JOURNAL OF
Veterinary Emergency
AND Critical Care

Original Study

Journal of Veterinary Emergency and Critical Care **00**(0) 2016, pp 1–8
doi: 10.1111/vec.12481

Assessment of systemic inflammation by time-trends of blood granulocyte count and plasma myeloperoxidase and elastase concentrations following colic surgery in horses

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Abstract

Objective – To determine changes in blood granulocyte counts and in plasma myeloperoxidase (MPO) and elastase (ELT) concentrations in surgical colic cases, and to determine the relationship between these changes and the surgical procedure performed, occurrence of postoperative ileus, and final outcome.
Design – Prospective clinical study conducted over a 12-month period.
Setting – University teaching hospital.
Animals – Fifty-three horses undergoing emergency laparotomy and surviving at least 12 hours postoperatively.
Interventions – Blood samples were taken before surgery, during surgery, at the recovery from anesthesia, and then serially until the 150th hour after the first blood sampling. Granulocyte counts were performed by an automated cell hematology analyzer. Specific ELISAs were performed for the MPO and ELT measurements. Mixed models were used to compare the time-trends of the 3 parameters.
Measurements and Main Results – Taking all horses together, the time-trends of MPO and ELT were not significantly different from each other, but they were significantly different from the granulocyte time-trend. The type of surgical procedure did not influence the time-trends of the 3 parameters. Significant changes in the granulocyte time-trends were associated with postoperative ileus and outcome. Significant changes in the MPO time-trends were associated with outcome. The ELT time-trends were not influenced by ileus or outcome.
Conclusions – Granulocyte counts and MPO change over time and are related to the severity of the inflammatory reaction in surgical colic cases. These time-trends may allow evaluation of treatment efficacy in an effort to modulate excessive granulocyte activation and degranulation.

(J Vet Emerg Crit Care 2016; 00(0): 1–8) doi: 10.1111/vec.12481

Keywords: blood, equine critical care, gastrointestinal colic, inflammation, statistical modeling, trends

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This study was partially funded by Fonds spéciaux de la Recherche 2009.

Preliminary results of this study were presented in abstract form at the 10th International Equine Colic Research Symposium (AAEP), Indianapolis, July 2011.

The authors declare no conflicts of interest.

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Submitted January 08, 2014; Accepted November 29, 2014.

Abbreviations

ELT	elastase
MPO	myeloperoxidase

Introduction

Despite considerable progress in the area of surgery and intensive care, colic remains a major cause of equine morbidity and mortality.[1–3] Hospital discharge rates of 54.5–70.3% have been reported for horses undergoing colic surgery,[4–8] and persistent pain/colic, postoperative ileus, endotoxemic shock, peritonitis, colitis/diarrhea, laminitis, jugular thrombophlebitis, or incisional drainage/herniation can complicate the postoperative period.[5,6,9–12]

Leukocytes, and especially neutrophils, are involved in the pathogenesis of ileus,[13] peritonitis,[14,15] laminitis,[16,17] and wound healing.[18] Tissue injury as occurs during colic and surgery may result in systemic neutrophil activation and systemic inflammatory response syndrome.[19–21] Systemic inflammatory response syndrome can be seen in horses suffering from colic and postoperative complications,[22–24] and can lead to life-threatening problems such as laminitis and renal failure. It has been shown in horses with colic that the kinetics of blood leukocytes during the perioperative period differ with outcome, and that postoperative blood leukocyte counts may predict outcome.[25]

Activation of neutrophils induces the production of reactive oxygen species and the release of inflammatory mediators and several oxidative and proteolytic enzymes such as myeloperoxidase (MPO) and elastase (ELT).[26,27] MPO exacerbates mitochondrial damage initiated by the ischemia-reperfusion phenomenon, and thereby alters mitochondrial function.[28] Increased concentrations of MPO and ELT in plasma, other biologic fluids, and tissues are considered markers of neutrophil activation and degranulation.[17,29–31] Plasma concentrations of MPO are higher in horses after colic surgery than at admission, are different in survivors and nonsurvivors of colic surgery,[32] and differ between strangulated and nonstrangulated intestinal pathology.[33] Plasma ELT concentrations are also increased in horses with colic.[34] However, investigations regarding MPO and ELT concentrations in horses with colic involved exclusively large intestinal pathology or reported measurements at limited points in time. Comparing serial blood granulocyte counts with plasma ELT and MPO concentrations during the perioperative period would improve the understanding of granulocyte activation and degranulation in surgical colic cases. This knowledge could help in the development of new therapies targeted against the degranulation products of granulocytes.

The aims of this study were to describe the time-trends of blood granulocyte counts and plasma MPO and ELT concentrations during the perioperative period in horses undergoing colic surgery, and to evaluate the relationship between such time-trends and the type of surgical procedure, the presence of ileus, and outcome. The authors hypothesized that granulocyte count and MPO and ELT plasma concentrations would be related to surgical procedure, ileus, and survival after abdominal surgery in horses.

Materials and Methods

Horses

The study included 53 horses that underwent colic surgery at the Equine Clinic of the Faculty of Veterinary Medicine, University of Liège, Belgium, over a period of 12 months. Only horses with a survival time of at least 12 hours following surgery were included. Signalment and clinical data were obtained from a review of the medical records. All horses underwent a complete physical and rectal examination as well as a nasogastric intubation at admission. Venous blood gas analysis and hematologic and biochemical profiles were performed. Peritoneal fluid was recovered when available. All horses were treated surgically by ventral midline celiotomy under general anesthesia. Surgical procedures entailed gas decompression, repositioning, small intestinal massage, enterotomy, and/or resection and anastomosis. Following recovery from anesthesia, the horses were kept in the ICU and treated with intravenous lactated Ringer's solution[a] supplemented with glucose and electrolytes as required, and removal of gastric reflux until progressive intestinal motility resumed. Routine postoperative medication included sodium penicillin[b] (20,000 UI/kg IV q 6 h) and gentamicin[c] (6.6 mg/kg IV q 24 h) for 8–10 days following surgery. Enoxaparin[d] (160 mg/horse SC q 24 h) and flunixin meglumine[e] (0.25 mg/kg IV q 8 h) were given for a minimum of 3 days after surgery, while phenylbutazone[f] (2 mg/kg IV q 12 h) was given during the first 5 days after surgery. Additional analgesia was provided as necessary. Gastrointestinal prokinetics were used in all cases with small intestinal involvement and in large intestinal cases that developed ileus. All cases received lidocaine[g] at 1.3 mg/kg + 0.05 mg/kg/min IV as a constant rate infusion. Some cases that did not respond to lidocaine received metoclopramide[h] or erythromycin[i] depending on the surgeon's preference. Antimicrobial therapy and other treatments were tailored to the patient's response to treatment.

The affected portion of the gastrointestinal tract, the disease process, the lesion type (strangulated vs nonstrangulated obstruction or inflammatory bowel disease) and the surgical procedure were recorded. Surgical procedure was classified as "resection and anastomosis and/or an enterotomy" versus "other manipulations without incising the intestinal wall." Postoperative complications were recorded. Ileus was defined as a nasogastric reflux volume >20 L during a 24-hour period after surgery or a volume >8 L at any single sampling time after surgery, as reported by Roussel et al.[35] The outcome of hospitalization was categorized as survival to discharge or death. Horses euthanatized solely for financial reasons were excluded from the study.

Blood sample collection and handling

Venous blood was collected into tubes containing EDTA[j] before surgery (at admission for most cases, and considered 0 h); during surgery after correction of the intestinal lesion (considered 3 h); and during anesthetic recovery (considered 6 h). During the postoperative period, samples were taken every 4 hours during the first 4 days (from 10 h until 102 h) and then every 12 hours until day 6 (150th h after the first blood sampling) or until euthanasia. Blood was analyzed hematologically[k] and then centrifuged at 1,000 × g[l] for 10 min at room temperature. The plasma was aliquoted and frozen at –20°C until assayed.

Granulocyte counts and MPO and ELT assays

Total and differential leukocyte counts were performed at the time of sampling by use of an automated impedance cell hematology analyzer. This automated impedance cell hematology analyzer has shown reliable and acceptable results for equine differential leukocyte counts.[36] The reference interval of granulocyte blood counts of 3–9.0 × 10^6/mL was established by our laboratory. MPO was assayed with a specific ELISA[m] developed by Franck et al[37] and validated for the assay of equine MPO in EDTA plasma diluted 40× before the assay. Neutrophil ELT was assayed with a specific ELISA[n] developed by de la Rebière de Pouyade et al[34] and validated for the assay of equine ELT in EDTA plasma diluted 6× before the assay. Normal MPO concentration of healthy horses of 181.80 ± 64.74 ng/mL (mean ± SD) was determined in our laboratory and previously reported by Franck et al.[37] Normal concentration of ELT of 32.53 ± 4.6 ng/mL was determined in our laboratory and reported previously by de la Rebière de Pouyade et al.[34]

Statistical analysis

Normality assumptions were tested using univariate analysis (Shapiro-Wilk, Kolmogorov-Smirnov statistic, the Anderson-Darling statistic, and the Cramér-von Mises). The value of the Shapiro statistic was 0.34 for MPO, 0.85 for granulocytes, and 0.56 for ELT. Next, a box-cox transformation (SAS procedure proc TRANSREG) was performed to identify a transformation to normality. The transformation was the log for all variables. Then, normality assumptions of the log-transformed variables were tested again with the univariate procedure. The associated P values were close to $P = 0.01$, much better than the P values ($P < 0.0001$) observed on the crude data. The histogram was analyzed. The values of the Shapiro statistic were 0.96 for log(MPO), 0.98 for log(granulocytes), and 0.99 for log(ELT).

Part 1 – The mixed model used to analyze whether time-trends of the 3 parameters (granulocytes, MPO concentration, ELT concentration) were different was as follows:

$$yijklt = \mu + Sl + b1Wikl + b2Aikl + b3\,Tiklt + b4kT^2iklt + Iikl + \varepsilon iklt \quad (1)$$

where $yijklt$ is the value at time t for the kth parameter ($k = 1, 2, 3$) for the ith individual ($i = 1, 2, \ldots, 53$); μ is the overall mean; Sl is the fixed effect of sex ($l = 1, 2, 3$); $Wikl$ and $Aikl$ are the values for weight and age at the start of the study; $Tiklt$ is the time in days since the entry in the study; $\varepsilon ijklt$ is the error term; and $b1$, $b2$, $b3k$, and $b4k$ are the regression coefficients. The variable $Iikl$ is a dummy variable taking the value of 1 if $Tiklt = 0$, and $Iikl = 0$ otherwise. The random effects $\varepsilon iklt$ were assumed normally distributed with null mean and var($\varepsilon iklt$) block diagonal with blocks corresponding to the animals and with each block having the compound-symmetry structure. This structure was chosen to allow cross-equation contemporaneous correlations. Contrasts between the estimates of $b3k$ (and $b4k$) were computed to test whether linear (and quadratic trends) were different across parameters.

Part 2 – Three separate mixed models were applied to analyze whether time-trends were similar according to each of the 3 binary factors under study, that is, intraoperative manipulations ("resection and anastomosis or enterotomy" or "other manipulations without incising the intestinal wall"), development of postoperative ileus (yes or no) or outcome (death or survival to discharge). Each model was as follows:

$$yijklt = \mu + Sl + b1Wijkl + b2Aijkl + b3jkTijklt + b4jkT^2ijklt + Iijkl + \varepsilon ijklt \quad (2)$$

where $yijklt$ is the value at time t for the kth parameter ($k = 1, 2, 3$); μ is the overall mean; Sl is the fixed effect of sex ($l = 1, 2, 3$); Wi and Ai are the values for weight and age at the start of the study; Tit is the time in days since the entry in the study; $\varepsilon ijklt$ is the error term; and $b1$, $b2$, $b3jk$, and $b4jk$ are the regression coefficients with j representing a category of the factor ($j = 1, 2$). The variable Iik is a dummy variable taking the value of 1 if $Tit = 0$ and $Iik = 0$ otherwise. Differences between the estimates of $b31k$ and $b32k$ were computed to test whether linear trend in the kth parameter was different between categories of the factor (as above, intraoperative manipulations; development of postoperative ileus; outcome).

 doi: 10.1111/vec.12481

Table 1: Predominant lesion of each horse diagnosed at the time of surgery for colic

Small intestine ($n = 22$)	**Volvulus ($n = 11$)**
	Incarceration in the epiploic foramen ($n = 4$)
	Enteritis ($n = 2$)
	Strangulation by a lipoma ($n = 2$)
	Ileocecal intussusception ($n = 1$)
	Incarceration in a mesenteric defect ($n = 1$)
	Ileal impaction ($n = 1$)
Large intestine ($n = 31$)	Large colon displacement ($n = 14$, of which 7 were nephrosplenic entrapments)
	Large colon torsion ($n = 8$)
	Typhlitis/colitis ($n = 3$)
	Cecocolic intussusception ($n = 2$)
	Cecal torsion ($n = 2$)
	Colitis with thromboembolism of the pelvic flexure ($n = 1$)
	Abdominal wall perforation with peritonitis and typhlitis ($n = 1$)

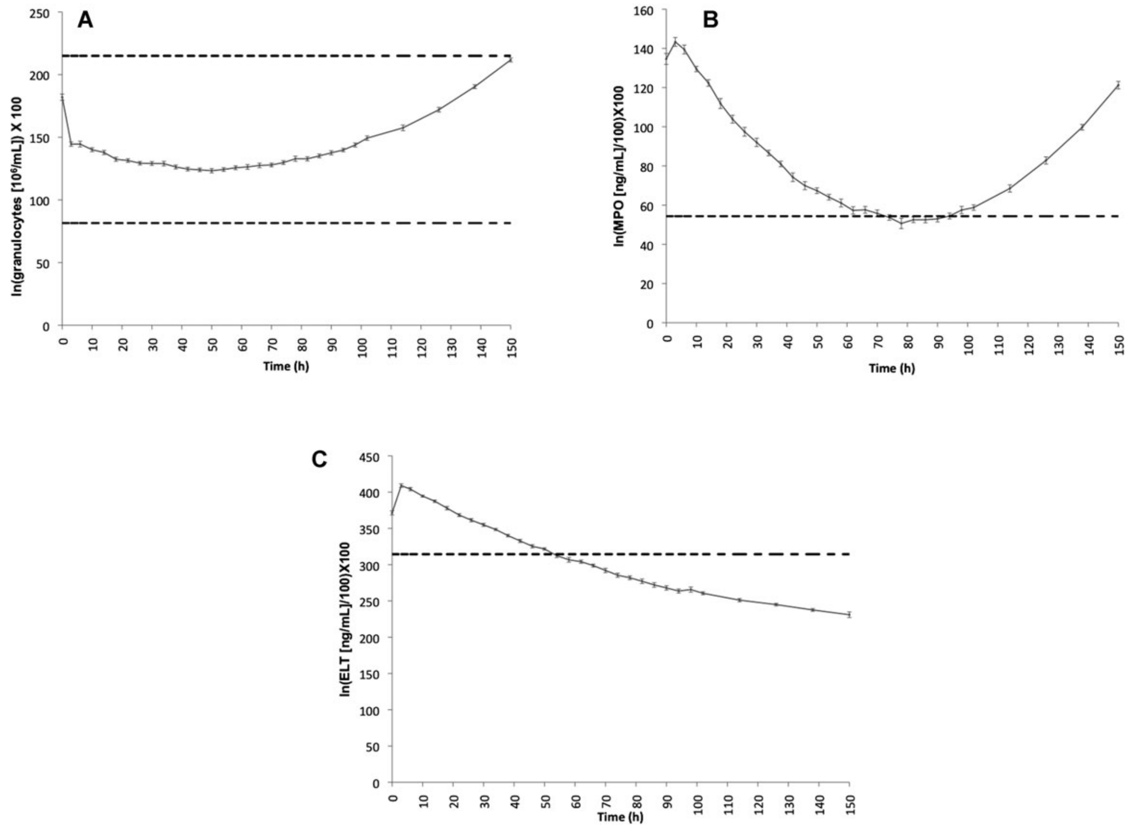

Figure 1: Time-trends (least squares means and standard errors – plain lines) for blood granulocyte counts (A), plasma MPO concentration (B), and ELT concentration (C) of horses with colic, and their corresponding reference intervals in healthy horses (hatched lines). ELT, plasma elastase concentration; ln, natural logarithm; MPO, plasma myeloperoxidase concentration.

The random effects $\varepsilon ijklt$ were assumed normally distributed with null mean and var($\varepsilon ijklt$) block diagonal with blocks corresponding to the animals and with each block having the compound-symmetry structure.

All computations were performed using commercial statistical software[o] using the procedure Proc Mixed, with Satterthwaite degrees of freedom. The significance level was set at $P < 0.05$.

Results

Fifty-three horses comprising 45 Warmbloods, 2 ponies, 2 Arabians, 1 crossbred Arabian, 1 Thoroughbred, 1 Purebred Spanish horse, and 1 Draft horse were included in the study. There were 32 mares, 16 geldings, and 5 stallions. Their age ranged from 0.5 to 20 years, with a median of 8.0 years. Their weight ranged from 203 to

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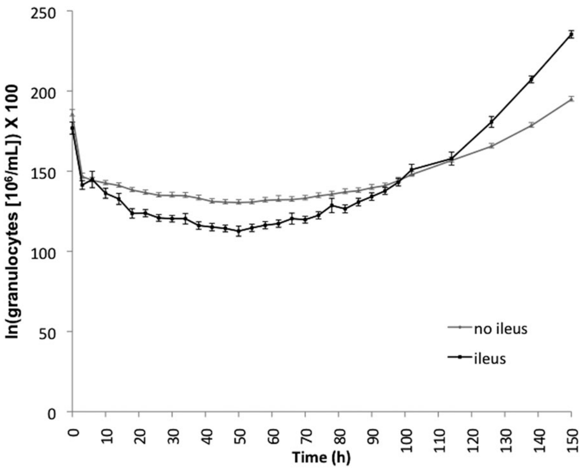

Figure 2: Comparison of time-trends (least squares means and standard errors – plain lines) of blood granulocyte counts in horses with colic that did or did not develop postoperative ileus. ln, natural logarithm; $P < 0.05$.

840 kg (median, 560 kg). Table 1 lists the predominant lesion diagnosed during surgery for each horse. A resection and anastomosis or enterotomy was performed in 8 horses. Resection and anastomosis of small intestine was performed in 3 cases, typhlotomy in 2 cases, and resection and anastomosis of the pelvic flexure was performed in 1 case. Two horses underwent a resection and anastomosis of the cecum (1 via colotomy and 1 without colotomy). Postoperative ileus was diagnosed in 21 horses. Forty-one horses survived to discharge from the clinic. Twelve horses were euthanized during the postoperative period beyond 12 hours after the surgery; 2 for severe shock; 6 for recurrent, uncontrollable pain; and 4 for combined shock and pain. For the horses euthanized during this period, the survival time varied from 0.5 day to 20 days with a median time of 8.0 days.

When all horses were considered together, the time-trends of MPO and ELT concentrations were significantly different from the time-trend of granulocyte counts. The time-trends of MPO and ELT concentrations were not significantly different from each other. The time-trends of granulocyte counts and MPO and ELT concentrations are shown in Figure 1. The type of surgical manipulation did not influence the time-trends of the 3 parameters. The time-trends of granulocyte count (Figure 2) were significantly different between horses that developed postoperative ileus and those that did not, whereas the time-trends of MPO and ELT concentrations were not associated with development of ileus. The time-trends of granulocyte count and MPO concentration were significantly different between survivors and nonsurvivors (Figure 3), whereas the ELT concentration time-trends were not.

Discussion

The search for inflammatory markers has been one focus of human and veterinary medicine over the past several decades.[38] Clinically, the number of circulating neutrophils is one marker of sepsis,[22,39,40] and other parameters such as acute phase proteins have also been described.[38]

The immediate and rapid decrease of circulating granulocytes in this population of horses may be explained by endotoxemia, by migration to the peritoneal cavity and intestines because of the intense stimulation of the laparotomy, or by the pathological process. These results are similar to those of a previous study in which a similar phenomenon was observed.[25] Weiss and Evanson[41] showed also that activated blood neutrophils are a negative prognostic indicator in horses with strangulating obstruction.

Interestingly, the time-trend of granulocyte count was significantly different from the time-trends of MPO and ELT concentrations. Indeed, the decrease in peripheral granulocyte count was temporally associated with an increase in plasma MPO and ELT concentrations during the first 3 hours. This increase in plasma MPO and ELT concentrations reflects intensive degranulation of the activated neutrophils.[51] Beyond 50 hours postadmission, the curve shows an increase of the number of circulating granulocytes, probably due to the recruitment of immature granulocytes.

The results of the present study corroborate the recent work of Schwarz and others,[42] who have demonstrated that a low MPO content in the neutrophils (MPO index) could be considered as an indicator of systemic inflammation in horses, especially when the blood leukocyte count is within the reference interval. The low content can be explained by the MPO release that occurs during activation.[42]

The lack of association between the time-trends of granulocyte count, MPO concentration, and ELT concentration and the type of surgical procedure suggests that resection and anastomosis or enterotomy is not a crucial element in the neutrophil-associated inflammatory response, when compared to other manipulations without incising the intestinal wall. However, these results should be interpreted with caution because only a small number of horses ($n = 8$) underwent resection and anastomosis or enterotomy in the present study.

Concerning the relationship between the postoperative complications and the time-trends of the studied parameters, it must be taken into account that treatments were adapted according to the patient's clinical status and complications. Some of these medications could modulate the neutrophil activation, for example, fractionated heparins.[43–45]

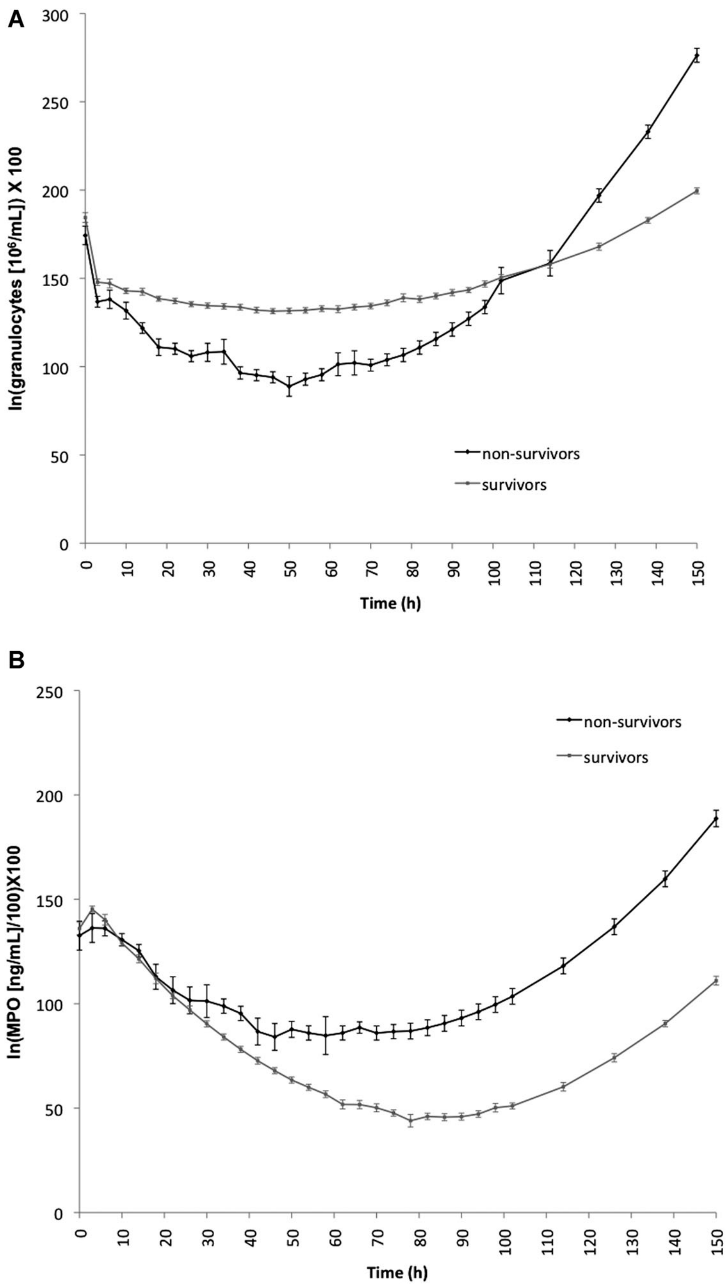

Figure 3: Significant differences in time-trends (least squares means and standard errors – plain lines) for blood granulocyte counts (A) and plasma MPO concentration (B) in horses with colic according to outcome. ln, natural logarithm; $P < 0.05$.

Postoperative ileus is considered a frequent complication after colic surgery in horses.[9,10,12,35,46,47] Although the exact etiology of postoperative ileus remains unclear, an inflammatory component is likely.[13,48] Curiously, when comparing horses with ileus to horses without ileus, only the granulocyte count time-trend was significantly different, while the time-trends of MPO and ELT concentrations were not significantly different between the 2 groups.

As shown in Figure 3, horses with adverse outcome had lower granulocyte count time-trends until 102 hours and higher MPO concentration time-trends starting at 10 hours than surviving horses. This difference could be explained by a more severe systemic inflammatory reaction and granulocyte activation. Studies in people have demonstrated that neutrophils and MPO play a central role in sepsis and multiple organ failure.[49,50] Contrary to the MPO concentration, serial measurements of

  doi: 10.1111/vec.12481

plasma ELT concentrations did not vary significantly between the different conditions studied here even though MPO and ELT are stored in the same neutrophil granules. Another major dissimilarity of the 2 enzymes is the divergence of their time-trends during the second part of the postoperative period, with the ELT concentration time-trend continuing to decrease while the MPO concentration time-trend increased (Figure 1). Different kinetics of plasmatic MPO and ELT concentrations were observed during experimental equine laminitis[16] and cardiopulmonary bypass in people,[51] where the increase in MPO concentration preceded that of ELT concentration by several hours. This observation was not made in the present study, as MPO and ELT concentrations changed almost simultaneously. The different kinetics of the 2 enzymes is generally explained by the binding of ELT to plasma antiproteases such as α2-macroglobulin. Such binding could impair recognition of some enzyme epitopes by the primary or secondary antibodies of the ELISA. Further studies are needed to confirm this possibility.

Unlike serial leukocyte counts,[25] MPO or ELT concentration should not be used alone to determine cut-off values for prognosis after colic surgery because of their wide daily variations observed in other studies[52] and in the current work before the log-transformation of the data. Nevertheless, blood granulocyte counts and serial plasma MPO concentration may help to evaluate the degree of the systemic inflammation following colic surgery in horses. Further studies are needed to elucidate all the cellular effects of MPO and its activity in the tissues. The time-trends described in this study might facilitate the evaluation of treatments aimed at modulating granulocyte activation.

Footnotes

[a] Ringer Lactate Solution, Baxter, Zurich, Switzerland.
[b] Penicilline 5.000.000 IE/UI, Kela Pharma, Sint-Niklaas, Belgium.
[c] Gentakel 5%, Kela Laboratoria, Hoogstraten, Belgium.
[d] Clexane 80 mg, Sanofi, Diegem, Belgium.
[e] Emdofluxin 50, Ecuphar, Oostkamp, Belgium.
[f] Fenylbutazon 20%, VMD, Arendonk, Belgium.
[g] Xylocaine 2%, Astra Zeneca, Bruxelles, Belgium.
[h] Primpéran 10 mg/2 mL, Sanofi, Diegem, Belgium.
[i] Erythrocine Vet. 200, Ceva Santé Animale, Libourne, France.
[j] Monovette 9 mL K3E, Sarstedt AG & CO, Nümbrecht, Germany.
[k] Medonic CA 530, Menarini, Zaventem, Belgium.
[l] Centrifuge BB VVV, Jouan SA, Saint-Herblain, France.
[m] Equine ELISA MPO, BiopTis, Liège, Belgium.
[n] Equine ELISA Elastase, BiopTis, Liége, Belgium.
[o] SAS Institute Inc., Cary, NC.

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