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Establishment of peritoneal liquid electrophoretogram from healthy horses and horses submitted to experimentally induced intestinal obstruction

[*Estabelecimento do eletroforetograma do líquido peritoneal de equinos hígidos e daqueles submetidos à obstrução intestinal experimental*]

A.F.S. *Nogueira*[1], P.A. *Di Filippo*[2], L.A. *Anai*[1], M.C. *Vieira*[1], K.M.M.G. *Simplício*[1], A.E. *Santana*[1]

[1]Faculdade de Ciências Agrárias e Veterinária/UNESP – Jaboticabal, SP
[2]Centro de Ciências e Tecnologias Agropecuárias – Universidade Estadual do Norte Fluminense "Darcy Ribeiro" – Câmpus do Goytacazes, RJ

ABSTRACT

The initial inflammatory stages of the colic syndrome include changes known as acute phase response. The aim of this study was to contribute with the establishment of reference values concerning the electrophoretogram of peritoneal liquid from healthy horses and horses submitted to experimentally induced intestinal obstruction. Twenty-one horses were allotted in four groups: duodenal obstruction (DG), ileum obstruction (IG), left-dorsal colon obstruction (MG), and control group (CG). Peritoneal liquid was sampled before obtruction (T0), with 3 hours of obstruction (T3) and 6, 30, 102 and 174 hours after desobstructing (T6, T30, T102 and T174, respectively). Total protein levels were determined by the biuret method and protein fractions were obtained by SDS-PAGE electrophoresis. The acute phase proteins (APP) identified were Immunoglobulin-A, ceruloplasmin, transferrin, albumin, α_1-antitrypsin, heavy and light chains of immunoglobulin-G, haptoglobin, α_1-acid glycoprotein and a still unnamed protein, which was called P24. There was no difference ($P>0.3$) in protein levels among groups, although a significant difference ($P>0.05$) was observed between distinct experimental moments in each group evidencing a higher response of the APP in the obstructed groups. The APP fractioning of the peritoneal liquid was standardized to establish a standard curve for healthy equines and those submitted to induced intestinal obstruction. Moreover, it was verified that the SDS-PAGE electrophoresis was sensitive and effective to help diagnose abdominal inflammatory processes.

Keywords: peritoneal fluid, equine, colic, acute phase proteins

RESUMO

Na cólica equina, os estágios iniciais da inflamação incluem alterações denominadas resposta de fase aguda. O objetivo deste estudo foi contribuir para o estabelecimento de valores de referência do proteinograma do líquido peritoneal de equinos hígidos e daqueles submetidos à obstrução intestinal experimental. Vinte e um animais foram distribuídos nos grupos: obstrução de duodeno (GD), íleo (GI), cólon dorsal esquerdo (GM) e controle instrumentado (GC). As colheitas das amostras de líquido peritoneal foram realizadas antes (T0), durante as obstruções (T3) e após as desobstruções (T6, T30, T102 e T174 horas). A proteína total foi determinada pelo método do biureto, e as frações proteicas obtidas por eletroforese em SDS-PAGE. Identificaram-se as proteínas de fase aguda (PFA): IgA, ceruloplasmina, transferrina, albumina, α_1-antitripsina, cadeias pesada e leve de imunoglobulina-G, haptoglobina, alfa-1-glicoproteína ácida e uma proteína nominalmente não identificada, que foi chamada P24. Não houve diferença ($P>0.3$) nas concentrações proteicas entre os grupos, somente entre tempos dentro de cada grupo ($P>0.05$), evidenciando uma resposta maior das PFA dos grupos obstruídos. O fracionamento eletroforético das PFA, presentes no líquido peritoneal, foi padronizado de modo a estabelecer a curva-padrão para equinos hígidos e para aqueles submetidos à obstrução intestinal; ademais, verificou-se que o referido fracionamento proteico mostrou-se sensível e eficaz no auxílio ao diagnóstico de processos inflamatórios abdominais.

Palavras-chave: eletroforese, líquido peritoneal, equinos, cólica, proteínas de fase aguda

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E-mail: dessafsn@yahoo.com.br

INTRODUCTION

Equine are predisposed to severe morphophysiological changes known as the colic syndrome due to their digestive anatomical peculiarities (Peiró and Mendes, 2004). It influences directly the organic fluid composition (Valadão *et al.*, 1996), posing a series of alterations according to when, where and how serious the obstruction process is (Nappert and Johnson, 2001).

Results from the analysis of peritoneal liquid obtained by abdominal paracentesis (Tulleners, 1983) is an important tool in the diagnosis of acute abdomen in horses and can be helpful to establish a prognosis and veterinarian clinical conduct (Messer, 1995). The electrophoretogram from body fluids represents one of the most reliable methods of protein fractioning, identification and quantification (Thomas, 2000; Kaneko *et al*., 2008). The acute phase proteins (APP) appear early in the blood stream (Carapeto *et al.*, 2006; Jacobsen, 2007) during inflammatory processes (Jacobsen, 2007) aiming to inhibit tissue damage as well as to activate the repairing process (Murata *et al.*, 2004). The APP electrophoresis can be helpful in the early diagnosis of inflammatory process (Di Filippo *et al.*, 2010) providing information about protein increase or decrease during the acute phase response (Thomas, 2000).

Despite the importance of the information obtained from peritoneal liquid electrophoresis from horses undergoing colic, few are the studies about the equine peritoneal fluid proteinogram. This study was idealized aiming to help establish range values for the peritoneal liquid electrophoretic profile in healthy horses and horses submitted to experimental intestinal obstruction.

MATERIALS AND METHODS

Twenty-one adult horses, twelve male and nine nonpregnant females, all from non-defined breeds, with an average age of 6.2-3.0 years were used. One week before the study a clinical evaluation was done, as well as endo and ectoparasites control using mebendazole (Platelmin Equine, 50mg kg^{-1}, Jaboticabal, São Paulo, Brazil) and deltametrine (Butox P, 0,025%, Cruzeiro, São Paulo, Brazil), respectively. Animals were kept in paddocks with a diet based on Coast cross (*Cynodon dactylon*) hay and water *at libitum*. Commercial concentrate feed (TecHorse, Ribeirão Preto, São Paulo, Brazil) was given twice a day, in an amount equivalent to 1% of body mass (2.5 to 3.4kg), as well as 50g/day of mineral supplement (Omolen Ephos, Ribeirão Preto, São Paulo, Brazil).

After adaptation all animals were identified and randomly allotted in three obstructing groups: duodenum obstruction (DG), ileum obstruction (IG) and major colon obstruction (MG). Three animals were used as control (GC), being submitted to the same anesthetic and surgical procedures as the other animals that had an intestinal segment obstructed.

A sedated state in the animals was achieved with acepromazine 1% (Acepran – 0.025mg/kg^{-1}, IV, São Paulo, São Paulo, Brazil), xylazine hydrochloride 2% (Virbaxil – 0.5mg/kg^{-1}, IV, Jurubatuba, São Paulo, Brazil) and meperidine (Dolosal - 4mg/kg^{-1}, IM, São Paulo, São Paulo, Brazil). Subsequently local infiltrating anesthesia was done using an association of 1:1 lidocaine 2% (Lidovet, Rio de Janeiro, Rio de Janeiro, Brazil) and bupivacaine 0.75% (Neocaine, São Paulo, São Paulo, Brazil), both with no vasoconstrictor. Laparotomy was conducted through the right flank to reach duodenum and ileum, and left laparotomy was used to reach the large colon. Obstruction was accomplished using a Penrose drain n.3, according to the technique described by Datt and Usenik (1975). In that exact moment animals received 1.5mg/kg^{-1}, IV, of tramadol chloridate (Tramal, São Paulo, São Paulo, Brazil). The Penrose drain was kept obstructing the intestinal segment during three hours and then it was removed. After surgery antimicrobial and analgesic/antiinflammatory therapy was performed using benzathine benzylpenicillin (Enhanced Veterinarian Pentabiotic, Campinas, São Paulo, Brazil), 30,000 IU/kg^{-1}, IM, with a 48 hour interval, during three days and Flunixin meglumine (Flunexina Injetável, Jaboticabal, São Paulo, Brazil), at the dose of 0.5mg/kg^{-1}, IV, daily, during 2 days. Surgical wounds were treated with polyvinylpyrrolidone-iodine topic at 1%, twice daily until stitches were removed, in the 10^{th} day post-surgery. This study was approved by the FCAV/UNESP-Jaboticabal

Committee for Experimental Animal Use (CEUA) under protocol number 023232-05.

Peritoneal liquid was sampled by abdominal paracentesis according to Neves *et al.* (2000) before surgery (T0), three hours after obstruction (T3), three hours after Penrose drain removal (T6) and 30 (T30), 102 (T102), and 174 (T174) hours post-surgery. Samples were stored at -20°C for posterior processing.

Total protein concentrations were obtained with the biuret method using commercial kits (Labtest, Lagoa Santa, Minas Gerais, Brazil) and spectrophotometric readings (Bioplus 2000, Lagoa Santa, Minas Gerais, Brazil).

For peritoneal fluid protein fractioning the electrophoresis in dodecyl sulfate polyacrylamide gel (SDS-PAGE) was done according to the technique described by Laemmli (1970), modified using the electrophoresis vertical system (PROTEAN II XI-VERTICAL ELETROPHORESIS CELLS® - BIO-RAD). Molecular weighs and protein fraction concentrations were determined by computerized densitometry (Fotodyne, Houston, Texas, United States), through scanning of the samples. Markers with known molecular weights of 200, 116, 97, 66, 55, 45, 36, 29, 24, and 20kDa were used (Marker 6.500 – 200.000, St. Louis, Missouri, United States), besides the purified proteins (Sigma Marker) albumine, α_1-antitrypsin, haptoglobin, ceruloplasmin, transferrin, and immunoglobulin G (IgG). To evaluate the protein band densitometry reference curves were established from the standard marker readings.

For better adaptation and standardization of the results, data was submitted to a logarithmic scale which was applied in the equation log (observation + 1) and then analyzed by variance analysis (ANOVA) and the Tukey test. When significance was found between the interaction "group-moment", the variance was fixed at $P<0.30$. On the other hand, when significance was observed within a certain group or within a certain experimental moment, variance was fixed at $P<0.05$. Statistical analysis was performed using the statistical program SAS.

RESULTS

Electrophoretic fractioning identified protein fractions from 20 to 53 in which the molecular weight varied from 15 to 318kDa.

Among proteins detected in the peritoneal liquid 10 were acute phase proteins, which are important to study the equine colic syndrome. Nine of them were identified as follows: immunoglobulin A (IgA) weighing 175kDa, ceruloplasmin (Cp - 110kDa), transferrin (Transfer - 80kDa), albumin (Alb - 65kDa), α_1-antitrypsin (α_1-antitryp - 58kDa), heavy chain immunoglobulin G (IgG-HC) weighing 55kDa, haptoglobin (Hp - 42kDa), α_1-acid glycoprotein (AGP - 39 kDa), and light chain immunoglobulin G (IgG-LC) weighing 29kDa. Only one protein was not identified by name, weighing 24kDa, denominated P24 (Figure 1).

There was no difference between protein concentrations among the groups studied. However, data and electrophoretic analysis evidenced a higher response of APP in the obstructed groups (DG, IG and MG) when compared to the control group. When experimental moments were compared in each group alone, the post-surgical periods T30, T102, and T174 showed significantly higher values ($p<0.05$) when compared to pre-surgical moments, T0 and T3 (Table 1). The protein α_1-antitrypsin was absent or not detectable in several experimental moments. For that matter the statistical analysis of this protein was not possible to achieve.

DISCUSSION

The peritoneal liquid electrophoretic profile of healthy and obstructed horses included albumin, alpha-globulins (α_1-acid glycoprotein, α_1-antitrypsin, ceruloplasmi and haptoglobin), beta-globulins (transferrin), and gamma-globulins (IgA, IgG-LC and IgG-HC). However, in the literature consulted there were no studies addressing this subject. When compared to the sub-fractions of serum proteins composed by albumin and globulins wich are subdivided into alpha (α_1 and α_2), beta (β_1 and β_2), and gamma (γ_1 and γ_2) chains, the findings for peritoneal liquid are similar to blood (Murata *et al.*, 2004; Petersen *et al.*, 2004; Cerón *et al.*, 2005).

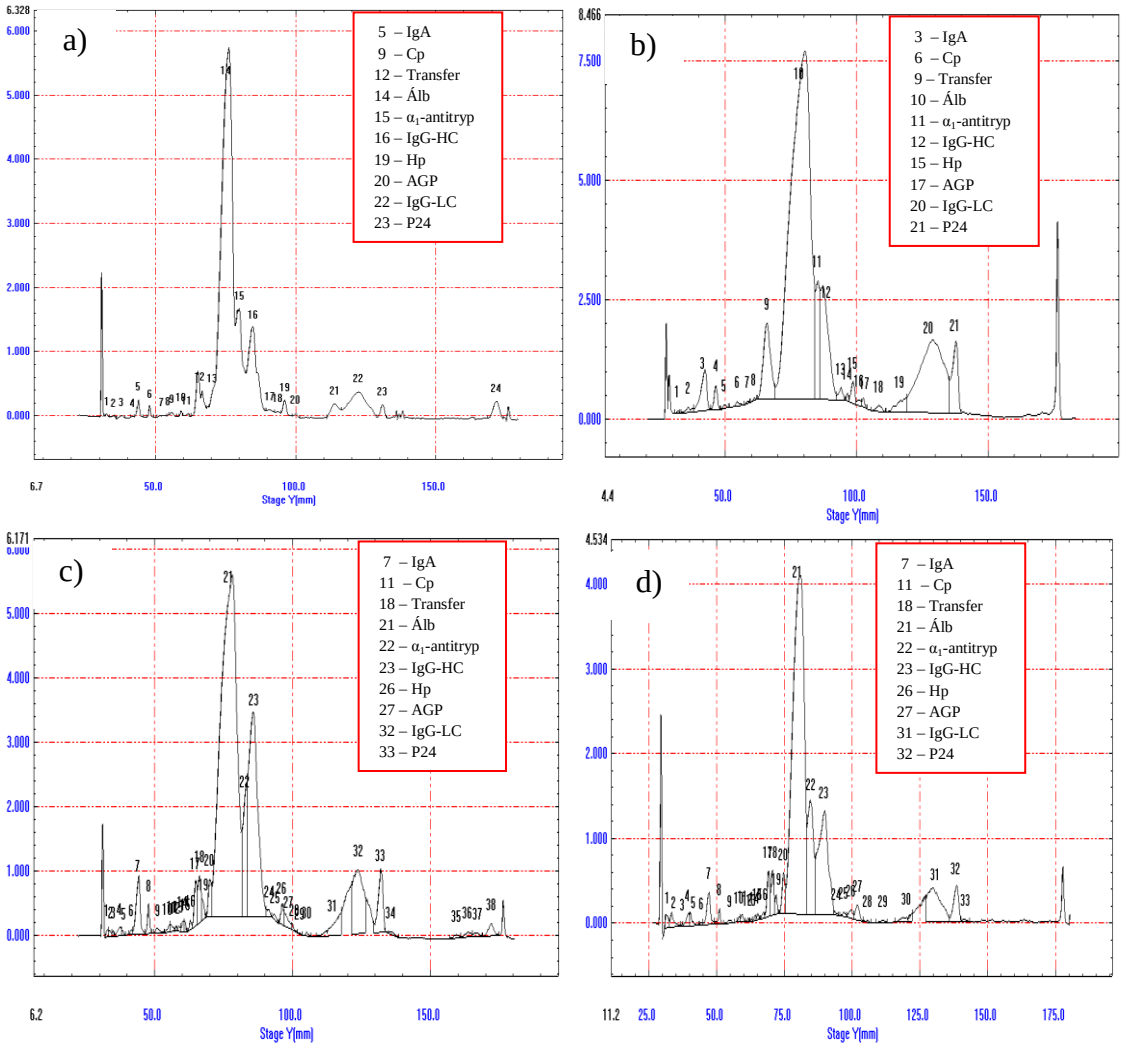

Figure 1. Graphical representation of the electrophoretic pattern trace from the peritoneal liquid proteinogram of healthy horses that belonged to the control group (a), from horses submitted to duodenum obstruction (b), ileum obstruction (c), and large colon obstruction (d), obtained by SDS-PAGE electrophoresis (FCAV/Unesp-Jaboticabal, 2009).

Total protein and albumin concentrations observed during surgey (T3 and T6) and after surgery (T30, T102, and T174) were higher than the ones found in the pre-surgical moment (T0), indicating that there was an inflammatory response stimulated by the surgical procedure in CG, DG, IG and MG, and by the enteric lesion itself in groups GD, GI, and GM (Thomassian, 1996; Lopes *et al.*, 1999; Fagliari and Silva, 2002). Total protein values found in the peritoneal liquid are somewhat proportional to the extension and intensity of the inflammatory process (Santschi *et al.*, 1988). Moreover, the surgical act itself, duration of surgery, type and severity of mechanical manipulations performed may influence the intestinal inflammatory reaction (Hopster-Iversen *et al.*, 2011).

Table 1. Averages and mean deviations of total protein, transferrin, albumin, ceruloplasmin, haptoglobin, α_1- acid glycoprotein, P24, IgA, IgG-HC, and IgG-LC of the peritoneal liquid from horses that belonged to the control group, CG (n=3) and from horses submitted to duodenum obstruction, DG (n=6), ileum obstruction, IG (n=6), and large colon obstruction, MG (n=6). FCAV/Unesp - Jaboticabal, SP, 2009

	Time (hours)					
Groups	T0	T3	T6	T30	T102	T174
			Total Protein (g/dL)			
CG	0.6±0.1a	0.9±0.4ab	1.3±0.4ab	4.2±1.3b	3.9±0.9b	2.9±1.4ab
DG	0.6±0.3a	1.3±0.6ab	2.3±0.8b	4.1±1.6b	3.4±1.1b	3.9±1.4b
IG	0.5±0.2a	1.8±1.6ab	2.9±1.6b	4.0±1.8b	3.6±1.2b	3.3±1.2b
MG	1.6±1.2	1.3±0.7	3.9±3.5	3.0±1.7	3.3±1.1	3.29±1.3
			Transferrin (10^{-2} g/dL)			
CG	2.0±1.0a	3.8±3.1ab	4.5±2.3ab	16.6±10.6b	14.5±5.1b	11.4±6.4b
DG	1.8±1.3a	3.6±2.3ab	6.1±2.9b	11.4±5.1b	9.1±3.7b	11.7±6.4b
IG	1.8±0.8a	5.8±4.8ab	9.4±4.7b	13.1±5.4b	11.3±6.4b	9.3±4.8b
MG	4±5.2a	4.2±2.4ab	10.2±7.9b	9.4±5.1b	11.1±2.5b	13.3±3.6b
			Albumin (10^{-2} g/dL)			
CG	42.0±14.6a	63.3±30.8ab	76.6±32.6ab	221±83.3b	207.3±69b	158±68ab
DG	42.7±23.2a	79.5±50.3ab	141±58.2b	230±78.5b	194±56.8b	196±51.9b
IG	32.5±17.2a	92.9±73.1ab	153±62.8b	224.5±90b	199±72.5b	197±90.2b
MG	72.1±70.2a	86.7±37.2ab	239±200b	199.5±97b	200.4±64b	213±47.9b
			Ceruloplasmin (10^{-2} g/dL)			
CG	0.1±0.1	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.06	0.2±0.05
DG	0.1±0.1	0.1±0.1	0.3±0.2	0.3±0.1	0.3±0.2	0.4±0.3
IG	0.2±0.2	0.3±0.3	0.7±0.5	0.3±0.2	0.3±0.2	0.1±0.1
MG	0.1±0.1a	0.2±0.1ab	0.9±0.9ab	0.9±1.6ab	1.1±1.7b	0.4±0.3ab
			Haptoglobin (10^{-2} g/dL)			
CG	0.2± 0.2a	0.6±0.04ab	1.3±0.4ab	8.5±2.7b	7.8±1.9b	4.4± 3.9b
DG	0.3± 0.3a	0.9±0.2ab	2.4±1.0b	6.1±8.7b	5.7±4.5b	10.4±10.6b
IG	0.1±0.08a	1.3±1.7ab	2.7±1.8b	6.2±4.0b	6.7±4.2b	5.1± 3.0b
MG	1.5±2.80a	0.6±0.08a	1.1±0.8ab	2.2±2.1ab	2.9±1.0b	4.4± 2.5b
			α_1-acid glycoprotein (10^{-2} g/dL)			
CG	0.07±0.01a	0.1±0.1ab	0.1±0.1ab	0.3±0.2ab	1.6±1.4b	0.7±0.5b
DG	0.05±0.04a	0.2±0.1ab	0.2±0.1ab	0.3±0.1ab	0.5±0.3b	1.2±1.0b
IG	0.10±0.08a	0.2±0.3ab	0.3±0.3ab	0.6±0.3b	0.9±0.6b	0.5±0.5b
MG	0.60±1.4ab	0.1±0.1a	0.3±0.3ab	0.3±0.3ab	0.8±0.6ab	0.7±0.2ab
			P24 (10^{-2} g/dL)			
CG	0.9±0.3	1.4±1.3	2.1±1.2	9.1±7.1	5.5±3.8	5.5±3.1
DG	1.3±1.5a	2.3±1.6ab	4.1±1.4ab	8.2±3.2b	6.7±4.0b	6.6±3.9b
IG	0.9±0.5a	4.9±4.9ab	9.8±7.2b	12.5±5.6b	9.4±3.4b	7.9±3.1b
MG	3.8±3.7	3±2.1.0	9.6±8.2	6.9±4.4	10.2±6.7	9.0±6.4
			IgA (10^{-2} g/dL)			
CG	0.3±0.1a	0.9±0.4ab	1.9±1.4ab	7.5±4.2b	6.7±3.3b	5.9±2.8b
DG	0.8±0.7a	2.3±1.6ab	4.1±1.4b	9.1±4.3b	8.5±4.6b	9.0±3.4b
IG	0.3±0.2a	3.1±3.7b	6.5±5.2b	7.5±4.1b	7.8±4.9b	5.5±2.6b
MG	1.6±2.7a	2.3±1.6ab	4.8±4.0b	4.0±1.6b	7.4±2.6b	7.3±1.8b
			IgG-HC (10^{-2} g/dL)			
CG	5.9± 2.0a	9.9± 5.8ab	16.4± 2.8ab	64.7±13.7b	68.0±16.1b	49.9±24.2b
DG	8.6± 6.1a	20.9± 9.4ab	37.2±13.5b	67.3±36.9b	60.3±21.1b	66.5±28.0b
IG	7.9± 5.7a	39.2±48.6ab	60.1±49.3b	66.6±38.4b	66.2±25.6b	63.3±30.5b
MG	16.1±16.3a	20.0±11.8ab	44.4±39.5ab	40.5±17.5b	46.6±10.5b	54.8±14.3b
			IgG-LC (10^{-2} g/dL)			
CG	4.0±0.4a	6.6± 4.6ab	8.7± 3.4ab	46.0±30.3b	35.6±12.7b	33.1±18.1b
DG	4.7±3.9a	10.0± 4.6ab	17.2± 7.9b	42.7±30.9b	26.1±14.4b	37.6±25.3b
IG	3.1±1.5a	16.5±17.1ab	29.2±19.3b	39.7±28.2b	33.6±13.2b	24.4±10.7b
MG	10.5±13a	11.6± 7.8ab	54.1±86.4b	31.8±22.4b	40.0±16.2b	39.8±20.6b

Averages followed by different lower-case letters in the same line differ between them (P>0.05) by the Tukey test at 5% probability to compare the averages between experimental moments. The absence of letters in the same row corresponds to similar average when comparing the averages (FCAV/Unesp-Jaboticabal, SP, 2009).

The electrophoretogram showed a higher increase of α-globulins (α_1-acid glycoprotein, ceruloplasmin and haptoglobin) in post-surgical moments T30, T102 and T174. Alpha globulins rose earlier, reaching concentration peaks starting few hours after the stimuli and up to 2-3 days (Kaneko *et al.*, 2008) and maintaining these peritoneal levels along several weeks after surgery. Therefore, it can be inferred that the protein weighing 24 kDa (P24) had an APP α-globulin behavior due to the fact that it increased progressively, reaching concentration peaks in the first day post-surgery. Flagliari and Silva (2002) and Fagliari *et al.* (2008) observed increased values in α_1- acid glycoprotein, haptoglobin, and ceruloplasmin in serum samples from horses undergoing colic and which were submitted to laparotomy. However, it is reasonable to remember that ceruloplasmin levels increase in some, but not in all, inflammatory diseases as postulated by Thomas (2000). This statement may be of importance when analyzing data from studies evaluating APP through different inflammatory processes.

The β fraction of the electrophoretic trace consists of numerous proteins. One of them is transferrin, which usually decreases right after an inflammatory stimuli to then elevate its levels showing peak concentrations at 7 to 10 days after the stimuli. It can maintain high levels for several weeks (Murata *et al.*, 2004), corroborating the findings in this study, in which the averages of transferrin presented a gradual increase along time. Inversely, in the studies of Fagliari and Silva (2002) and Carapeto *et al.* (2006), there was a progressive increase of γ-globulin IgA along the experimental period as well as in the IgG fraction with its sub-fractions IgG-HC and IgG-LC. The γ-fraction proteins in domestic animals include mainly immunoglobulin IgA, IgM, IgG, and IgE synthesized by the immunologic system in response to antigenic and viral stimuli (Thomas, 2000; Kaneko *et al.*, 2008). The gamma-globulin concentration increase may be related to the unspecific polyclonal activation in B cells. That happens because interleukin-6 (IL-6) production increases as well as other interleukins causing unspecific polyclonal activation of the B cells, resulting in antibody production from different origins (Kaneko *et al.*, 2008).

Results observed in the peritoneal proteinogram are due to the acute phase protein extra-hepatic synthesis from heterogeneous cell types, from organs or tissues including leucocytes (Fournier *et al.*, 2000) and especially by endothelial and epithelial cells from organs that communicate with the external environment, such as the gastrointestinal tract. According to Jacobsen (2007), the determination of local APP concentrations increases the precision in the establishment of the diagnosis by providing information about the inflammatory/infectious status of a specific organ. In studies done by Eurell *et al.* (1993) the changes in peritoneal APP levels were more sensitive in setting an abdominal disorder diagnose when compared to serum samples, and were strongly correlated to clinical signs due to post-surgical complications.

Based on the results obtained it is possible to conclude that all horses evaluated showed an inflammatory response, where higher response intensity was seen in animals submitted to intestinal obstruction. This response was characterized by changes in peritoneal APP levels associated to the enteric lesion from the obstruction model and from laparotomy itself. This study allowed us to standardize an APP electrophoretic fractioning of the peritoneal liquid from both healthy and obstructed horses proving to be sensitive and effective in the diagnosis of abdominal inflammatory processes.

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