USE OF HEMOGLOBIN AS AN ADJUVANT DURING EXPERIMENTAL INTRAPERITONEAL INFECTION WITH *Escherichia coli* AND/OR *Bacteroides fragilis* IN HORSES

UTILIZAÇÃO DA HEMOGLOBINA COMO ADJUVANTE DURANTE INFECÇÃO EXPERIMENTAL EM EQÜINOS, VIA INTRAPERITONERAL, COM *Escherichia coli* E/OU *Bacteroides fragilis*

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SUMMARY

The goals of this study were to evaluate if hemoglobin played a role as adjuvant in experimental peritonitis in horses and could cause clinical and haematological alterations that could be used for diagnosis and prognosis of cases of peritonitis. Fifteen adult horses were randomly divided into 5 equal groups, which were injected intraperitoneally with the following suspension: GI: $1x10^9$ colony-forming units (CFU) of E. coli diluted in 500 mL of 0.9% saline solution plus 5 g of hemoglobin; GII: $1x10^9$ CFU of B. fragilis diluted in 500 mL of 0.9% saline plus 5 g of hemoglobin; GIII: $1x10^9$ CFU of E. coli in combination with $1x10^9$ CFU of B. fragilis diluted in 500 ml of 0.9% of saline plus 5 g of hemoglobin; GIV: 500 mL of 0.9% saline plus 5 g of hemoglobin and GV: 500 mL of 0.9% saline. Leukopenia with neutropenia was observed in GI and GIII and a significant increase in plasma fibrinogen concentration occurred in horses of GI. There was a significant increase in total nucleated cell count in peritoneal fluid in horses of GI, GII, GIII and GIV. Fever, tachycardia, tachypnea, abdominal wall sensibility and tension, diarrhoea, colic, and decreased borborygmi sounds were the most frequent clinical signs observed in horses of GI, GII, GIII and GIV. In conclusion, hemoglobin was able to cause chemical peritonitis in horses, it had an adjuvant effect when associated to E. coli in experimental peritonitis in horses potentiating pathogenic effects of E. coli, but was not lethal to horses.

KEY-WORDS: Peritonitis. Horses. Escherichia coli. Bacteroides fragilis. Hemoglobin.

RESUMO

Os objetivos deste estudo foram avaliar se a hemoglobina atua como adjuvante nas peritonites experimentais em eqüinos e se pode causar alterações clínicas e laboratoriais que podem ser utilizadas no diagnóstico e prognóstico de casos de peritonite. Quinze eqüinos adultos foram aleatoriamente divididos em 5 grupos e foram inoculados por via intraperitoneal com a seguinte suspensão: GI: $1x10^9$ unidades formadoras de colônias (UFC) de *E. coli* diluídas em 500 mL de solução salina a 0.9% adicionada de 5 g de hemoglobina; GII: $1x10^9$ UFC de *B. fragilis* diluídas em 500 mL de solução salina a 0.9% adicionada de 5 g de hemoglobina; GIII: $1x10^9$ UFC de *E. coli* em associação com $1x10^9$ UFC de *B. fragilis* diluídas em 500 mL de solução salina a 0.9% adicionada de 5 g de hemoglobina; GIV: 500 mL de solução salina a 0.9% adicionada de 5 g de hemoglobina e GV: 500 mL de solução salina a 0.9%. Leucopenia com neutropenia foram observadas nos animais dos GI e GIII e aumento da concentração de fibrinogênio plasmático ocorreu nos eqüinos do GI. Houve um aumento na contagem de células nucleadas totais no líquido peritoneal dos eqüinos nos GI, GII, GIII e GIV. Febre, taquicardia, taquipnéia, tensão e sensibilidade da parede abdominal, diarréia, cólica e diminuição dos ruídos intestinais foram os sinais clínicos mais observados nos animais dos GI, GII, GIII e GIV. Concluímos que a hemoglobina foi capaz de causar peritonite química nos cavalos inoculados e possuiu efeito adjuvante nas peritonites experimentais potencializando a patogenicidade da *E. coli*, mas sem ser letal para os eqüinos.

PALAVRAS-CHAVE: Peritonite. Eqüinos. *Escherichia coli. Bacteroides fragilis.* Hemoglobina.

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INTRODUCTION

Peritonitis or inflammation of the peritoneum may be induced by any contaminant or irritating agent. It is a complex disease state characterized by multiple organ involvement (HOSGOOD and SALISBURY, 1989). Horses have always been considered to be highly susceptible to peritonitis (SCHNEIDER, 1992) and their susceptibility has been tentatively attributed to the small size of the equine omentum compared with that of other species, and to the inability to wall off peritoneal contamination effectively (MAIR, HILLYER, TAYLOR, 1990). It is considered a potentially fatal or permanently incapacitating condition, and has been identified as a major postoperative complication of surgical colic (RICKETTS, 1987).There have been few detailed studies on peritonitis in adult horses (MAIR, HILLYER, TAYLOR, 1990).Much of what we know about peritonitis has been determined through studies on laboratory species and humans (TRENT, 1995).

The clinical signs of horses with naturally acquired peritonitis are abdominal pain, ileus, hyperthermia or normothermia, abdominal distension, anorexia, weight loss and diarrhoea or constipation (COFFMAN and TRISCHLER, 1972, DYSON, 1983, CLABOUGH and DUCKETT, 1992, MOLL and SCHUMACHER, 1992). Horses with acute diffuse peritonitis also show prolonged capillary refill time, dehydration, increased heart and respiratory rates, pawing, depression, anorexia, red-to-purple mucous membranes and muscle fasciculations (MAIR, HILLYER, TAYLOR, 1990). Abdominal pain is most evident in the early stages of disease and is characterized by immobilization, reluctance to move, splinting of the abdominal wall, and sensitivity to external abdominal pressure (SEMRAD, 1990).

Escherichia coli is a bacteria present in the normal flora of gastrointestinal tract and most cases of peritonitis in horse are caused by this agent (COFFMAN and TRISCHLER, 1972, MAIR, HILLYER, TAYLOR, 1990, MOLL and SCHUMACHER, 1992).

Hemoglobin has long been known to be an important virulence factor in infected peritoneal fluid (LEE et al., 1979, DUNN et al., 1983, DUNN et al., 1983a, PRUETT et al., 1984, PRUETT et al., 1985, LANGERMANS et al., 1996). In experimental peritonitis models, examination of the infection potentiating effect of hemoglobin reveals several facts: (1) hemoglobin and bacteria must be physically in contact for increased mortality to occur; (2) the death is preceded by accelerated bacterial proliferation in the peritoneal cavity; (3) other substances that carry coordinated iron (i.e., heme, myoglobin) are adjuvants in proportion to the amount of iron they contain; and (4) the infection potentiating efect of hemoglobin can be blocked if haptoglobin is added in sufficient amounts to bind the hemoglobin (LEE et al., 1979, DUNN et al., 1983, DUNN et al., 1983a, PRUETT et al., 1984, PRUETT et al., 1985, LAW and KELLY, 1995, LANGERMANS et al., 1996).

The major goals of the present experiment were to study the clinical and laboratory alterations of equine experimental peritonitis caused by *E. coli* or by the combination of this bacteria to an adjuvant such as hemoglobin.

MATERIALS AND METHODS

This study was in accordance with the rules of the Local Animal Care Committee.

Fifteen adult horses of various breeds, ranging from 3 to 10 years old were used. They were randomly divided into 5 equal groups which were injected intraperitoneally with the following suspension: Group I, 1 x 10^9 colony-forming units (CFU) of *E. coli* diluted in 500 mL of 0.9% saline plus 5 g of hemoglobin[1], Group II, 1 x 10^9 CFU of *B. fragilis* diluted in 500 mL of 0.9% saline plus 5 g of hemoglobin, Group III, 1 x 10^9 CFU of *E. coli* in combination with 1 x 10^9 CFU of *B. fragilis* diluted in 500 mL of 0.9% saline plus 5g of hemoglobin, Group IV, 500 mL of 0.9% saline plus 5 g of hemoglobin and Group V,500 ml of 0.9% saline. During the study the horses were housed in individual stalls, fed commercial ration (3 kg/animal/day), coast-cross (*Cynodon dactilon L.*) hay and water *ad libitum.*

All animals were physically examined at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 120, 168 and 216 hours after inoculation (HAI). Clinical records included rectal temperature, heart and respiratory rates, mucous membrane colour, capillary refill time, skin elasticity, tension and sensitivity of the abdominal wall, intestinal sounds and other observations, if necessary.

Blood was collected from the jugular vein into tubes[2] containing EDTA for complete blood cell (CBC) counts, determined by use of an automated cell counter[3] at the same intervals above mentioned. Smears were prepared and stained with Rosenfeld. Plasma fibrinogen concentration was determined by heat precipitation and total plasma protein was determined by refractometry (SCHALM et al., 1975).

Abdominal fluid was collected into sterilized tubes with EDTA[b] in all animals at the same moments as blood samples according to the technique described by White II (1990) and physical (colour and turbidity) and laboratory aspects (total nucleated cell count and differential cell counts, total protein and fibrinogen) were determined using the same techniques for blood parameters.

Data were submitted to analysis of variance, for comparisons between groups at each time, and the significant difference was evaluated at level of $P < 0.05$ by Tukey test to compare means using a computer program[4].

[1] Hemoglobin n,n-dimethylated from bovine blood – SIGMA - USA
[2] Vacutainer – Becton-Dickinson, NJ, USA.
[3] CC-510 Celm, Barueri, SP, Brazil.
[4]SAS Program, v.8, Statistical Analysis System, SAS Institute, Cary, NC, USA.

RESULTS

Contrary to many studies performed in rats and humans, no horse died during the experimentation.

Clinical signs - Physical examination of the animals before inoculation did not show any abnormality in rectal temperature, heart and respiratory rates, mucous membrane color, capillary refill time, skin elasticity, tension and sensitivity of the abdominal wall. Fever, tachicardia, tachipnea, abdominal wall sensitivity and tension, diarrhoea, colic, decreased borborigmi sounds were the most frequent clinical signs observed in horses of GI, GII, GIII and GIV similarly to others in natural and experimental peritonitis. Animals of Group V showed no physical signs alterations. All the animals inoculated showed other clinical signs which are listed in Tables 1 to 4.

Hematologic changes (Table5) - Significant leukopenia in GI at 2 and 4 hours after inoculations (HAI) and in GIII from 2 until 6 HAI. Neutropenia was detected in GI at 2 HAI and in GIII at 2 and 4 HAI followed by neutrophilia in GI at 24 and 48 HAI. Lymphopenia was seen in GI at 24 HAI and 36 HAI and in GIII at 6 HAI. Band neutrophils significantly increased in GI at 24 HAI, from 24 until 48 HAI in GIII and at 8 HAI in GIV. No alterations in basophils, monocytes and eosinophils were observed.

Total plasma protein did not show any alterations during the experiment. A significant increase in plasma fibrinogen concentration in horses of GI was observed at 48, 60 and 120 HAI and at 60 HAI in GIII.

RBC increased significantly in GI at 8 and 10 HAI, at 6 HAI in GII, from 2 to 24 HAI in GIII and at 6 and 8 HAI in GIV. Hematocrit significantly increased in GI from 4 to 12 HAI, at 6 HAI in GII, at 2, 4, 6, 10, 12 and 72 HAI in GIII, and from 72 untill 168 HAI in GIV. Hemoglobin levels were significantly increased in GI at 4, 6 and 10 HAI, at 24 HAI in GII, at 72 HAI in GIII and at 120 HAI in GIV.

Peritoneal fluid findings (Table 6) - Leukocytes in the peritoneal fluid were significantly increased from 10 to 120 HAI in GI, from 10 to 60 HAI in GII and from 24 to 60 HAI and at 216 HAI in GIII, and in GIV at 10, 24 and 36 HAI. Polymorphonuclear cells (PMNs) were the predominant cells in the peritoneal fluid in groups I and III at 4 HAI. No increase in mononuclear cells (non-reactive macrophages, reactive macrophages and mesothelial cells) were observed in the peritoneal fluid.

Total protein in peritoneal fluid had a significant increase from 2 HAI till the end of the study in GI, from 10 HAI till 72 HAI in GII, from 6 HAI till 72 HAI in G III and from 4 HAI till 60 HAI in G IV, while there was a significant increase in fibrinogen levels from 4 to 168 HAI in GI, at 120 HAI in GII and 72 HAI in GIII.

The reisolation of *E. coli* was possible in the peritoneal fluid of animals in GI and GIII from 2 to 6 HAI and of *B. fragilis* in the peritoneal fluid in GII and GIII at 2 HAI. No bacterial isolation was possible in GIV and GV.

Table 1- Clinical signs observed in group I horses inoculated intraperitoneally with $1x10^9$ CFU of *E. coli* + 5 g of hemoglobin.

Horse	Clinical Signs	Hours after inoculation
1	Sensitivity of abdominal wall	6 , 8, 10, 12, 24, 36, 48, 72 and 120
	Tension of abdominal wall	4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 120,168 and 216
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72 and 120
	Diarrhoea	2, 4
	Cyanotic mucous membranes	24
	Recumbence	2
2	Sensitivity of abdominal wall	24
	Tension of abdominal wall	4, 6, 8, 10, 12, 24, 36, 48, 60, 72 and 168
	Increased intestinal sounds	2
	Decreased intestinal sounds	4, 6, 8, 10, 12, 24, 36, 48 and 60
	Diarrhoea	2
	Colic (pawing)	2
3	Sensitivity of abdominal wall	8 and 10
	Tension of abdominal wall	4, 6, 8, 10, 12, 24,36, 48, 60, 72, 120 and 168
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72
	Diarrhoea	2

All animals were physically examined at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 120, 168 and 216 hours after inoculation (HAI)

Table 2- Clinical signs observed in group II horses inoculated intraperitoneally with $1x10^9$ CFU of *B. fragilis* + 5 g of hemoglobin.

Horse	Clinical Signs	Hours after inoculations
4	Sensitivity of abdominal wall	4
	Tension of abdominal wall	4, 6, 8
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12
	Diarrhoea	2, 4
	Dry mucous membranes	6
	Recumbence	2
5	Sensitivity of abdominal wall	4, 6
	Tension of abdominal wall	4, 6, 8
	Decreased intestinal sounds	2, 4, 6, 8
	Diarrhoea	2, 36
	Depression	4
6	Sensitivity of abdominal wall	6
	Tension of abdominal wall	4, 6, 8, 10, 12
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12
	Pale mucous membranes	4, 12, 24
	Endotoxic line in oral mucosa	10
	Colic (looking at the flank and pawing)	2, 4, 6, 8, 48
	Diarrhoea	36

All animals were physically examined at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 120, 168 and 216 hours after inoculation (HAI)

Table 3- Clinical signs observed in group III horses inoculated intraperitoneally with $1x10^9$ CFU of de *E. coli* + $1x10^9$ CFU of *B. fragilis* + 5 g of hemoglobin.

Horse	Clinical Signs	Hours after inoculations
7	Sensitivity of abdominal wall	4, 6 , 8
	Tension of abdominal wall	2, 4, 6, 8, 10, 12, 24, 36, 48, 60
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72 e 168, 216
	Diarrhoea	2
	Depression	2
8	Sensitivity of abdominal wall	2, 4, 6, 8, 10, 12, 24
	Tension of abdominal wall	2, 4, 6, 8, 10, 12, 24
	Increased intestinal sounds	2
	Decreased intestinal sounds	4, 6, 8, 10, 12, 24, 36, 48, 60, 72
	Diarrhoea	2, 10, 12, 24
	Colic (pawing)	2
	Depression	2
	Recumbence	2, 4, 6, 8, 12, 24, 36
	Congested mucous membranes	4, 6, 8, 10, 12, 24, 48, 60, 72
	Cyanotic mucous membranes	6, 24

	Sensitivity of abdominal wall	2, 4, 6, 8, 10, 12
	Tension of abdominal wall	4, 6, 8, 10, 12, 24, 36, 48, 60, 120
	Depression	2, 4, 6, 8, 10, 12
	Recumbence	2
	Colic (pawing)	2, 6, 10, 12, 24
9	Congested mucous membranes	4, 6, 8, 10, 12, 24, 36, 48, 60, 72
	Petechia	6, 8
	Endotoxic line in oral mucous	8
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12, 24, 36, 48
	Diarrhoea	4, 12

All animals were physically examined at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 120, 168 and 216 hours after inoculation (HAI)

Table 4- Clinical signs observed in group IV horses inoculated intraperitoneally with 5 g of hemoglobin.

Horse	Clinical Signs	Hours after inoculations
	Tension of abdominal wall	2, 4, 6, 8
10	Decreased intestinal sounds	2, 4, 6, 8
	Depression	2
	Tension of abdominal wall	2, 4, 6
11	Decreased intestinal sounds	2
	Depression	4
	Tension of abdominal wall	6, 8, 10
12	Recumbence	2, 4, 48
	Decreased intestinal sounds	2, 4, 6, 8, 10, 12, 48, 60

All animals were physically examined at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 120, 168 and 216 hours after inoculation (HAI)

Table 5 – Hematology data from the five groups of horses in this study.

		Hours after inoculation														
		0	**2**	**4**	**6**	**8**	**10**	**12**	**24**	**36**	**48**	**60**	**72**	**120**	**168**	**216**
Red Blood cells (x10³/µL)	GI	6066^{A}	6200^{AB}	7600^{AB}	7266^{AB}	9433^{A}	9266^{A}	8700^{AB}	7500^{AB}	6400^{A}	6033^{A}	6233^{A}	6366^{A}	5666^{A}	5833^{A}	6233^{A}
	GII	6406^{A}	5350^{A}	7110^{AB}	6086^{A}	6783^{BC}	6726^{BC}	6700^{A}	6316^{A}	6420^{A}	5876^{A}	6240^{A}	6473^{A}	7840^{A}	6833^{A}	6526^{A}
	GIII	7366^{A}	8936^{B}	9193^{A}	8156^{A}	8756^{A}	8530^{AC}	9160^{B}	8360^{B}	7320^{A}	6770^{A}	7516^{A}	7733^{A}	7463^{A}	7240^{A}	7516^{A}
	GIV	7843^{A}	6556^{AB}	7060^{AB}	7733^{A}	7866^{AB}	7060^{BC}	7156^{AB}	7040^{AB}	7520^{A}	6970^{A}	7436^{A}	7506^{A}	7963^{A}	8353^{A}	8410^{A}
	GV	6243^{A}	6310^{A}	5910^{B}	5720^{B}	5880^{C}	5986^{B}	6546^{A}	5890^{A}	5580^{A}	5983^{A}	5876^{A}	6140^{A}	5703^{A}	5856^{A}	5966^{A}
Packed cell volume (%)	GI	30,67^{A}	33,67^{AB}	39,33^{C}	37,33^{C}	37,33^{A}	39,33^{A}	39,67^{A}	34,33^{A}	32,33^{A}	29,33^{A}	28,67^{A}	28,00^{AC}	28,33^{A}	30,33^{AB}	31,00^{A}
	GII	29,67^{A}	27,67^{BC}	29,33^{A}	29,33^{B}	30,67^{AB}	30,00^{BC}	29,00^{B}	27,00^{A}	26,00^{A}	26,67^{A}	27,00^{A}	28,33^{AC}	30,00^{AB}	29,33^{AB}	29,67^{A}
	GIII	33,33^{A}	36,33^{A}	36,67^{BC}	35,00^{BC}	35,00^{AB}	36,67^{A}	38,33^{A}	34,67^{A}	31,67^{A}	30,67^{A}	31,00^{A}	37,67^{B}	32,67^{AB}	33,33^{AB}	32,33^{A}
	GIV	34,67^{A}	29,33^{BC}	31,67^{AB}	34,3^{ABC}	35,33^{AB}	33,7^{ABC}	32,00^{AB}	32,67^{A}	33,67^{A}	32,33^{A}	32,67^{A}	36,67^{AB}	36,67^{B}	36,00^{B}	36,33^{A}
	GV	31,67^{A}	28,67^{BC}	27,67^{A}	28,67^{A}	29,00^{B}	27,67^{C}	27,33^{B}	27,33^{A}	27,67^{A}	27,33^{A}	25,67^{A}	25,00^{C}	26,67^{A}	27,67^{A}	27,33^{A}
Leukocytes (x10³/µL)	GI	9,43^{A}	4,23^{A}	4,10^{A}	6,03^{AB}	8,33^{AB}	9,37^{AB}	10,90^{A}	12,83^{A}	11,90^{A}	10,77^{A}	10,37^{A}	8,13^{A}	9,10^{AB}	11,67^{A}	11,27^{A}
	GII	9,73^{A}	7,40^{AB}	10,40^{B}	11,87^{A}	14,17^{A}	14,27^{A}	13,43^{A}	11,33^{AB}	10,67^{A}	10,13^{A}	10,40^{A}	10,10^{A}	7,53^{A}	10,57^{A}	10,67^{A}
	GIII	10,60^{A}	4,03^{A}	3,47^{A}	2,93^{B}	4,80^{B}	5,70^{B}	6,93^{A}	8,30^{B}	8,60^{A}	9,83^{A}	9,87^{A}	8,30^{A}	9,17^{AB}	11,23^{A}	14,10^{A}
	GIV	9,16^{A}	7,10^{AB}	8,80^{AB}	11,67^{A}	12,90^{AB}	12,63^{AB}	11,43^{A}	10,13^{AB}	11,13^{A}	10,67^{A}	10,93^{A}	10,70^{A}	9,57^{AB}	11,17^{A}	11,57^{A}
	GV	9,13^{A}	9,77^{B}	10,60^{B}	10,77^{A}	11,03^{AB}	10,47^{AB}	10,27^{A}	10,70^{AB}	10,83^{A}	8,83^{A}	10,00^{A}	10,73^{A}	11,70^{A}	11,50^{A}	11,63^{A}
Lymphocytes (x10³/µL)	GI	3,36^{A}	2,62^{A}	1,80^{A}	1,79^{AB}	1,91^{A}	1,27^{A}	1,77^{A}	1,20^{A}	1,52^{A}	1,39^{A}	1,74^{A}	1,89^{A}	3,15^{A}	3,11^{A}	2,64^{A}
	GII	3,77^{A}	3,10^{A}	3,18^{A}	2,90^{AB}	2,22^{A}	2,80^{A}	3,55^{A}	3,81^{AB}	3,58^{AB}	3,57^{A}	3,81^{A}	4,05^{A}	3,28^{A}	3,65^{A}	4,09^{A}
	GIII	5,08^{A}	3,02^{A}	2,32^{A}	1,29^{A}	1,43^{A}	1,40^{A}	1,35^{A}	1,84^{AB}	2,58^{AB}	3,09^{A}	4,23^{A}	3,55^{A}	3,38^{A}	4,85^{A}	4,87^{A}
	GIV	3,91^{A}	2,66^{A}	2,87^{A}	2,21^{AB}	2,42^{A}	2,40^{A}	2,83^{A}	2,83^{AB}	4,33^{B}	3,68^{A}	3,64^{A}	3,13^{A}	4,00^{A}	4,43^{A}	4,79^{A}
	GV	4,64^{A}	3,43^{A}	3,48^{A}	3,73^{B}	2,84^{A}	3,43^{A}	3,14^{A}	4,47^{B}	4,78^{B}	3,96^{A}	3,99^{A}	3,71^{A}	4,73^{A}	4,68^{A}	4,25^{A}
Segmented neutrophils (x10³/µL)	GI	5,40^{A}	1,26^{ABC}	2,12^{AB}	4,04^{AB}	6,15^{A}	7,55^{AB}	8,64^{A}	10,86^{A}	10,01^{A}	8,97^{A}	8,52^{A}	6,01^{A}	5,36^{A}	8,28^{A}	7,95^{A}
	GII	5,57^{A}	4,02^{BE}	6,69^{A}	7,30^{A}	10,60^{A}	9,58^{A}	8,85^{A}	7,11^{AB}	6,38^{A}	5,97^{AB}	5,82^{A}	5,71^{A}	3,97^{A}	6,57^{A}	6,13^{A}
	GIII	5,00^{A}	0,64^{C}	0,94^{B}	1,48^{B}	2,63^{A}	3,27^{B}	4,13^{A}	4,92^{B}	4,75^{A}	6,04^{AB}	4,91^{A}	4,26^{A}	4,78^{A}	5,67^{A}	8,24^{A}
	GIV	4,48^{A}	3,45^{BDE}	4,61^{AB}	6,76^{AB}	7,57^{A}	8,23^{A}	6,85^{A}	6,56^{B}	5,74^{A}	5,64^{AB}	5,90^{A}	6,23^{A}	4,27^{A}	5,47^{A}	5,82^{A}
	GV	4,30^{A}	5,90^{E}	6,63^{A}	6,50^{AB}	7,61^{A}	6,53^{AB}	6,63^{A}	5,94^{B}	5,68^{A}	4,02^{B}	5,44^{A}	6,18^{A}	6,31^{A}	6,33^{A}	6,07^{A}
fPlasma fibrinogen (g/dL)	GI	0,40^{A}	0,27^{A}	0,20^{A}	0,47^{A}	0,53^{A}	0,53^{A}	0,60^{A}	0,67^{A}	0,77^{A}	0,97^{A}	0,97^{B}	0,60^{AB}	1,06^{A}	0,80^{A}	0,43^{A}
	GII	0,20^{A}	0,23^{A}	0,20^{A}	0,20^{A}	0,27^{A}	0,20^{A}	0,20^{A}	0,33^{AB}	0,27^{B}	0,40^{B}	0,33^{A}	0,33^{B}	0,40^{B}	0,37^{AB}	0,27^{A}
	GIII	0,17^{A}	0,13^{A}	0,27^{A}	0,33^{A}	0,20^{A}	0,20^{A}	0,20^{A}	0,20^{B}	0,40^{AB}	0,40^{B}	0,93^{B}	1,33^{A}	0,60^{B}	0,47^{AB}	0,40^{A}
	GIV	0,20^{A}	0,13^{A}	0,23^{A}	0,20^{A}	0,20^{A}	0,20^{A}	0,20^{A}	0,20^{B}	0,17^{B}	0,20^{B}	0,13^{A}	0,33^{B}	0,27^{B}	0,20^{B}	0,20^{A}
	GV	0,40^{A}	0,43^{A}	0,30^{A}	0,37^{A}	0,33^{A}	0,43^{A}	0,47^{A}	0,50^{AB}	0,53^{AB}	0,23^{B}	0,43^{A}	0,47^{AB}	0,47^{B}	0,63^{AB}	0,53^{A}

Different letters within column indicate significant difference at P< 0.05 by Tukey's test.
Results are expressed as means

Table 6 – Laboratory parameters in the peritoneal fluid from the Five groups of horses in this study.

		0	**2**	**4**	**6**	**8**	**10**	**12**	**24**	**36**	**48**	**60**	**72**	**120**	**168**	**216**
Leucocytes (x10³/µL)	GI	2,17^{A}	4,50^{A}	23,53^{A}	30,33^{A}	45,43^{AB}	66,67^{A}	106,67^{A}	185,7^{A}	220,00^{A}	242,00^{A}	290,33^{A}	278,00^{A}	93,80^{A}	36,70^{A}	19,00^{AB}
	GII	1,17^{A}	1,40^{A}	23,03^{A}	38,33^{A}	52,20^{A}	66,40^{A}	82,77^{A}	120,7^{B}	113,00^{B}	112,10^{B}	63,27^{B}	32,70^{B}	11,10^{B}	8,90^{A}	12,77^{AB}
	GIII	1,60^{A}	7,70^{A}	8,60^{A}	16,10^{A}	24,67^{AB}	40,93^{AB}	53,17^{AB}	97,7^{BC}	120,17^{B}	121,27^{B}	190,37^{C}	91,67^{B}	33,40^{AB}	20,35^{A}	28,20^{A}
	GIV	2,10^{A}	1,43^{A}	25,87^{A}	38,53^{A}	54,60^{A}	53,20^{A}	75,80^{AB}	111,70^{B}	79,53^{B}	40,83^{C}	17,37^{BD}	10,80^{B}	7,23^{B}	6,37^{A}	4,93^{B}
	GV	1,57^{A}	1,47^{A}	1,87^{A}	3,33^{A}	4,13^{AB}	4,17^{B}	4,67^{B}	4,33^{D}	3,63^{C}	2,33^{C}	2,13^{D}	2,23^{B}	1,40^{B}	1,20^{A}	2,17^{B}
Total proteins (g/dL)	GI	0,20^{A}	2,27^{A}	2,87^{A}	3,07^{A}	3,13^{A}	3,33^{A}	3,87^{A}	5,73^{A}	6,00^{A}	5,67^{A}	5,67^{A}	6,60^{A}	4,93^{A}	3,64^{A}	2,25^{A}
	GII	0,07^{A}	0,13^{B}	0,97^{BC}	1,27^{BC}	1,40^{BC}	1,73^{B}	2,00^{BC}	2,87^{B}	3,03^{B}	2,53^{B}	2,27^{B}	1,73^{B}	1,30^{B}	1,20^{AB}	1,40^{AB}
	GIII	0,07^{A}	0,47^{B}	0,93^{BC}	1,47^{B}	2,07^{AB}	2,73^{AB}	3,13^{AB}	5,33^{A}	5,70^{A}	5,73^{A}	4,80^{A}	4,20^{C}	2,20^{B}	1,30^{AB}	1,30^{AB}
	GIV	0,60^{A}	0,87^{AB}	1,53^{B}	1,77^{B}	1,93^{AB}	2,20^{AB}	2,33^{AB}	2,40^{B}	2,00^{B}	1,93^{B}	1,73^{B}	1,47^{BD}	1,07^{B}	1,00^{B}	0,80^{AB}
	GV	0,13^{A}	0,07^{B}	0,13^{C}	0,07^{C}	0,67^{C}	0,07^{C}	0,47^{C}	0,10^{C}	0,13^{C}	0,13^{C}	0,27^{C}	0,20^{D}	0,07^{B}	0,20^{B}	0,20^{B}
Fibrinogen(g /dL)	GI	0,00	0,33^{A}	0,20^{A}	0,27^{A}	0,23^{A}	0,33^{A}	0,53^{A}	0,30^{A}	0,27^{A}	0,23^{A}	0,20^{A}	0,40^{A}	0,67^{A}	0,33^{A}	0,05^{A}
	GII	0,00	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{B}	0,00^{A}	0,00^{B}	0,30^{B}	0,00^{B}	0,00^{A}
	GIII	0,00	0,07^{AB}	0,03^{B}	0,07^{B}	0,07^{AB}	0,00^{B}	0,00^{B}	0,03^{B}	0,00^{B}	0,07^{B}	0,27^{A}	0,33^{A}	0,13^{BC}	0,00^{B}	0,00^{A}
	GIV	0,00	0,13^{AB}	0,03^{B}	0,00^{B}	0,07^{AB}	0,00^{B}	0,00^{B}	0,00^{B}	0,03^{B}	0,00^{B}	0,13^{A}	0,03^{B}	0,07^{BC}	0,03^{B}	0,00^{A}
	GV	0,00	0,07^{AB}	0,00^{B}	0,00^{B}	0,00^{B}	0,07^{B}	0,07^{B}	0,00^{B}	0,03^{B}	0,00^{B}	0,00^{A}	0,00^{B}	0,00^{C}	0,00^{B}	0,00^{A}

Different letters within column indicate significant difference at P< 0.05 by Tukey's test.
Results are expressed as means

DISCUSSION

Viable bacteria injected intraperitoneally are not lethal to dogs, rabbits, rats, or mice (HAU et al., 1978) neither to horses as we concluded in this study and in a previous one (MENDES et al., 1999). However, when bacteria are injected in combination with certain adjuvant substances, the combinations are highly lethal. These adjuvant substances are innocuous when injected alone, but apparently they enhance the growth or toxicity of bacteria or interfere with natural defense mechanisms (HAU et al., 1978, YOO et al., 1999). In contrast to other authors statements, hemoglobin potentiated the pathogenicity of *E. coli* despite not being lethal to these animals.

Sensitivity and tension of the abdominal wall, diarrhoea, an increase in intestinal sounds followed by a decrease and hyperthermia were the clinical signs more frequently observed in horses inoculated with *E. coli* associated to hemoglobin. These signs were similar to those occuring in natural peritonitis in horses described by Dyson (1983), Mair et al. (1990), Clabough and Duckett (1992) and by Mendes et al. (1999) in experimental peritonitis in horses.

Horses inoculated with *E. coli* associated to hemoglobin showed hyperthermia for a longer period when compared to horses inoculated only with *E. coli* as shown by Mendes (1996). This finding reinforces

the hypothesis that hemoglobin acts potentiating the pathogenic effects of *E. coli* when associated to peritonitis in horses, as described for mice and rats (BORNSIDE and COHN Jr., 1968, LEE et al., 1979, DUNN et al., 1983, DUNN et al., 1983a, PRUETT et al., 1984, PRUETT et al., 1985, LANGERMANS et al., 1996). Release of exogenous pyrogens occurs in infectious or inflammatory processes stimulating neutrophils and eosinophils to produce interleukin-1 (IL-1) (WHITE II, 1990). Hyperthermia is a central response for IL-1 release. In addition, release of prostaglandin E_2 occurs in inflammation and endotoxemia and is responsible for increases in rectal temperature (WHITE, 1990). Fever and dehydration had a longer duration in groups I and III, even if when compared to other studies with *E. coli* and *B. fragilis* inoculation only, indicating that hemoglobin increases *E. coli* pathogenicity in peritonitis in horses.

Several investigators have suggested that tachycardia occurs in response to fluid loss, toxin absorption and release of inflammatory mediators (KUNESH, 1984, HOSGOOD and SALISBURY, 1989, MAIR et al., 1990, BONOUS, 1993).

Increases in red cell counts, packed cell volume and hemoglobin occurred at different times. An elevated packed cell volume and polycythemia with a proportionate increase in total protein may be seen early in the disease process, reflecting the degree of dehydration present, splenic contractions and hyperglobulinemia (TYLER et al., 1987, SPURLOCK and FURR, 1990). In the horses of GI in this experiment, diarrhoea and fluid loss into the abdominal cavity were responsible for alterations of the water-electrolyte status.

Leukopenia with lymphocytosis and relative neutropenia appeared in all animals inoculated with bacteria and hemoglobin during the first hours of the experiment followed the same response pattern described by Mendes (1996) during experimental peritonitis in horses. Leukocytosis with neutrophilia has been reported to occur in natural peritonitis in horses (DYSON, 1983, MAIR, HILLYER, TAYLOR, 1990, MOLL and SCHUMACHER, 1992). Probably leukopenia with lymphocytosis is not detected in equine peritonitis because laboratory evaluation is performed several hours after the beginning of the inflammatory process in the peritoneum. In any moment of this study leukocytosis was observed in agreement with observations of Mendes (1996).

Fibrinogen is a plasma protein that, when transported to the extravascular space, plays an important role in organism defence and aids in finding the pathologic process. Plasma levels of fibrinogen are considered to be important for the evaluation of the inflammatory response. Variations in plasma fibrinogen levels occurred in GI but differed from those reported in literature (DYSON, 1983, MAIR, HILLYER, TAYLOR, 1990). Increased plasma fibrinogen levels in equine peritonitis have also been demonstrated (CLABOUGH and DUCKETT, 1992). The present data stated that the evaluation of plasma fibrinogen was of little value for the prognosis of equine peritonitis, similar data were reported previously (HAWKINS et al., 1993).

Analysis of peritoneal fluid has been used for the diagnosis and prognosis of abdominal diseases in horses (TULLENERS, 1983). The paracentesis technique is safe (White II, 1990) with no complications even after repeated collections (TULLENERS, 1983, SCHUMACHER et al., 1985, JUZWIAK et al., 1991).

A significant increase in peritoneal fluid leukocytes was observed from 10 HAI. These findings reinforces the importance of this ancillary method, able to indicate a peritoneal injury very early. Mendes et al. (1999a) also observed, during experimental peritonitis in horses, a high leukocyte count in peritoneal fluid being 516×10^3 leukocytes/mm^3. According to Ricketts (1987) in peritonitis, nucleated cell counts are higher than 10×10^3 and in septic peritonitis, they are higher than 50×10^3. Dyson (1983) also observed a considerable number of leukocytes (385×10^3) in horses with natural peritonitis. Despite leukocyte high counts no horse died, indicating that this parameter is very important to evaluate the inflammatory response but not for the prognosis of peritonitis. Leukocyte high counts without lethality was also observed by SUSKO et al. (1994) and Mendes et al. (1999a).

Moll and Schumacher (1992) and Clabought and Duckett (1992) also reported increased total protein in peritoneal fluid and total protein levels higher than 2,5 g/dl in inflammation as a consequence of increased capillary permeability in abdominal viscera (WILSON and GORDON, 1987). MENDES et al. (1999a) also observed increased values for total protein in peritoneal fluid during experimental peritonitis.

Fibrinogen was significantly increased in peritoneal fluid from 4 to 120 HAI. Wilson and Gordon (1987) considered fibrinogen values of 0.1 g/dL or more in peritoneal fluid to indicate vascular and/or inflammatory injuries. Thus, our data suggest that the evaluation of this parameter in horses with peritonitis could be an important ancillary method for diagnosis.

The reisolation of *E. coli* and *B. fragilis* from inoculated animals confirms the idea that the clinical and laboratory alterations observed in these animals are due to installation of septic peritonitis. The reisolation only in the first hours of peritonitis evolution indicates that, even in the presence of an adjuvant (hemoglobin), the bacterial proliferation was limited by the organic defenses. The peritoneal fluid presents antimicrobian activity in association with polymorphonuclear and mononuclear cells, beyond the possibility of the mechanical elimination of the agent. The specific immune system, which is mediated by the activity of lymphocytes, provides a secondary amplification system that may be of great importance for the clearance of the agent (HEEL and HALL, 1996).

Jennings et al. (1980) showed that in rats the uptake of bacteria from the peritoneum begins nearly instantaneously after peritoneal inoculation and that large numbers are either destroyed or sequestered by 4h, when an equilibrium between host and invader becomes established. Later, a second defense response occurs and bacteria will be eliminated by immunologically effective cells.

The physiopathological alterations observed in animals from GII cannot be attributed to endotoxemia, but to the bacterial action because according to Moore (1993) *B. fragilis* produces endotoxins that lack the lipid A of conventional endotoxin and thus are less potent and do not induce significant biological responses in host.

Animals from GI and GIII, which contained *E. coli* in their inoculum, showed clinical signs and laboratory alterations more evident and for a longer period. This severe signs could be imputed to the potentiated effects of hemoglobin over *E. coli* growth.

The role of hemoglobin during peritonitis has been studied extensively (KLAERNER et al., 1997), but the reasons why certain *E. coli* strains become highly virulent when injected with hemoglobin or other soluble iron sources remain unknown (TELANG et al., 2001). This phenomenon is certainly due, in part, to promotion of bacterial growth by the presence of iron, which is normally scarce in mammalian body fluids (TELANG et al., 2001). Bacteria require iron for growth. They have developed specific mechanisms for competing for host iron (ALLEN et al., 2000). Apparently, our results with animals from GI support this idea.

It was shown by Hau and Simmons (1980) that the addition of hemoglobin to an intraperitoneal *E. coli* inoculum simultaneously interferes with the normal rapid clearance of bacteria, and the normal influx of granulocytes into the peritoneal cavity in response to the inoculum. However, our results showed a major influx of polymorphonuclear cells into the peritoneal cavity and phagocytic activity in a great number of samples in contrast with a previous report (HAU et al., 1978).

One of the hypotheses to explain the role of hemoglobin in peritonitis is that the presence of hemoglobin may facilitate hydroxyl-radical generation from activated oxygen species, mainly hydroxyl radical (YOO et al., 1999). However, other studies are necessary to confirm this hypothesis with our experimental model.

Bovine hemoglobin was used because it is the only purified hemoglobin available and it was previously used in experimental model systems in rats (DUNN et SIMMONS, 1983). When using hemoglobin without proper purification it was shown that many of the results obtained experimentally were not reproductible due to the presence of substances in the inoculum other than hemoglobin. Lee et al. (1979) showed that fresh lysates and commercially isolated hemoglobins from various animals are virtually equivalent.

Beyond facilitating the growth of *E. coli*, hemoglobin alone could provoke a chemical peritonitis as shown in animals from GIV. This finding goes against what Hau et al. (1978) observed in rats, who stated that the intraperitoneal injection of hemoglobin did not produce any ill effects. Probably this could be considered as variations between the studied species, since horses are very sensitive to peritonitis, by this way responding more aggressively to small stimuli.

No animal died although the marked severity and duration of the symptoms and laboratory alterations observed in animals inoculated with *E. coli* plus hemoglobin. We believe that the host defense proteins responded to the threat of bacterial infection by binding free iron in an attempt to make it unavailable to potential pathogens since bacteria require iron for growth (ALLEN et al., 2000).

Although horses have always been considered highly susceptible to peritonitis (SCHNEIDER, 1992, MAIR et al., 1990), some evidences show that the severity of pathogenicity is not dependent only of causes but also from a complex series of physiopathological interactions between infective agents and host defenses.

In conclusion, hemoglobin was able to cause chemical peritonitis in horses, it had an adjuvant effect when associated to *E. coli* in experimental peritonitis in horses potentiating the pathogenic effects of *E. coli* but was not lethal to horses. However, hemoglobin in association with *B. fragilis* showed more attenuated clinical and haematological alterations when compared to the association with *E. coli*. Finally, this attenuated signs seemed to be due to the interference of hemoglobin on the synergism between *B. fragilis* and *E. coli*.

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