 5 onwards in the apical cytoplasm of the regenerative epithelial cells. Although the evolution of COX expression could not be assessed in this present in vitro study, the observations from rodent studies suggest that in chronic ulcers COX-1 and COX-2 may have different locations and different times of expression. If COX-2 is up-regulated in chronic gastric ulcers and inhibitors of COX-2 prevent the healing of ulcers then COX-2 may play an important role in acceleration of ulcer healing. Prostaglandins inhibit leukocyte adherence to the vascular endothelium[39], improving the resistance of the gastric mucosa to injury through the down regulation of inflammatory responses[40,41]. PGD_2 derived from the COX-2 pathway decreased granulocyte infiltration in experimentally induced colitis in rats[42]. COX-2 derived PGD_2 metabolite was shown to mediate PMNs and macrophages apoptosis during resolution of acute inflammation[43]. Recent findings in rats further support this hypothesis as selective COX-1 inhibition was shown to result in a reduction in gastric mucosal blood flow while selective COX-2 inhibition increased leukocyte adherence in mesenteric venules suggesting that the two COX isoforms differ in their biological activity [44]. It is unknown if these mechanisms apply as well to nonglandular ulcers.

In conclusion, this *in vitro* study suggests that, as demonstrated for glandular gastric tissue of rodents [28,45,46], COX-2 may play an important role in equine gastric ulcer healing in the nonglandular mucosa. Further studies are required to elucidate the clinical relevance of these findings and the potential impact of the administration of selective COX-2 inhibitors (such as firocoxib) on gastric ulcer healing in horses.

Footnotes

[a] **Picavet M-T. The Equine Gastric Ulcer Syndrome (EGUS) and preventive feeding. Proceedings of First European Equine Health and Nutrition Congress 2002.**

[b] Merck Frosst Centre for Therapeutic Research, Point-Claire, Dorval, Québec, Canada.

[c] Vectastain ABC kit, Vector Laboratories, Burlingame, CA.

[d] Sigma-Aldrich, Oakville, Ontario, Canada.

[e] Amersham Life Sciences, Arlington Heights, IL.

[f] Eastman Kodak, Rochester, NY.

[g] SAS Institute, Cary, NC.

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Table 1: **Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) Scoring Frequency According to Mucosal Status**

Table 1

	COX-1				COX-2			
Staining Score	0	1	2	3	0	1	2	3
Normal (n=10)	0	0	0	100	80	20	0	0
Ulcerated (n=38)	2.6	57.9	29	10.5	7.9	47.4	31.6	13.2

Frequencies are shown in percentages.

Figure 1: Cyclooxygenase Expression in Normal Equine Squamous Gastric Mucosa

A) Section stained with antibody MF241 (selective against COX-1) shows immunoreactivity mostly in fibroblast-like cells in the submucosa.
B) Section stained with antibody MF243 (selective against COX-2) shows no positivity. DAB substrate and hematoxylin counterstain. Bar = 50 μm.

Figure 1

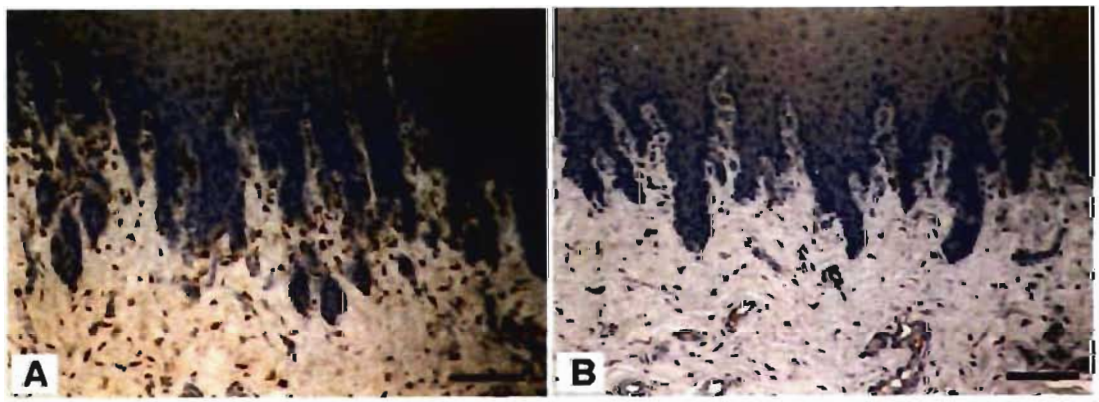


Figure 2: Cyclooxygenase-1 Expression in Ulcerated Equine Squamous Gastric Mucosa

A) COX-1-positive cells are reduced in number in many ulcers.
B) Numerous COX-1 positive cells are still present in some ulcerated stomach. DAB substrate and hematoxylin counterstain. Bar = 50 μm.

Figure 2

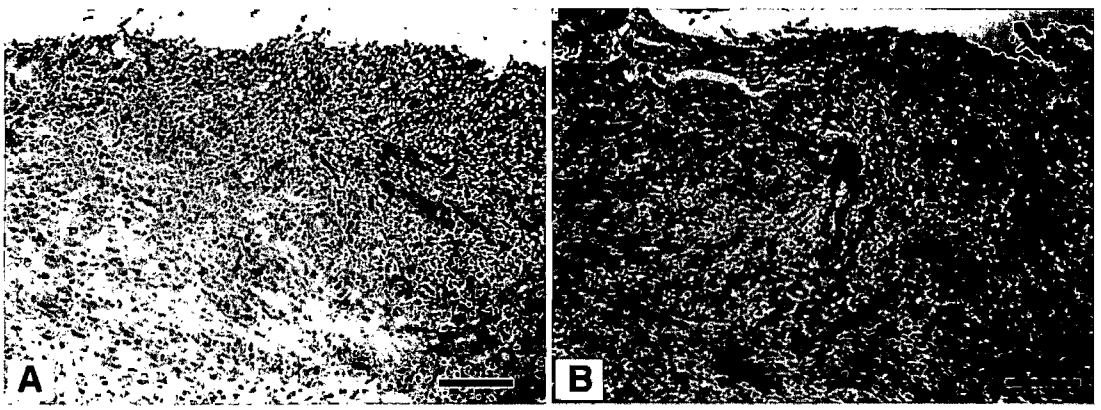


Figure 3: Cyclooxygenase-2 Expression in Ulcerated Equine Squamous Gastric Mucosa

A-B) Two ulcerated mucosa are shown with several COX-2 positive fibroblast-like cells;

C) COX-2 expression is also present in epithelial cells bordering the ulcer. D) Negative control staining with normal rabbit serum (without primary antibody). DAB substrate and HE counterstain. Bar = 70 μm.

Figure 3

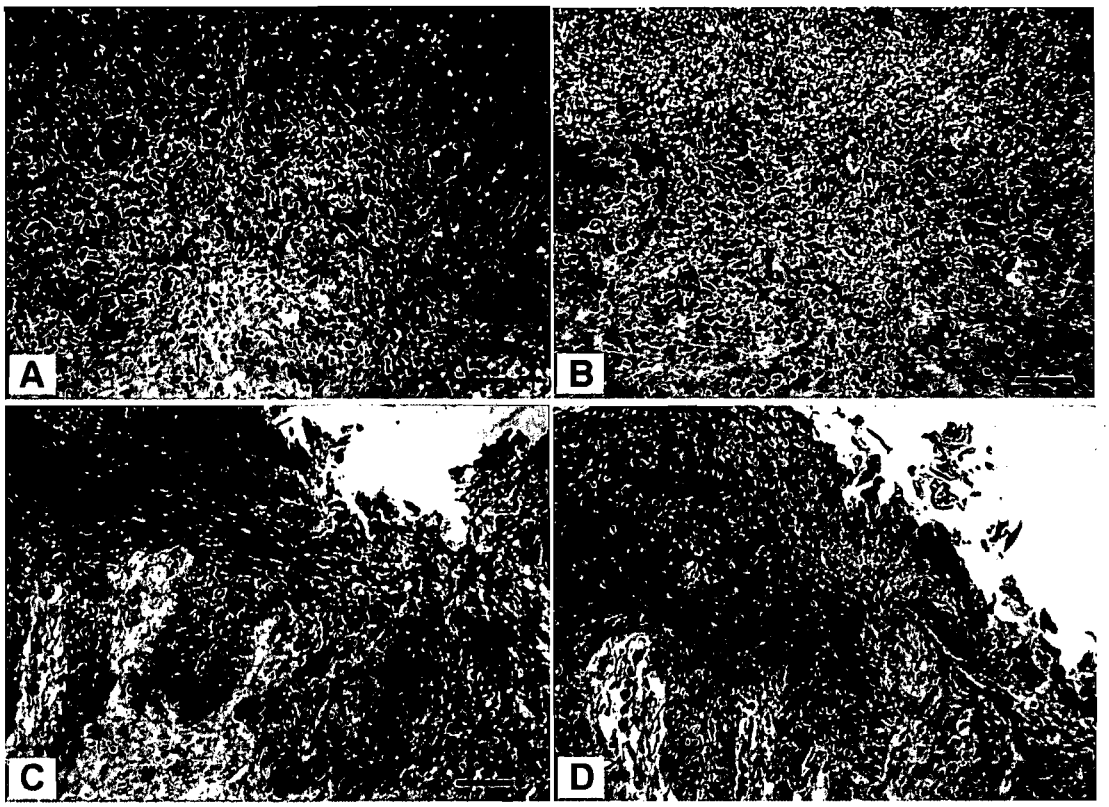
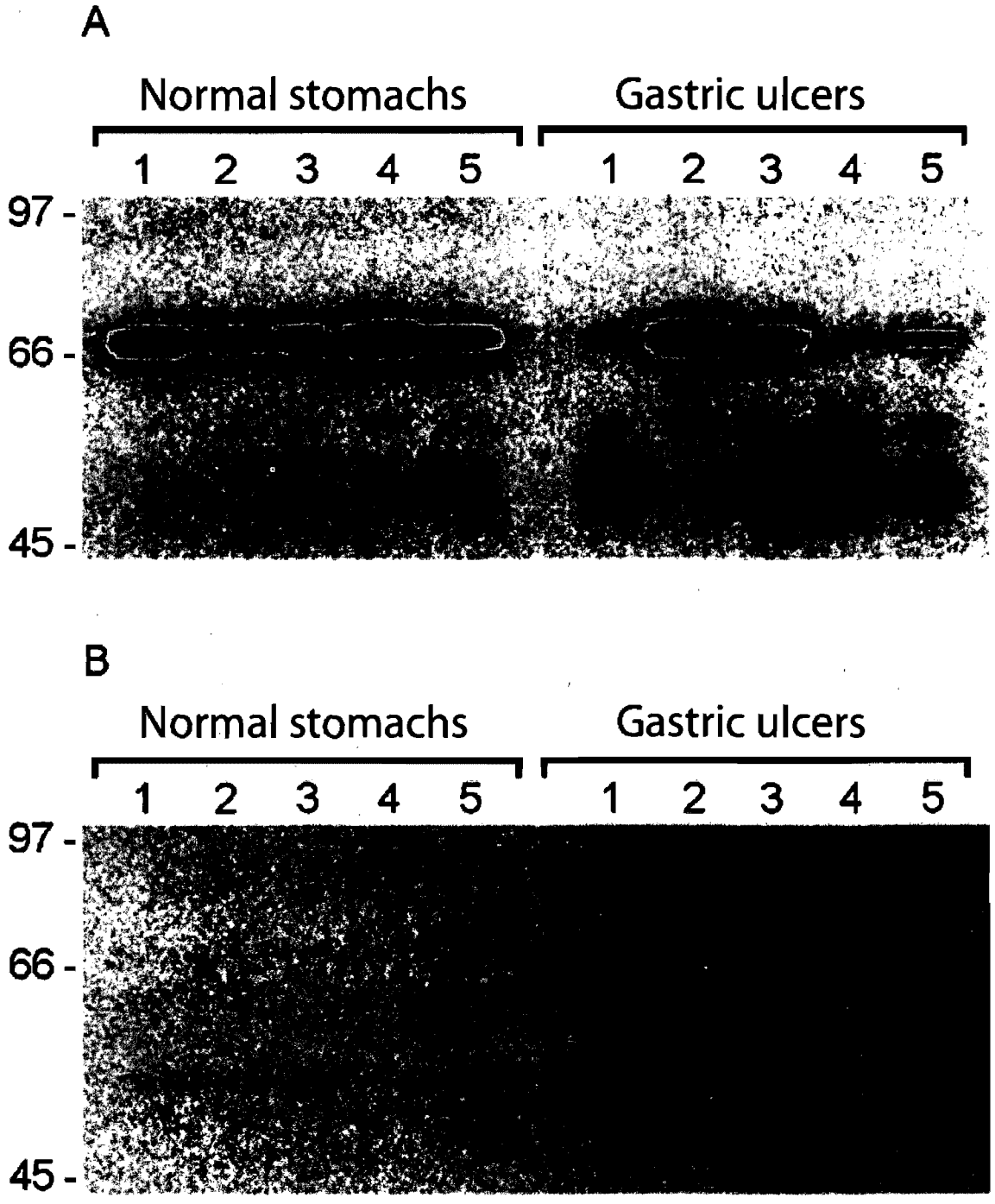


Figure 4: Western Blot Analysis of COX-1 and COX-2 Isoforms in Normal and Ulcerated Equine Squamous Gastric Tissues

A) Blots probed with antibody MF241 (selective for COX-1).COX-1 protein is strongly expressed in normal stomachs but markedly diminished in 3 out of 5 ulcerated samples.
B) Blots probed with antibody MF243 (selective for COX-2). No COX-2 protein is detected in normal stomachs but a strong COX-2 induction is present in all ulcers.

Figure 4


A
Normal stomachs
Gastric ulcers
1 2 3 4 5
1 2 3 4 5
97 -
66 -
45 -
B
Normal stomachs
Gastric ulcers
1 2 3 4 5
1 2 3 4 5
97 -
66 -
45 -

DISCUSSION

The equine stomach is composed of two distinct types of mucosa which have very different causes for ulceration. While the glandular portion tends to ulcerate due to non-steroidal anti-inflammatory drug use in horses of all ages or to stress in foals, risk factors for ulceration of the nonglandular portion are mostly related to feeding habits and exercise intensity in adult horses (The Equine Gastric Ulcer Council 1999; Merritt 2003). The current study performed in adult horses, focused on ulceration of the nonglandular portion of the stomach. While the stomachs of most monogastric animal species including humans have only glandular type mucosae, the only domestic animal species that shares a similar gastric histology with the horse is the pig. As a direct comparison, findings from this study will therefore be compared to limited data available for the porcine stomach while some similarities with human oesophageal ulceration (esophageal reflux disease) will also be made since human oesophagus is covered in squamous or nonglandular type mucosa as well. In light of limited data concerning the role of COX isoforms in nonglandular mucosa, studies performed in rats, mice and humans which all have exclusively glandular type gastric mucosae will also be discussed in an attempt to elucidate the mechanisms by which the expression of COX isoforms is modulated in general but indirect fashion.

This study demonstrates that normal nonglandular equine gastric mucosa expresses COX-1, and that most specimens do not express COX-2. Previous studies performed on human squamous oesophageal mucosa and on porcine nonglandular gastric mucosa (Lajoie, Sirois et al. 2002; Kase, Osaki et al. 2004; Martins, Artigiani Neto et al. 2007) reported similar observations and are in agreement with the basic understanding that COX-1 is the constitutive and COX-2 the inducible isoform of cyclooxygenase in most tissues (Herschman 1996; Williams and DuBois 1996). Although general theory would predict otherwise, COX-1 expression was significantly decreased in ulcerated equine nonglandular mucosa as it has also been reported in porcine naturally-occurring gastric ulcers (Lajoie, Sirois et al. 2002) and in rats after experimentally-induced gastric ulceration (Schmassmann, Peskar et al. 1998). While the rat stomach is lined

exclusively with glandular mucosa, porcine gastric ulcers are located in the nonglandular portion of the stomach, which is similar in structure and function to equine squamous mucosa and therefore has a pathophysiology comparable to equine squamous gastric ulcers.

The explanation for this decrease in COX-1 expression remains unclear at the moment. It could be hypothesized that this is a consequence of the loss of normal submucosa containing COX-1 positive cells and its replacement by granulation tissue containing COX-2 expressing fibroblasts. Alternatively, it could result from a negative feedback following the increase in COX-2 expression. However, the latter theory appears less plausible since no correlation was found between COX-1 and COX-2 expression in ulcerated gastric mucosal samples.

COX-2 expression was significantly induced in the ulcerated mucosa of adult horses. In pigs, COX-2 was also strongly expressed in naturally occurring porcine ulcers of the nonglandular portion of the stomach, compared to normal mucosa where COX-2 expression was absent or very low (Lajoie, Sirois et al. 2002). Cyclooxygenase-2 (COX-2) expressing cells in the current study were principally fibroblasts and epithelial cells located in the granulation tissue under the ulcer bed. Hardly any inflammatory cells were COX-2 positive and no correlation was found between either COX isoform expression and the intensity of the inflammatory reaction. Nonetheless, the score used to assess inflammation was only semi-quantitative. Similarly, in a study in human patients presenting symptoms of gastroesophageal reflux disease (Hamoui, Peters et al. 2004), no correlation was found between inflammation and COX-2 levels. COX-2 expression in the granulation tissue in equine ulcers suggests that this enzyme could be involved in the healing process of equine gastric ulcers. Despite that, it cannot be forgotten that COX-2 is responsible for PG production during inflammatory processes and is upregulated during an inflammatory response (Lee, Soyoola et al. 1992; Xie, Robertson et al. 1992; Meade, Smith et al. 1993; Mitchell, Akarasereenont et al. 1993; O'Sullivan, Huggins et al. 1993). Therefore COX-2 expression could simply be induced on the ulcerated gastric mucosa of horses because of its inflammatory functions.

Although very little is known of the role of COX-1 and COX-2 in nonglandular mucosal healing, the development of COX-1 and COX-2 knockout mice and selective COX-1 and COX-2 inhibitors has helped to elucidate the role of COX-1 and COX-2 in ulcer healing in glandular-type gastric mucosa. Wild-type, COX-$1^{-/-}$ and COX-$2^{-/-}$ mice with gastric ulcers were treated with selective COX-1 (SC-560), COX-2 (celecoxib, rofecoxib, and valdedoxib) and non-selective COX (piroxicam) inhibitors (Schmassmann, Zoidl et al. 2006). Healing was moderately impaired by COX-2 gene disruption and COX-2 inhibitors. Severe healing impairment was observed in dual (SC-560 + rofecoxib) and unselective (piroxicam) COX inhibition and combined COX impairment (in COX-$1^{-/-}$ mice with COX-2 inhibition and COX-$2^{-/-}$ mice with COX-1 inhibition). Inhibition of COX-1 or gene expression had no effect on ulcer healing. In rats with esophageal ulcers, it was shown that delayed ulcer healing caused by selective COX-2 inhibition was due to a reduction in epithelial cell proliferation (Baatar, Jones et al. 2002). Hepatocyte growth factor induction is alleged to be a very important contribution of prostaglandins to ulcer healing (Bamba, Ota et al. 1998). Since COX-2 mRNA and protein were increased in gastric ulcers induced by subserosal injection of acetic acid in mice (Mizuno, Sakamoto et al. 1997), cyclooxygenases have also been shown to be involved in ulcer healing, whilst no effect on COX-1 mRNA expression was observed in ulcerated or non-ulcerated mucosae. Prostaglandin increase (PGA_2, PGE_2, $PGF_{2\alpha}$) was demonstrated in ulcerated tissues when compared to normal ones and was inhibited by exposure to the COX-2 inhibitor NS-398 (Mizuno, Sakamoto et al. 1997) demonstrating that those prostaglandins were synthesized principally via the COX-2 pathway. All of the above suggests that, at least in glandular-type mucosae, COX-2 is an important mediator in promoting ulcer healing and COX-1 becomes imperative when COX-2 is impaired. Whether the same mechanisms can be extrapolated to nonglandular type mucosa such as in horses has yet to be clarified.

In this study, COX-2 immunoreactivity was primarily located in fibroblasts at the ulcer base. In rats with chronic ulcers, the immunoreactivity of COX-2 was low in normal gastric wall and strongly increased in the tissue of the ulcer base like in our equine specimens (Schmassmann, Peskar et al. 1998), where it was identified in the cytoplasm

of different cell types situated in regions of maximal repair activity. COX-1 immunoreactivity was located mainly in the non-ulcerated mucosa and was reduced after gastric ulceration in the mucosa adjacent to the ulcer crater. In the same experiment, COX-1 immunoreactivity reappeared from day 5 onwards in the apical cytoplasm of the regenerative epithelial cells. Even though the evolution of COX expression could not be assessed in this present *in vitro* study, the observations from rodent studies suggest that in chronic ulcers COX-1 and COX-2 may have different spatial and temporal expression patterns. If COX-2 is up-regulated in chronic gastric ulcers and inhibitors of COX-2 put off the healing of ulcers then COX-2 may play an important role in acceleration of ulcer healing. Other findings in rats sustain this hypothesis as selective COX-1 inhibition was shown to result in a reduction in gastric mucosal blood flow whereas selective COX-2 inhibition increased leukocyte adherence in mesenteric venules suggesting that the two COX isoforms differ in their biological activity (Wallace, McKnight et al. 2000).

Overall, this study suggests that, as established for glandular gastric tissue of rodents (Shigeta, Takahashi et al. 1998; Takahashi, Shigeta et al. 1998; Schmassmann, Zoidl et al. 2006), COX-2 may play an important role in equine gastric ulcer healing of the squamous mucosa. Complementary studies are essential to reveal the clinical consequence of these findings and the potential impact of the administration of selective COX-2 inhibitors on gastric ulcer healing in horses.

Tracking immunohistochemical changes in gastric specimens before and after ulceration, with and without the administration of selective COX-2 NSAIDs is a compelling way to study the role of COX-2 on EGUS healing. Trying to do so, the use of an endoscopic technique to take multiple biopsies of equine gastric mucosa *in vivo* was evaluated as a secondary pilot project (Appendix 1). The validation of an endoscopic biopsy technique that would be safe, repeatable and non-invasive in horses would be a step forth to facilitate the study of EGUS pathophysiology over time, because it could allow researchers to take multiple samplings with minimal inconvenience to the study subjects. Although the technique was safe, simple to execute, and appropriate for the glandular-type mucosae, it was not possible to obtain samples of

sufficient size and depth from the nonglandular gastric mucosae of horses used in this pilot study. Another technique should be investigated to perform repeated transendoscopic biopsies of the nonglandular mucosa of horses with the purpose to disclose the pathophysiology of EGUS *in vivo*.

CONCLUSION

Gastric ulcers have a high occurrence in athlete horses because of intensive training practices. Even though several hypotheses have been discussed, the pathogenesis of EGUS remains unclear. COX-2 up-regulation associated with gastric ulceration has been demonstrated in humans, pigs and now in horses.

Under physiologic conditions, COX-1 is strongly expressed in the equine gastric squamous mucosa, while COX-2 is barely discernible. However, when ulcers occur the expression of COX-1 diminishes and of COX-2 increases. Describing COX-1 and COX-2 expressions in the gastric mucosa of horses is the first step towards understanding the role of these isoenzymes in EGUS pathogenesis and healing.

Future research should investigate the role of the cyclooxygenase isoforms on equine gastric ulcers through an experimentally-induced model where endoscopic gastric biopsies would be obtained from healthy and ulcerated mucosa and analyzed by immunohistochemistry on a temporal basis. Until then, care is advised when treating horses at risk of having EGUS with selective anti-COX-2.

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APPENDIX I

Validation of a Transendoscopic Glandular and Nonglandular Gastric Biopsy Technique in Horses

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Summary

Reasons for performing the study: In order to study the evolution of histopathologic and immunohistochemical changes in the gastric mucosa of horses with EGUS (Equine Gastric Ulcer Syndrome), a feasible, useful, valid, and safe *in vivo* gastric biopsy technique was required.

Objectives: To determine the average gastric mucosal healing time following endoscopic gastric biopsy sampling and evaluate the feasibility, safety and usefulness of samples obtained by this method for histopathological analysis.

Methods: Six (6) adult mares from the Faculté de Médecine Vétérinaire research herd were used. Trans-endoscopic gastric biopsy was performed on days 0 and 9 using a flexible forceps with oval and fenestrated jaws to obtain gastric mucosal samples from 4 different sites: cardia (C), fundus (F), margo plicatus (MP) and glandular mucosa (GL). A maximum of 4 samples per site was taken. Samples were routinely processed for histopathological examination and evaluated by a pathologist. On days 1 to 4 and 9 to 11 the lesions created by the biopsies were evaluated by gastroscopy. Lesions were evaluated over time based on a score from 0 to 4, where a score of 4 was considered the most severe.

Results: Biopsy samples could be obtained from all targeted sites except the cardia. No abnormal clinical signs were observed up to 7 days post-biopsy. The average biopsy lesion scores decreased significantly with time for all sites. The average lesion score was significantly higher for the MP site compared to the other sites at days 1 and 2. Samples taken from the nonglandular portion of the stomach were considered inadequate for histopathology, while those taken from the glandular mucosa were adequate.

Conclusion: The transendoscopic gastric biopsy technique described here is a feasible, safe and useful technique for obtaining samples from the equine gastric glandular mucosa. Although biopsy samples could be obtained from several areas in the non-

glandular mucosa, these were very small, took longer to heal and were not considered adequate for histopathological evaluation. Hence, another technique will need to be validated for the nonglandular gastric mucosa.

Introduction

Endoscopic gastro-biopsy has been used as a reliable diagnostic method in human medicine since the 1970's (Gear and Dobbins 1969; Hatfield *et al.* 1975; Kasugai 1970) and in companion animal practice for approximately the last ten years (Mansell and Willard 2003; Sonea *et al.* 1999; Willard *et al.* 2001). Compared to full-thickness surgery techniques, endoscopic biopsy is generally preferred because it is less invasive, can be done under direct vision and allows multiple samples to be taken safely (van der Gaag 1994).

Flexible biopsy forceps are long and thin instruments which can be passed through the full length of the endoscope insertion tube. These forceps have a clenching bivalved cup at the end which is used to grasp and avulse the tissue from its biopsy site (Golden 1993; Waye 1981). Most authors agree that forceps with elongated, fenestrated cups yield superior quality biopsy specimens than those with round, closed cups. Also, it is considered that the absence of a needle results in deeper specimens compared to forceps with a needle (Mansell and Willard 2003; Woods *et al.* 1999). To ensure an accurate diagnosis by fiberoptic endoscopic biopsy of the gastrointestinal tract, it has been recommended to take six to eight samples for each anatomic region since pathological lesions are often multifocal and heterogeneous in these tissues (Mansell and Willard 2003; Waye 1981). In accordance with the same studies, each sample should contain mucosa and submucosa (i.e., muscularis mucosa) in order to allow an accurate interpretation of histologic findings by the pathologist.

Regrettably, only a few researchers have reported use of this technique in equine patients. In 2002, Murray and colleagues (Murray *et al.* 2002) presented an abstract on endoscopic duodenal biopsy. In this study, biopsies were performed on the duodenal ampulla of 15 foals and 54 mature horses using a 3 meter endoscope with a biopsy channel diameter of 2.8 mm. Although details of the comparison between endoscopic biopsies and full-thickness tissues were not reported, this procedure appeared to provide good quality mucosal specimens in most subjects and comparison with full-thickness

biopsies obtained in 6 horses seemed to indicate good reliability. In another abstract (Jean *et al.* 2004), endoscopic duodenal biopsy was performed in 8 horses with malabsorption syndrome and 6 control horses. Unfortunately, the instrument used for the procedures was not described. Specimens containing both mucosal and submucosal tissues were obtained and were adequate for histopathologic diagnosis of several types of enteritis within the malabsorption syndrome group. This study also showed that duodenal endoscopic biopsy was well tolerated by the horses.

The only full paper publication on equine gastric endoscopic biopsy (Murray *et al.* 2004) described a novel technique for obtaining larger sized biopsies in the gastric antrum of horses for microscopic diagnostic purposes. Fifteen (15) mature horses were used. The technique consisted of attaching a polypectomy snare on the outer part of the endoscope and connecting it to a unipolar electrocautery unit. A biopsy forceps was used to grab the mucosa while the loop was closed around it and the electrocautery was activated. Generally, the samples were of reasonable size, included the entire mucosa and were considered satisfactory for histological examination. The majority of horses had negligible bleeding and no discomfort related to this biopsy technique however the healing process was not assessed past day 1.

To date, all information published regarding endoscopic gastro-intestinal biopsy in horses has been favourable with regards to safety and acceptability of samples obtained for histopathologic evaluation. However, none of the studies discussed above have assessed the healing process of the lesions created on the gastric or intestinal mucosa which is an important criterion to evaluate when considering multiple sampling over a short period of time for research purposes. Furthermore, evaluation of sample quality in various regions of the gastric mucosa has not yet been reported in the horse. For the study of the pathophysiology of EGUS or other gastric pathologies *in vivo*, samples from different anatomic regions in the equine stomach could be of great value. The purpose of this study was therefore to investigate the feasibility, healing time, safety and usefulness of a trans-endoscopic gastric mucosal biopsy technique for multiple sampling of various sites in the stomach in adult horses.

Materials and methods

Study subjects

Six (6) Standardbred and Arab breed mares from the research herd of the Faculté de médecine vétérinaire (FMV), aged between 8 and 15 years and weighing between 420 and 540 kg were used. Mares were housed at the FMV equine research facility, kept in their normal environment and fed their standard ration for the duration of the study. Horses that presented clinical signs of systemic disease and/or behavioural problems were considered unsuitable. In order to confirm inclusion in the study, a complete physical examination was performed on Days 0 and 9 including assessment of temperature, heart and respiratory rates as well as a complete physical examination.

Study design

Endoscopic biopsies were performed on days 0 and 9. Daily gastroscopic examinations were carried out on days 0 to 4 and 9 to 11 in order to evaluate mucosal healing and clinical safety of the gastric biopsy technique. All horses were fasted for a minimum of 12 hours prior to each gastroscopic examination. On days 1 to 4 and 9 to 11 the lesions created by the biopsies were evaluated by endoscopy in order to assess their evolution over time. All gastroscopies, biopsies and evaluations of the lesions were recorded with a digital video camera for further evaluation. The experimental protocol was approved by the Institutional Animal Care and Use Committee (CEUA, Faculté de médecine vétérinaire, Université de Montréal).

Trans-endoscopic gastric biopsy technique

Endoscopy was performed as previously described (Roy *et al.* 2005). Xylazine hydrochloride (AnaSed, 0.5mg/kg, i.v.)[1] was administered as needed for minimal

sedation. Four distinct sites, namely cardia (C), fundus (F), margo plicatus (MP) and glandular mucosa (GL) were targeted for sampling. At most, 4 specimens were taken per site. A reusable, 3 m long, oval shaped, fenestrated distal cup forceps (model KW2430S)[2] (Figure 1) measuring 2,4 mm in diameter was inserted via the 2.9 mm diameter working channel of the endoscope[2]. The tip of the endoscope was placed at a nearly 90 degree angle with the gastric mucosa, and the forceps was advanced to grasp the mucosa with its open jaws. The forceps was then closed quickly and tightly while the endoscope was held as straight as possible to protect the biopsy channel and allow the retraction of the forceps. Next, the forceps was briskly pulled back through the endoscope thus sectioning the tissue in the process. The biopsy samples were carefully removed and immediately fixed in 10% neutral buffered formalin while the endoscope remained in position for collection of other samples.

Evaluation of clinical safety

Daily records assessing health status, appetite, feces appearance, concomitant medications, health problems, temperature, pulse and respiration were completed from Days 0 to 13 in order to assess clinical safety of gastric biopsies.

Evaluation of biopsy lesions

The healing process of the gastric mucosa was assessed, using the recorded videotapes of gastroscopies performed on Days 1, 2, 3, 4, 9, 10 and 11, by two independent evaluators. The worst lesion per site was considered for evaluation, using a newly established visual scoring system based on macroscopic changes observed during gastroscopy described in Table 1 and illustrated in Figures 2 and 3.

Evaluation of biopsy sample usefulness

All formalin-fixed tissues were routinely processed, embedded in paraffin and cut into sections of 3-μm thickness. Tissues sections were stained with hematoxylin, eosin, phloxin and safran, and evaluated by a pathologist. Specimens were classified as adequate or inadequate for histopathologic diagnosis based on quality, size and depth.

Data analysis

Statistical analyses were performed using computer software (SAS V.9.1)[3]. The kappa coefficient was used to determine the agreement between the evaluators for the assessment of lesion healing scores. The Cochran-Mantel-Haenszel (CMH) test for repeated measurements, using site as a categorical variable and day and score as ordinal variables was used to establish the effect of time and site on the lesion score. The effect of day (1 versus 9) of biopsy was also evaluated using the CMH test. Values were considered significant at $p < 0.05$.

Results

Five horses completed the study. One horse was removed at day 7 due to behavioural problems that were unrelated to the study. No other adverse events were observed during the period of this study.

Biopsy samples could be obtained from all targeted sites except the cardia region because of technical impediment. On day 0, 24 and 23 samples were collected respectively from the fundus and the margo plicatus of 6 mares. Because of significant post-biopsy bleeding of the glandular gastric mucosa in the first two horses, a total of 4 samples (2 per mare) were collected from this site on Day 0 until further assessment could be made of short-term healing and side-effects. Furthermore, due to their very small size, some nonglandular mucosal samples were lost in the process of preparing the histology slides leading to decreased sample numbers from Day 1. On day 9, in order to increase or complete the number of samples at each site, ten (10) samples were collected from the fundus of 4 mares while 8 samples from the margo plicatus and 17 samples from the glandular gastric mucosa were collected from 5 subjects. All new samples obtained on day 9 were obtained in regions where biopsies had not been performed on Day 0 in any given subject. Daily median lesion scores for each site are presented in Figure 4. As statistical analyses confirmed that there was no effect of day of sampling on lesion score change over time, scores from biopsies performed on day 0 were combined with those taken on Day 9. A kappa coefficient of 0.66 indicated a good agreement between evaluators for lesion scores while; only data from the senior evaluator were used for the Cochran-Mantel-Haenszel test.

Lesion scores presented a significant temporal linear decrease for all sites ($p=0.02$, $p<0.0001$ and $p=0.0002$ for sites GL, MP and F, respectively).

Significant differences were found between average lesion scores by site on Days 1 and 2 ($p = 0.005$ and $p = 0.007$, respectively). Further two by two comparisons indicated

that the average lesion score was significantly higher in site MP than in site GL or site F while there was no significant difference between site GL and site F.

Histopathologic evaluation revealed that all biopsy specimens obtained from the nonglandular squamous mucosa were inadequate and samples from the glandular mucosa were adequate for diagnostic purposes. In general, samples obtained from all sites were very small, in the range of 2x2 mm for the nonglandular samples and 3x3 to 4x3 mm for the glandular samples (Figures 5A and 5C). Samples collected from the glandular portion of the stomach contained the entire mucosa and the superficial portion of the lamina propria, while the nonglandular samples contained only the stratified squamous epithelium (Figure 5).

Discussion

In accordance with previous studies on equine gastro-intestinal endoscopic biopsies, no adverse clinical signs were observed, indicating that the technique described in this study is clinically safe when repeated twice within 9 days (Jean *et al.* 2004; Murray *et al.* 2004; Murray *et al.* 2002).

The transendoscopic biopsy technique was generally simple to execute for all regions, except the cardia. At this site, it became impossible to direct the forceps close enough to the mucosa because of the flexed position the endoscope takes when observing the cardia.

Furthermore, it is of note that, as others have reported in dogs (Tams 1990), when the gastric folds are flattened it becomes more difficult for the forceps to hold and grasp the mucosa in horses. Therefore, it is recommended to avoid over distending the stomach with air, as is frequently done with most gastroscopic examinations in horses.

Although it has not been validated using post-mortem tissue samples as a gold standard, the lesion scoring system used on this study was found to be useful as a visual reference and estimate of lesion evolution over time. Healing time was significantly delayed in the nonglandular margo plicatus compared to the nonglandular fundus or the glandular mucosal sites in this study. The glandular mucosa, for one, is histologically different and physiologically better adapted to healing in an acidic environment compared to the nonglandular mucosa in general. In fact, defence mechanisms such as surface mucus secretion, bicarbonate secretion into mucus, mucosal blood flow, apical surface membrane transport, epithelial regenerative capacity and elaboration of prostaglandins (Crawford and Kumar 2003; Gelberg 2007; Herdt 2007; Sjaastad *et al.* 2003) most likely contributed to the relatively rapid healing of biopsy lesions performed at this site. Although, the nonglandular mucosa also benefits from defence mechanisms to aid in repair, primarily associated with its histological characteristics (Murray 1994; Picavet 2002; The Equine Gastric Ulcer Council 1999), it is not well adapted to continual

exposure to destructive elements such as acid and pepsin. Hence, biopsy lesions created along the nonglandular *margo plicatus* may have healed slower because of the constant proximity of the gastric juices. This has also been proposed to explain why naturally occurring gastric ulcers of the nonglandular mucosa are more predominantly found along the *margo plicatus* in adult horses (Bezdekova *et al.* 2007; Dionne *et al.* 2003; The Equine Gastric Ulcer Council 1999).

The fact that samples obtained in this study were generally of very small size could be deemed as one disadvantage of using the transendoscopic gastric biopsy technique since the size of the biopsy sample is limited by the size of the biopsy channel of the endoscope (Tams 1990). This also likely contributed to the poor quality of the histologic samples obtained from the nonglandular mucosa compared to the larger ones obtained from the glandular mucosa.

In conclusion, the transendoscopic technique described in this study yielded samples of adequate size and depth for histopathologic assessment of gastric biopsy samples taken in the glandular mucosa of horses. However, in order to perform repeated transendoscopic biopsies of the nonglandular mucosa for diagnostic or investigative purpose, other techniques using different instruments should be investigated with the intention of obtaining larger and deeper samples. Serrated edge biopsy cups have been reported to provide larger samples than straight edge forceps (Tams 1990). The addition of serrated forceps to the multiple bite approach could possibly grant enough material for satisfactory histological diagnosis of the nonglandular squamous mucosa in horses as well. The use of the polypectomy snare, as proposed by Murray and colleagues for obtaining samples from the gastric antrum could be another interesting option for obtaining biopsies in this region of the equine stomach (Murray *et al.* 2004).

Acknowledgements

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Manufacturers' addresses

[1] Novopharm Limited, Toronto, ON, Canada.
[2] Pentax Canada Inc, Mississauga, ON, Canada.
[3] SAS Institute Inc, Cary, NC, USA.

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Authors' contributions: All authors contributed to all aspects of this study.

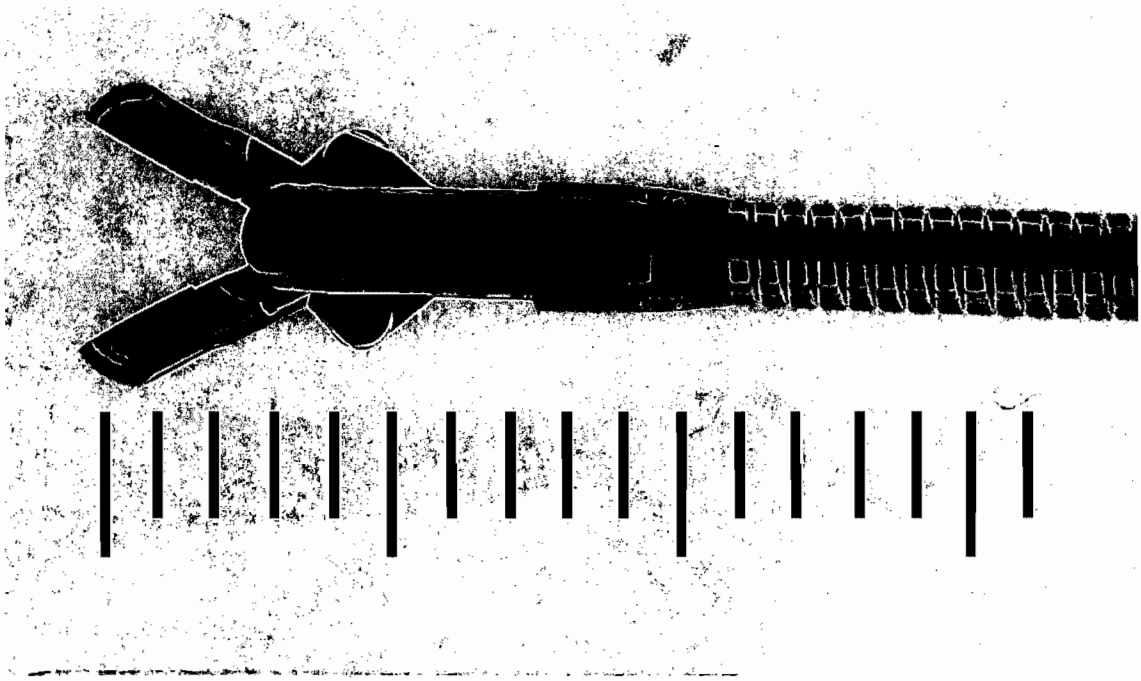
Table 1: Gastric Biopsy Lesion Scoring System

Table 1

Score	Description
0	Intact mucosa (lesion no longer detectable)
1	Intact mucosa with some redness
2	Granulation tissue covers entire lesion although healing remains incomplete
3	Some granulation tissue and contraction of lesion (initial healing)
4	Fresh lesion with or without bleeding (no appearance of healing)

Figure 1: Biopsy Forceps Model (Pentax, KW2430S) used in this study.

Figure 1



Ruler is in millimetres.

Figure 2: Examples of Endoscopic Visual Healing Scores Used for Biopsies Performed in the Nonglandular Portion of the Equine Stomach

Examples of lesions scored 1 (A), scored 2 (B), scored 3 (C) and scored 4 (D).

Figure 2

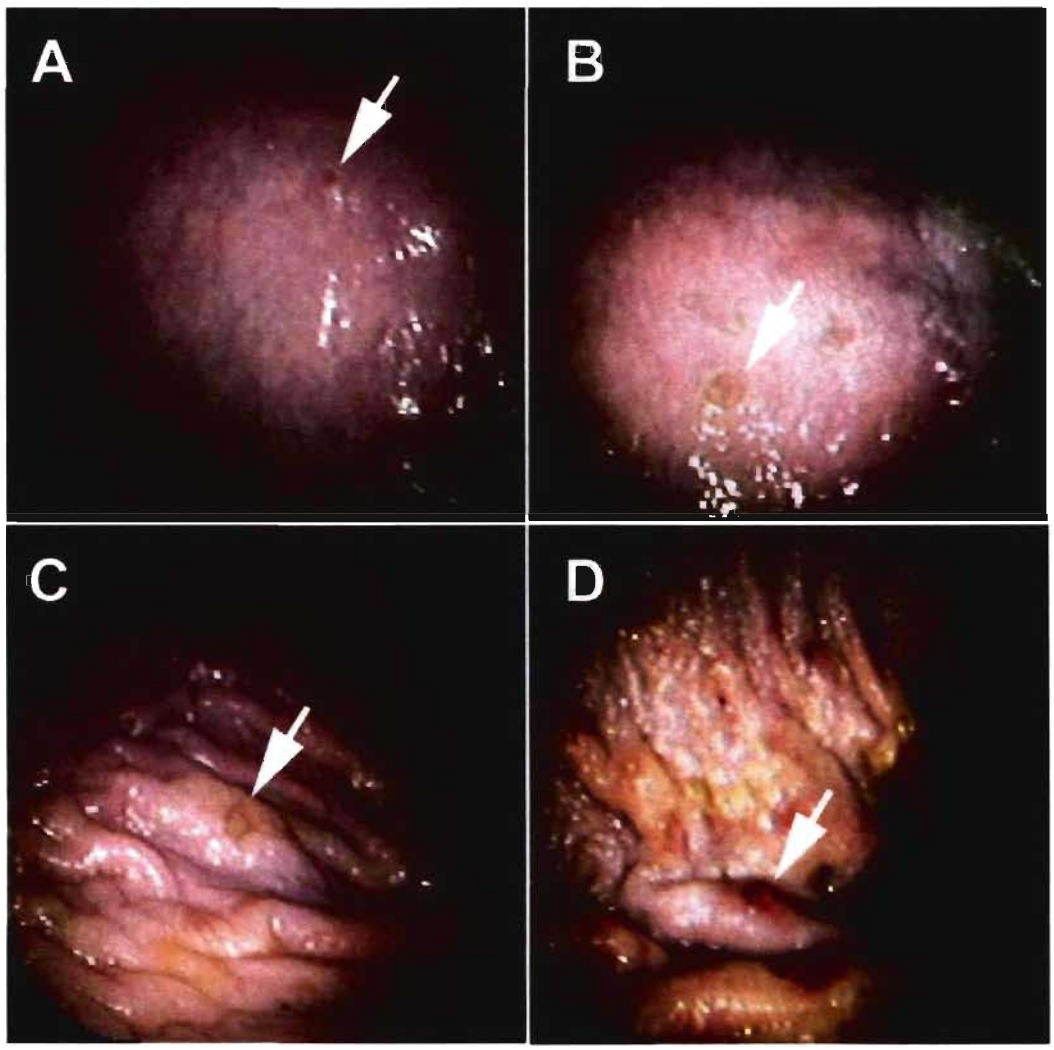


Figure 3: Examples of Endoscopic Visual Healing Scores for Biopsies Performed in the Glandular Portion of the Equine Stomach

Examples of lesions scored 1 (A), scored 2 (B), scored 3 (C) and scored 4 (D).

Figure 3

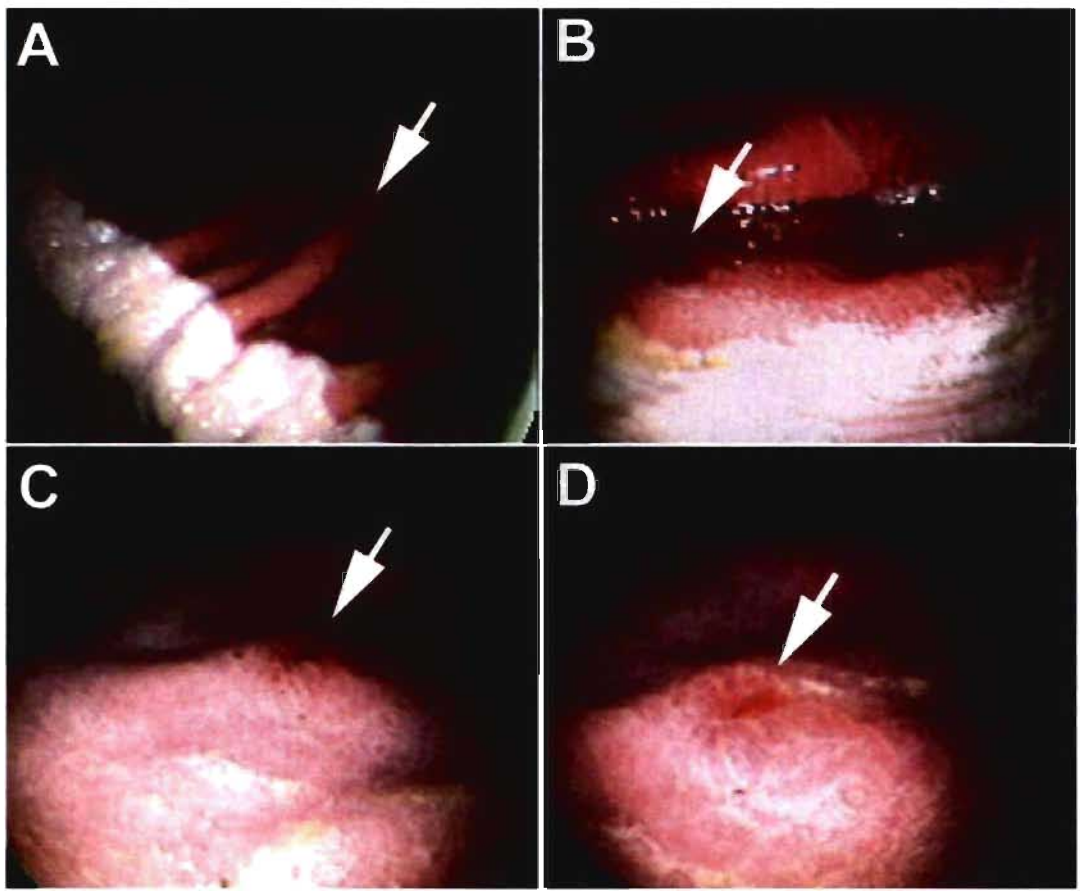
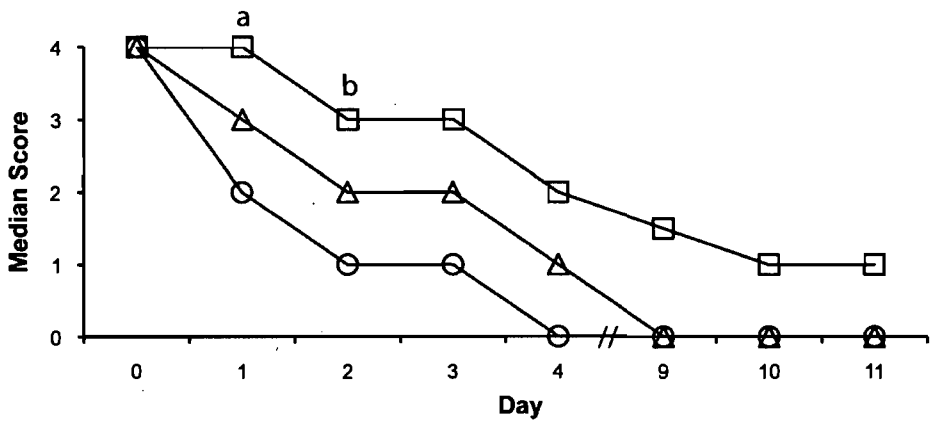


Figure 4: Median Gastric Biopsy Lesion Healing Scores for the Margo Plicatus (MP, Squares), Fundus (F, Triangles) and Glandular Mucosa (GL, Circles)

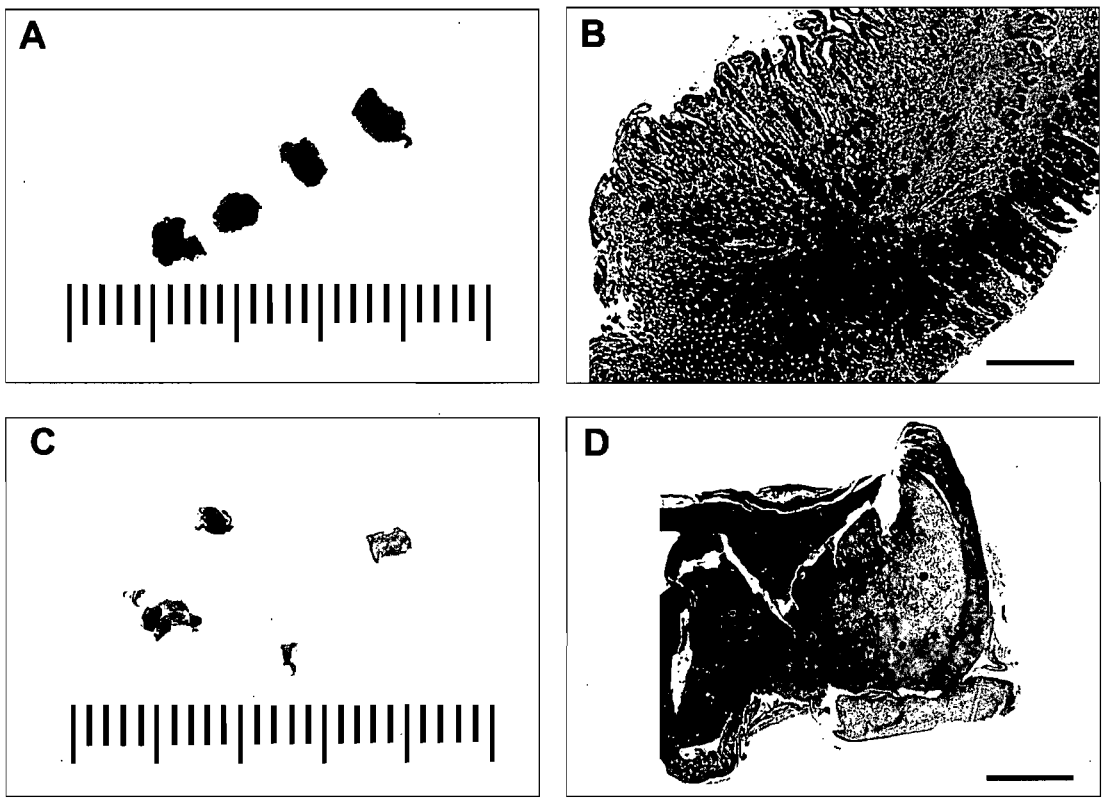
Figure 4



a: Scores for MP significantly greater than for F and GL at p = 0.005. b: Scores for MP significantly greater than for F and GL at p = 0.007.

Figure 5: Macroscopic Views (A and C) and Photomicrographs (B and D) of Equine Gastric Glandular Mucosa (A and B) and Squamous Mucosa (C and D) Samples

Figure 5:



Ruler in macroscopic views is in millimetres. Bar in photomicrographs represents 500 μm.

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DESCRIPTION DES ARTICLES

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Titre
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Auteurs
Natalia Rodrigues, Monique Doré et Michèle Y. Doucet
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