

# Leukocyte infiltration and anti-myeloperoxidase immunoreactivity in granulocytes of the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload

## *Infiltração de leucócitos e imunorreatividade antimieloperoxidase em granulócitos da mucosa e submucosa do intestino grosso de equinos submetidos à sobrecarga dietética com amido*

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### Abstract

This study was carried out to evaluate leukocyte infiltration and anti-myeloperoxidase immunoreactivity in granulocytes of the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload. Eight adult horses were allocated randomly in three treatments: Treatment I (Control) (n = 2), animals euthanized without starch overload; and Treatments II (n = 3) and III (n = 3), animals undergoing starch overload, with gastric infusion of 17.6 g starch per kg of body weight, euthanized after 24 and 36 hours, respectively. Only afflux of neutrophils in the intestinal mucosa and submucosa blood vessels (*leukocyte stasis*) was observed. Eosinophils were the predominant cells in the mucosa and submucosa in all horses, independent of dietetic overload, with infiltration grade from mild to moderate. Lymphocyte infiltration was also observed in all horses, but with lower intensity when compared to eosinophils. Congestion, edema and dilatation of lymphatic vessels were the main circulatory alterations observed, with more intensity in the submucosa. Higher immunoreactivity to the anti-myeloperoxidase antibodies was observed in the mucosa and submucosa of horses 36 hours after overload. Horses submitted to dietetic starch overload showed intestinal inflammatory response with prevalence of eosinophils, leukocyte stasis and circulatory alterations, varying from discreet to moderate.

**Keywords:** Equines. Dietetic Starch Overload. Inflammation, Immunohistochemistry. Myeloperoxidase.

### Resumo

Este estudo teve como objetivo avaliar a infiltração de leucócitos e a imunorreatividade antimieloperoxidase em granulócitos da mucosa e submucosa do intestino grosso de equinos submetidos à sobrecarga dietética com amido. Oito equinos adultos foram distribuídos aleatoriamente em três tratamentos: Tratamento I (Controle) (n = 2), equinos eutanasiados sem sobrecarga com amido; Tratamento II (n = 3) e III (n = 3), equinos submetidos à sobrecarga com amido, com infusão gástrica de 17,6 g de amido/kg de peso vivo e eutanasiados após 24 e 36 horas, respectivamente. Observou-se apenas afluxo de neutrófilos (leucocitoestase) nos vasos sanguíneos da mucosa e submucosa intestinal. Eosinófilos foram as células predominantes na mucosa e submucosa em todos os equinos, independente da sobrecarga dietética, com grau de infiltração de leve a moderada. Infiltração por linfócitos também foi observado em todos os equinos, porém com menor intensidade quando comparado aos eosinófilos. Congestão, edema e dilatação de vasos linfáticos foram as principais alterações circulatórias observadas, com maior intensidade na submucosa. Maior imunorreatividade para anticorpos antimieloperoxidase foi observado na mucosa e submucosa dos equinos 36 horas após a sobrecarga. Equinos submetidos à sobrecarga dietética com amido apresentam resposta inflamatória intestinal com predominância de eosinófilos, leucocitoestase e alterações circulatórias variando de discreta a moderada.

**Palavras-chave:** Equinos. Sobrecarga Dietética com Amido. Inflamação. Imuno-histoquímica. Mieloperoxidase.

### Introduction

Large amounts of hydrolyzable carbohydrates in horse diets promote alterations in the large intestinal microbiota and proliferation of lactic acid-producing bacteria, which result in reduction of pH<sup>1</sup> and an increase in the concentration of lactate and endotoxins in

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the cecal fluid<sup>2,3</sup>. Krueger et al.<sup>4</sup> noticed histopathological alterations in the cecal mucosa of horses, indicating that the dietetic starch overload promotes cellular degeneration, characterized by sloughing of the mucosal epithelium. The damaged intestinal mucosa absorbs endotoxins (lipopolysaccharides), which activate neutrophils<sup>5</sup>, resulting in a sudden increase in oxygen consumption, production of reactive oxygen metabolites (ROMs) and liberation of enzymes such as proteases and myeloperoxidase (MPO)<sup>6</sup>. Studies have indicated oxidative stress with liberation of ROMs as one of the main mechanisms of tissue lesions<sup>7</sup>.

Myeloperoxidase is a specific enzyme of the *azurophilic* granules of granulocytes, mainly neutrophils and eosinophils<sup>8</sup>. Analysis of the MPO in the intestinal mucosa and submucosa has been used as a quantitative index of intestinal inflammation in rats and hamsters<sup>9</sup> as well as horses<sup>10,11,12</sup>. It has also been used recently in studying the neutrophils infiltration in seromuscular layer of small colon of horses submitted to intestinal injuries<sup>13</sup>. There are no records in the literature of immunolabeling with anti-human MPO antibodies for quantification of the inflammatory response in the intestinal mucosa and submucosa of horses, despite the fact that anti-human MPO antibody reaction with horse tissue has already been described<sup>14</sup>. According to Ruiz et al.<sup>15</sup>, the presence of anti-human MPO antibodies can be used for immunohistochemical diagnosis in veterinary pathology.

This study was carried out to evaluate the leukocyte infiltration and anti-MPO immunoreactivity in granulocytes of the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload.

## Material and Method

*Experimental design* – The experimental design was completely randomized with three treatments and eight adult horses, with body weight (BW) ranging from 335 to 393 kg and age from 17 to 27 years. In Treatment I (n = 2), the animals fed a control diet

composed by concentrate and *coastcross* hay, and euthanized without starch overload. In Treatment II (n = 3) and Treatment III (n = 3), the animals fed the same diet, submitted to starch overload, and euthanized after 24 and 36 hours, respectively. This study was approved by the Ethics Committee of the Veterinary Institute of UFFRJ: 23083.011750/2006-33.

*Animals, nutritional management and dietetic starch overload* – The horses were maintained in stalls measuring 6 m<sup>2</sup> with *ad libitum* access to mineral salt and water and receiving a diet composed by hay and commercial concentrate, at a proportion of 60:40, equivalent to 2.5% of body weight (BW) based on dry matter<sup>16</sup>. The overload was performed on the horses of treatments II and III, after a 12-hour fast, through gastric infusion of cornstarch (Maizena - Duryea®), via a nasogastric tube. The amount of cornstarch was 17.6 g /kg BW<sup>17</sup> diluted in water at the rate of 757 g of starch per liter of water. The infusion occurred during an hour in three stages, with the administration of 1/3 of the diluted starch per stage.

*Euthanasia, necropsy and sample collecting* – The euthanasia was performed with previous sedation using 1% acepromazine (0.2 mg/kg BW i.v.) followed by administration of sodium thiopental (8.2 mg/kg BW i.v.) until general anesthesia and, intravenous administration of a 30% potassium chloride solution until death. During necropsy, two fragments each of the cecum, right ventral colon (RVC), left ventral colon (LVC), right dorsal colon (RDC), transverse colon (TC) and descending colon (DC) were collected. One of them was immediately fixed in 20% neutral buffered formalin for histopathological evaluation and the other was washed in water to withdraw the intestinal contents and fixed in 10% neutral buffered formalin solution for immunohistochemical evaluation.

*Histopathological evaluation* – The fragments of the gastrointestinal tract were routinely processed for histopathology. Five-micrometer cross-sections were stained with hematoxylin and eosin (HE) for histo-

logical evaluation<sup>18</sup>. Infiltration of neutrophils, eosinophils and lymphocytes and circulatory alterations in the mucosa and submucosa of the intestinal segments were evaluated according to a scale of 0 to 3, an adaptation of the methodology described by Dabareiner, White and Donaldson<sup>19</sup>: grade 0, no lesion; 0.5, discreet; 1, mild; 1.5, mild to moderate; 2.0, moderate; 2.5, moderate to accentuated and 3, accentuated.

*Immunohistochemical evaluation* – The sections were deparaffinized, rehydrated and treated with a 6% hydrogen peroxide solution for 30 minutes before undergoing a water bath at 96 °C for 30 minutes in antigen retrieval solution (Target Retrieval Solution 10X concentrated - DAKO Corporation, Carpinteria, CA, USA). Then, the sections were incubated in milk and bovine albumin solution at 37 °C for 30 minutes, followed by incubation with rabbit anti-human MPO polyclonal antibodies, in a dilution of 1:500 during 30 minutes. The sections were washed with buffered saline (TBS) before incubation with Envision + System, anti-rabbit HRP (DAKO Corporation, Carpinteria, CA, USA) for 30 minutes at room temperature. The reactions were visualized with diaminobenzidine (DAKO Corporation, Carpinteria, CA, USA) and counterstained with hematoxylin. Sections of human bone marrow were used as positive controls and for negative controls the primary antibodies were omitted. The cells marked in the mucosa and submucosa of the large intestine were counted in five random fields at 400x and classified in grades from 0 to 4 according to the number of granulocytes marked, with adaptation of the Sidney System<sup>20</sup>. 0 - no cells marked; 1 - from 1 to 10 cells marked per field; 2 - from 11 to 50 cells marked per field; 3 - from 51 to 100 cells marked per field; 4 - >100 cells marked per field.

*Statistical analyses* – The histopathological alterations and immunoreactivity to the anti-human MPO antibodies observed in the mucosa and submucosa of the intestinal segments of the horses were submitted to descriptive analysis and compared through of the mean and standard deviation (s.d.).

## Results

Any neutrophil infiltration was observed in the mucosa and submucosa of large intestine of the horses, irrespective of overload. Only afflux of neutrophils (leukocyte stasis) was observed. After 24 hours of overload, a discreet neutrophils leukocyte stasis was observed in the submucosa followed by an increase to the 36 hours after overload. The grade of eosinophils and lymphocytes infiltration in the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload can be observed in figure 1.

The eosinophils were the cells predominantly observed in the intestinal mucosa and submucosa of all horses, regardless of starch overload, with infiltration grade from mild to moderate. The submucosa presented higher infiltration of eosinophils in the horses submitted to starch overload than in the control horses. The mucosa had a higher infiltration grade by lymphocytes, from mild to moderate, compared to the discreet infiltration in the submucosa of the cecum until RDC, followed by reduction in the TC and DC. Furthermore, there was great variability in the grade of leukocyte infiltration in the mucosa and submucosa of the large intestine among horses of the experimental groups.

The main circulatory alterations observed in the intestinal mucosa and submucosa of the horses submitted to dietetic starch overload were congestion, edema and dilatation of lymphatic vessels (data not shown). Submucosa presented a higher flux of neutrophils in the blood vessels and the circulatory alterations were more evident. The RDC showed the largest change due to starch overload, with congestion of the blood vessels, mild to moderate edema and a mild dilatation of the lymphatic vessels.

The immunohistochemical technique revealed brownish marking of granulocytes by diaminobenzidine, indicating immunoreactivity to the anti-MPO antibodies in the mucosa and submucosa of large intestine of the horses submitted to dietetic starch overload (Figure 2).

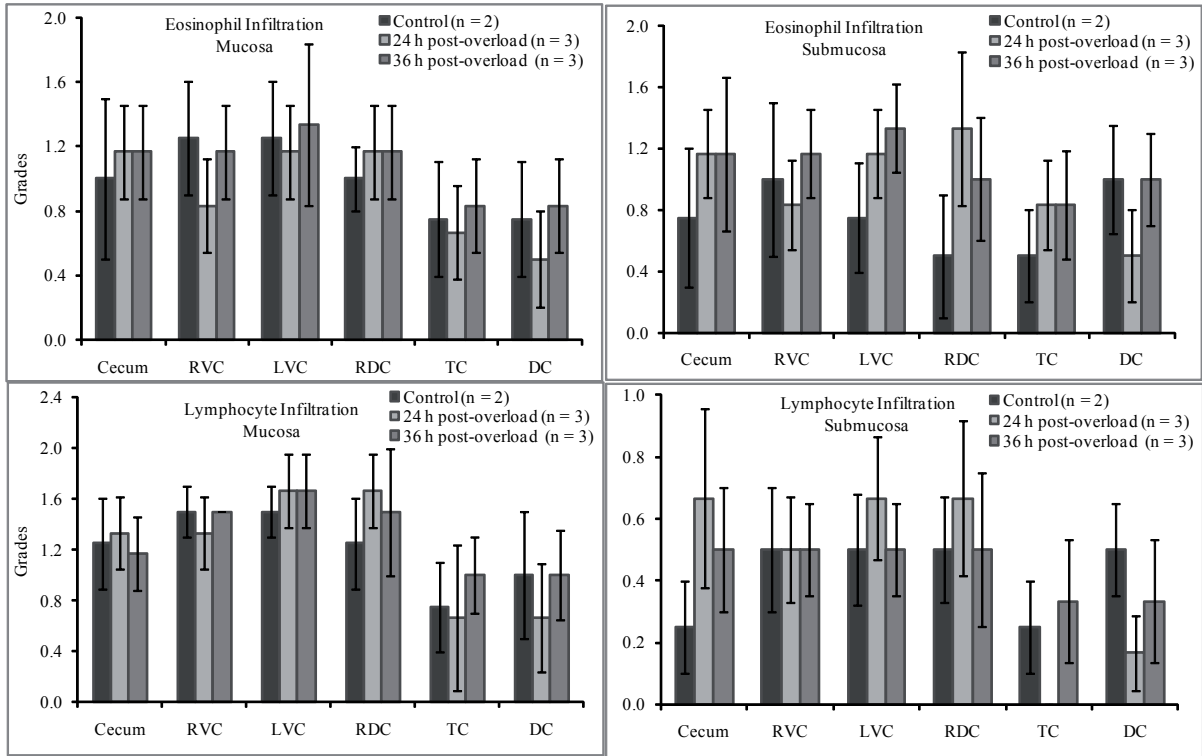


Figure 1 - Grades (mean  $\pm$  s.d.) of eosinophil and lymphocyte infiltration in the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload. RVC: Right ventral colon; LVC: Left ventral colon; RDC: Right dorsal colon; TC: Transverse colon; DC: Descending colon

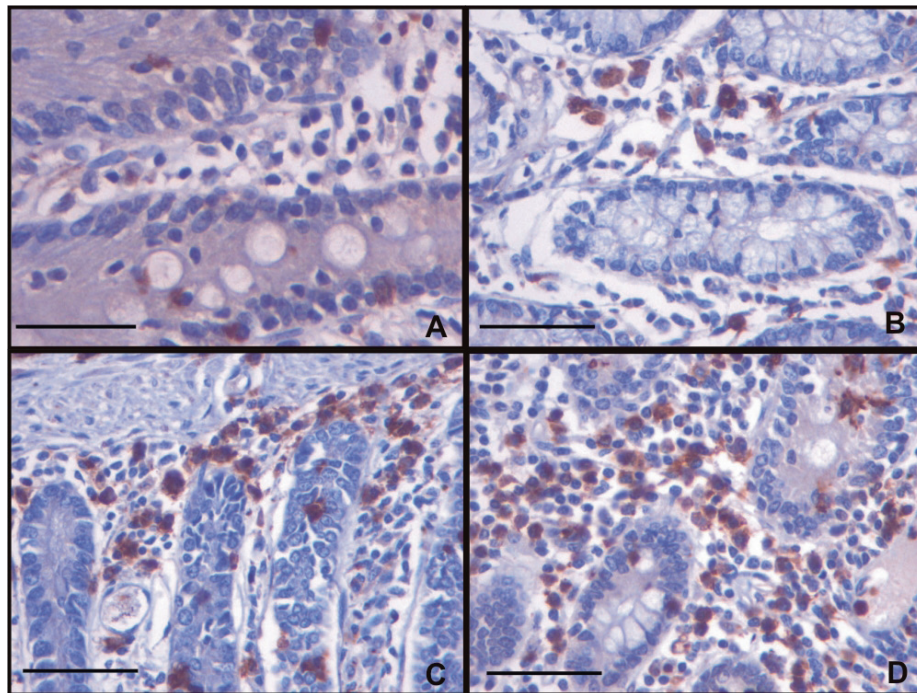


Figure 2 - Immunoreactivity to anti-human MPO antibodies in grades from 1 to 4 according to the number of marked granulocytes in the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload. Grade 1 – from 1 to 10 cells per field (A) right ventral colon; 2 – from 11 to 50 cells per field (B) transversal colon; 3 – from 51 to 100 cells per field (C) transversal colon; 4 - >100 cells per field (D) right dorsal colon. Fields at 400x. Bar = 100  $\mu$ m

The horses submitted to dietetic starch overload and euthanized 36 hours after the overload showed a higher grade of immunoreactivity to the anti-MPO antibodies in the mucosa and submucosa of all segments of the large intestine, except in the DC (Figure 3).

## Discussion

The predominance of infiltrated eosinophils in the mucosa and submucosa of the segments of the horses' intestine, irrespective of the overload, agrees with other findings in the literature<sup>11,21</sup>. Eosinophils are commonly observed in horses' intestinal mucosa and submucosa under normal and inflammatory conditions<sup>21</sup>. Studies suggest that products released after the activation of eosinophils, in particular chemoattractants for neutrophils as ROMs and leukotrienes, are responsible for the neutrophilic infiltration observed in ponies with castor oil-induced acute colitis<sup>22,23</sup>. Other studies have reported high levels of eosinophils infiltrated in the intestinal mucosa of wormed horses. These eosinophils probably

play a role in the interaction between immune response and parasite, but the specific mechanism is unknown<sup>24</sup>. Eosinophils can have an important role at the start of the pathophysiological process in a model of intestinal inflammation in horses<sup>11</sup>.

There are no studies that have evaluated the infiltration of inflammatory cells and the MPO activity in the intestinal mucosa and submucosa of horses submitted to dietetic starch overload. It is known that horses submitted to overload develop metabolic acidosis, endotoxemia and laminitis<sup>2,4,17,25</sup>. In this context, the fermentation of the starch in the hindgut reduces the pH and makes the intestinal contents more acidic, which causes irritation in the mucosa, leading to an increase of vascular permeability<sup>26</sup>. The lipopolysaccharides originating from the death of gram-negative bacteria are absorbed, activating circulating neutrophils<sup>5,27</sup> and causing tissue lesion due to the oxidative stress and liberation of ROMs<sup>6,7</sup>. Based on the studies described above, the eosinophilic infiltration and neutrophils leukocyte stasis observed in

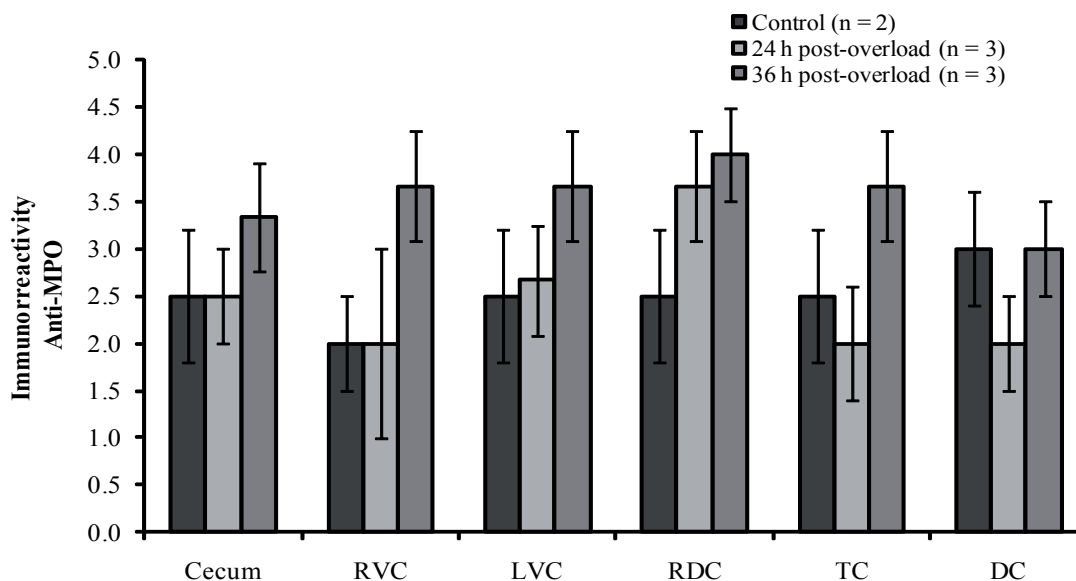


Figure 3 - Grades (mean  $\pm$  s.d.) of immunoreactivity to anti-human MPO antibodies in the mucosa and submucosa of the large intestine of horses submitted to dietetic starch overload. RVC: Right ventral colon; LVC: Left ventral colon; RDC: Right dorsal colon; TC: Transverse colon; DC: Descending colon

this study can represent the beginning of an inflammatory process of the intestinal mucosa and submucosa. However, the evaluation was limited up to 36 hours after overload. Besides this, the horses used in this study were adapted to the diet with concentrate at a level of 40% of total diet, which could have made them more adapted to the effect of the excessive starch intake.

Alterations in the cecal mucosa of the horses submitted to dietetic starch overload, although without adaptation to high-concentrate diet, have been reported<sup>4</sup>. In that study, the authors observed ballooned cells around the opening of the intestinal crypts 24 hours after the overload, but only after 32 hours did these alterations become more evident. They also observed cellular degeneration with sloughing of the epithelium of the cecal mucosa 40 hours after overload, becoming more evident at 48 hours and remaining that way until 72 hours after overload. Histological changes have also been reported in horses with several causes of colic<sup>21</sup>. Some circulatory changes described in the literature<sup>21</sup> were also observed in this study. The congestion, edema and dilatation of lymphatic vessels observed in the intestinal mucosa and submucosa of horses 36 hours after dietetic starch overload may indicate the onset of an inflammatory process. This is reinforced by the absence of neutrophilic infiltration and by the increased neutrophils leukocyte stasis until 36 hours after overload.

There was no neutrophilic infiltration while neutrophils leukocyte stasis was observed in the horses' intestinal mucosa and submucosa. The infiltrate observed was mainly composed of eosinophils and lymphocytes. The histopathological results observed in this study were discreet when compared to those reported in the literature<sup>4,21</sup>. We suggest that eosinophils may play an important role in the inflammatory process in horses submitted to dietetic starch overload, since the neutrophil stasis observed 36 hours after overload may indicate a chemotaxy process. The individual variability observed in the grade of leukocyte infiltration should be considered for inflammatory re-

sponse quantification in horses submitted to dietetic starch overload.

Studies indicate the utilization of the MPO activity as an means to estimate neutrophilic infiltration in intestinal tissue, particularly in animal models of intestinal inflammatory disease<sup>9,10,28</sup>. Unlike usually reported in the literature eosinophils constituted the main cells observed in the horses' intestinal mucosa and submucosa in models of colitis, correlated positively with increased activity of the MPO in injured tissues<sup>11,12</sup>. There was any correlation between the grades of immunoreactivity to anti-MPO antibodies in the mucosa and submucosa of the large intestine segments 36 hours after overload with the eosinophils infiltration in these segments. However, the immunolabeling of MPO in eosinophils was more evident 36 hours after overload, which may indicate tissue injury. The discrepancy observed between the grade of eosinophil infiltration observed by histopathology and immunohistochemistry can be due to the higher sensitivity the latter technique in the quantification of cellular infiltration, since immunolabeling facilitates visualization.

## Conclusions

The present study demonstrated that horses adapted to a diet with high level of concentrate and submitted to dietetic starch overload presented intestinal inflammatory response, with predominance of eosinophils. Any neutrophilic response was observed, but leukocyte stasis and circulatory alterations were observed, varying from discreet to moderate, related to the pathophysiology of the starch overload in the horses' intestinal mucosa and submucosa. Immunoreactivity to the anti-MPO antibodies as a method to evaluate the inflammation in horses' intestinal mucosa and submucosa was satisfactory. More studies are necessary with immunohistochemical analysis by anti-MPO antibodies during periods longer than 36 hours, after which neutrophil infiltration probably occurs, allowing better analysis of the inflammatory process in the intestinal mucosa of horses.

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