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Peritoneal fibrinolytic activity in equines subjected to small colon enterotomy and treated with heparin

J.M. Alonso[a],*, R.K. Takahira[a], T.F. Bachiega[b], K.A. Rodrigues[a], A.L.M. Yamada[a], A.L.G. Alves[a], M.J. Watanabe[a], C.A. Rodrigues[a], C.A. Hussni[a]

[a]UNESP, Univ. Estadual Paulista, School of Veterinary Medicine and Animal Science. Botucatu, São Paulo, Brazil.
[b]UNESP, Univ. Estadual Paulista, Biosciences Institute. Botucatu, São Paulo, Brazil.

*Corresponding author; UNESP, Univ. Estadual Paulista, School of Veterinary Medicine and Animal Science. Botucatu, São Paulo, Brazil.

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ABSTRACT

Study rationale: Heparin is routinely administered postoperatively in abdominal surgery to prevent the formation of adhesions; however, there is no consensus in the literature indicating the effectiveness of such use. Objectives: This study sought to assess peritoneal fibrinolytic activity post-enterotomy of the small colon in equines treated with heparin. Methods: In the present study, 10 adult equines were divided into 2 groups of 5 animals each: the control group (CG) and treated group (TG). Both groups underwent laparotomy and enterotomy of the small colon through the right paralumbar fossa in quadrupedal position. In addition, the animals received combinations of flunixin meglumine, gentamicin and penicillin. The TG also received subcutaneous heparin (150 IU/kg, bwt q. 12 hours, 5 days). The animals were evaluated for the peritoneal concentrations of tissue plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1) and D-dimer at the following time-points: prior to enterotomy (M0); 12 hours after (M1); 1 day after (M2); 2 days after (M3); 4 days after (M4); 6 days after (M5); 10 days after (M6) and 14 days after enterotomy (M7). Results: A significant difference in tPA level was observed between the groups when all time-points were combined, with a median value of 2.59 IU/mL for the CG and 2.03 IU/mL for the TG. Although no significant difference was observed when the groups were compared

at different time-points, smaller tPA and D-dimer values were observed for the TG during heparin treatment. Conclusions: In addition to the finding that the TG showed a lower tPA concentration and reduced D-dimer formation, it was concluded that heparin treatment decreased the formation of fibrin clots and peritoneal fibrinolytic activity.Relevance: Because elevated D-dimer levels are directly related to a poor prognosis and high mortality rate, this study reinforced the relevance of the use of heparin in hypercoagulable states and following abdominal surgery.

1. Introduction

Mesothelial cells actively participate in the processes of peritoneal inflammation and repair through the release of inflammatory mediators such as procoagulants and fibrinolytic agents (Delgado et al., 2009). Fibrinolysis is activated by tissue plasminogen activator (tPA) and inhibited by tissue plasminogen activator inhibitor type 1 (PAI-1), which is stimulated in the presence of trauma, infection or endotoxins (Holmdahl et al., 1997; Ivarsson et al., 2001; Mitchell et al., 2005; Caldwell and Mueller, 2010; Shimomura, 2012).

The expression of tPA and PAI-1 in non-inflamed tissues occurs in the mesothelium and submesothelial layer, respectively (Holmdahl, 1997). Surgery results in abrasion of the mesothelium, thereby eliminating the source of tPA, exposing the submesothelial layer and increasing the source of PAI-1 (Holmdahl, 1997). As a result, after surgery, there is a decrease in the degradation and an increase in the production of fibrin, which may result in the formation of abdominal adhesions (Shimomura, 2012).

D-dimer is a fragment that is exclusively released during fibrin lysis via the action of plasmin. In the serum, plasmin levels are correlated with the destruction of fibrin after hypercoagulable and hyperfibrinogenic states and serve as a marker of coagulation and fibrinolytic activity (Stokol et al., 2005; Delgado, 2009; Cesarini, 2010).

For D-dimer analysis, antibodies against the degradation products of human fibrin are used, and these are available in commercial ELISA, immunofiltration, immunoturbidimetry and latex agglutination kits. In particular, this latter type of kit is often favoured due to its availability, ease of use and rapid results (Stokol et al., 2005).

D-dimer evaluation is a sensitive approach for the diagnosis of thromboembolic disease and disseminated intravascular coagulation in humans and is considered a good predictor of mortality. In equines, hypercoagulable states can be detected in cases of colic associated with ischemia, inflammation and peritonitis (Stokol et al., 2005; Cesarini, 2010).

The normal plasma value of D-dimer in equines is approximately 1000 ng/mL (Stokol et al., 2005), although this concentration in the peritoneal fluid can range from 4.0 to 88.0 ng/mL (Delgado et al., 2009). Moreover, in cases of peritonitis, the literature describes average peritoneal D-dimer concentrations of 24301 ng/mL (Delgado et al., 2009). Few studies have been conducted using peritoneal fluid, and most of these have focused on human patients. However, measurement of the D-dimer concentration in the peritoneal fluid of equines with severe gastrointestinal disorders demonstrated marked hyperfibrinolysis related to increased fibrin formation and degradation (Delgado et al., 2009). A D-dimer concentration > 4000 ng/mL has been established as the cut-off point for predicting poor prognosis in equines with abdominal afflictions (Cesarini, 2010).

The most commonly used medications during the postoperative period following abdominal surgery include anti-inflammatory drugs, antibiotics and anticoagulant agents (Eggleston and Mueller, 2003; Claunch and Mueller, 2012). Anticoagulants are used to prevent the formation of fibrin clots and thus inhibit the formation of adhesions (Eggleston and Mueller, 2003; Ward and Panitch, 2011).

Heparin is an acid-sulphated proteoglycan, and a small portion of this molecule is responsible for its anticoagulant effect; in particular, the fraction that binds to antithrombin III acts as a slow inhibitor of thrombin, plasmin and coagulation factors. Heparin catalyses the inhibition of antithrombin III, thereby stimulating tPA and inhibiting coagulation factors in both the intrinsic and common pathways, including thrombin (Andrade and Strickland, 1986; Moore and Hinchliff, 1994; Majerus, 2006).

There is no consensus in the literature regarding whether heparin can inhibit adhesion formation (Claunch and Mueller, 2012). Several studies have evaluated the use of heparin in ponies subjected to experimentally induced adhesions and reported a significant reduction in the formation of adhesions at the dose of 40 IU/kg every 12 hours for 48 hours (Parker et al., 1987). However, the use of low-molecular-weight heparin at a dose of 66 IU/kg every 12 hours for 5 days in equines subjected to laparotomy showed no reduction in postoperative complications and failed to increase the survival rate (Young et al., 1989).

In rats, the use of heparin alone (Kutlay et al., 2004; Bahadir et al., 2007; Kement et al., 2011) or in combination with other prophylactic therapies after laparotomy and the induction of adhesions (Kutlay et al., 2004; Aysan et al., 2010) promoted favourable results. However, some authors have reported that the use of heparin in rats (Kaptanoglu et al., 2008) or humans (Jansen, 1998; Watson et al., 2000; Opitiz et al., 2003) did not inhibit the formation of adhesions.

Moreover, the recommended heparin dose remains controversial, ranging from 20 to 150 IU/kg every 6-12 hours for 2-3 days (Eggleston and Mueller, 2003). Although no specific studies have been performed with equines, the intraperitoneal application of 30,000 IU heparin diluted in saline solution is routinely used in horses and has been suggested to be effective (Eggleston and Mueller, 2003).

The aim of the present study was, therefore, to evaluate the effect of heparin on peritoneal fibrinolytic activity in horses subjected to colon laparotomy and enterotomy due to the frequent use of this drug as a prophylactic agent for inhibiting the formation of abdominal adhesions.

2. Materials and methods

2.1. Animals

Ten mixed-breed adult equines were subjected to small colon laparotomy and enterotomy in the quadrupedal position. The animals were divided into 2 groups of 5 animals each: the control group (CG) and treated group (TG). The TG received heparin subcutaneously at a dose of 150 IU/kg immediately after surgery and then every 12 hours for 5 days.

2.2. Surgical preparation

Prior to surgery, abdominal trichotomy was performed on the right side, and penicillin potassium (Ariston)1 at a dose of 30000 IU/kg, gentamicin (Gentatec)2 at 6.6 mg/kg and flunixin meglumine (Chemitec)3 at 1.1 mg/kg were administered intravenously to both groups. The procedure was performed with the animals in the quadrupedal position and under sedation via continuous infusion of detomidine (Dormium V)4 with a 5 μg/kg bolus followed by continuous infusion of 20 μg/kg/h combined with butorphanol (Turbogesic)5 in a 20 μg/kg bolus followed by continuous infusion of 13 μg/kg/h. After disinfection with chlorhexidine digluconate (Riohex)6, local anaesthesia was administered along the incision line and the adjacent deep tissues at the surgical site using 2% lidocaine (Xylestesin)7 combined with a vasoconstrictor.

2.3. Surgical procedure

A skin incision of approximately 10 cm was made on the right paralumbar fossa, the subcutaneous tissue was pulled back, the muscle was sectioned, the peritoneum was perforated, and the abdominal cavity was accessed. The small colon was identified by direct palpation, and the most aboral segment was externalised. Two coprostatic forceps were applied to interrupt the faecal flow after emptying the segment and delimiting the enterotomy region. Subsequently, 2 Allis clamps were used to hold the segment in place, and enterotomy of the teniae coli region was performed. A simple interrupted suture was then administered with catgut thread (chromic Catgut 3-0)8, followed by abrasion of the serous layer with dry gauze in 50 movements that were repeated for each portion adjacent to the teniae coli; this was done to make the surgical region haemorrhagic. The segment was repositioned, and the abdominal wall was closed in a Sultan pattern using number 2 polyglactin 910 thread (Vicryl)9. The skin was closed in a Wolff pattern using 0.60 nylon monofilament thread (Dourado)10.

After surgery, both groups received benzathine penicillin (Mogipen)11 intramuscularly at a dose of 30000 IU/kg every 72 hours for 10 days, gentamicin at a dose of 6.6 mg/kg IV, every 24 hours for 5 days, flunixin meglumine at a dose of 1.1 mg/kg intramuscularly every 24 hours for 5 days and anti-tetanus serum (Vencosat)12 at a dose of 10000 IU subcutaneously in a single application.

2.4. Assessment time-points

The animals underwent peritoneal fluid collection to measure the concentrations of tPA, PAI-1 and D-dimer at the following time-points: prior to enterotomy (M0); 12 hours after (M1); 1 day after (M2); 2 days after (M3); 4 days after (M4) 6 days after (M5); 10 days after (M6) and 14 days after enterotomy (M7).

2.5. Sample collection and processing

The peritoneal fluid samples were collected at the most ventral region of the abdomen using a 40 x 12 mm hypodermic needle and a tube containing 3.8% sodium citrate at a 9:1 (liquid:anticoagulant) ratio (v/v). The samples were centrifuged for 15 minutes immediately after collection, using a 1000G rotation for the D-dimer samples, 3000G for the PAI samples and 2500G for the tPA samples.

After centrifugation, the samples were aliquoted into Eppendorf tubes for storage. The D-dimer samples were stored at -20°C for a maximum of 1 month prior to processing, the tPA samples were stored for 4 months at -70°C, and the PAI-1 samples were stored for 1 month at -70°C.

The D-dimer analysis was performed using the latex agglutination test (D-dimer test13) according to the manufacturer's instructions. The tPA and PAI-1 concentrations were determined by ELISA (human tPA activity assay14 and human PAI-1 activity assay15), according to the manufacturer's instructions.

2.6. Statistical analysis

The distribution of the response variables was analysed as a criterion for the choice of analytical method. Due to the presence of varying degrees of asymmetry and deviations from a normal (or Gaussian) distribution, the Wilcoxon test (Pagano and Gauvreau, 2000) was used to compare the median of each response variable between the study groups. Then, the comparison between groups was performed for each time-point. For each group, the Wilcoxon test for paired samples (Pagano and Gauvreau, 2000) was used to compare the median of each variable between each time-point and the initial time-point. Statistical analysis was performed using the PROC NPAR1WAY procedure (SAS Institute Inc., 2009), and the statistical significance was defined as $p < 0.05$.

3. Results

The baseline tPA values for both groups (Table 1) were greater than values previously reported in the literature (0.27 to 0.45 IU/mL) (Delgado et al., 2009). After surgery, the tPA level was significantly increased in both groups above the normal range when comparing the groups across all time-points ($p = 0.0069$), with median values of 2.6 IU/mL (CG) and 2.0 IU/mL (TG) (Table 2). This comparison between the groups at different time-points showed that, although the tPA concentrations were lower in the TG throughout the treatment period, there were no significant differences between time-points (Table 1).

The baseline values for PAI-1 were also higher than those reported in the literature (0 to 2.37 IU/mL) (Delgado et al., 2009). However, there was no change in this concentration after surgery, with no significant difference ($p = 0.7022$) between groups or time-points (Tables 1 and 2).

The baseline values of D-dimer were below the reference values (4.0 to 88.0 ng/mL) (Delgado et al., 2009), with detected concentrations < 0.5 ng/mL. After surgery, considerably higher values were obtained for the CG, with median values of 4000 ng/mL (CG) and 1500 ng/mL (TG) (Table 2). However, there were no significant differences ($p = 0.0745$) between groups when all time-points were combined (Table 1).

4. Discussion

The baseline peritoneal values of tPA (0.27 - 0.45 IU/mL) and PAI-1 (0 - 2.37 IU/mL) were higher than the reference values for healthy equines; however, these values were reported in the literature by only a single study (Delgado et al., 2009), which used the same kits that were used in the present study. It was noted, however, that the centrifugation step carried out in the previous study (Delgado et al., 2009) was at 1000G for 15 minutes for D-dimer, tPA and PAI-1, and these samples were stored at -70°C, whereas in the present study, the experiments were performed in duplicate and according to manufacturer's instructions. Thus, the values obtained at M0 were chosen as baseline reference values.

The significantly higher tPA concentration in the CG may have been due to reduced availability of fibrin for degradation among non-heparinised animals in the TG, as well as increased tPA activation among CG animals for degradation of the fibrin clots. Although the TG showed higher values at M0 than the CG, these values were lower at the end of the assessment period, although this difference was not significant.

This difference in tPA concentration was most evident between M3 and M5. This observation was likely due to an increase in fibrinogen 48 hours after the inflammatory stimulus as well as the maintenance treatment with heparin until the 5th day, as there was an increase in the concentration of peritoneal tPA in the TG following its suspension, reaching a level equivalent to the CG.

Table 1
Comparison of the peritoneal concentrations of tPA, PAI-1 and D-dimer between the CG and TG at different time-points.

	tPA (IU/mL)					
	Control Group (n = 5)			**Treated Group (n = 5)**		
Time-point	**Median**	**Q1**	**Q3**	**Median**	**Q1**	**Q3**
M0	0.78	0.67	0.93	1.10	0.67	1.25
M1	2.78	2.62	2.84	2.65	2.65	2.77
M2	2.82	2.67	2.83	2.60	0.92	2.86
M3	2.83	2.79	2.91	1.82	1.66	2.50
M4	2.72	1.65	2.77	1.18	1.04	1.76
M5	2.54	1.64	2.59	0.93	0.00	2.41
M6	2.46	1.97	2.55	2.62	0.93	2.63
M7	2.16	1.72	2.67	2.31	1.28	2.59
	PAI-1 (IU/mL)					
	Control Group			**Treated Group**		
Time-point	**Median**	**Q1**	**Q3**	**Median**	**Q1**	**Q3**
M0	17.45	16.02	21.57	16.81	12.53	17.45
M1	18.72	18.40	28.23	17.76	16.50	18.83
M2	23.48	19.67	34.12	28.26	20.89	34.32
M3	24.43	18.70	24.43	20.57	12.73	27.21
M4	19.10	17.13	25.70	18.40	15.88	25.70
M5	16.50	15.21	19.35	18.70	14.81	23.79
M6	18.72	17.13	21.11	24.11	18.56	31.97
M7	16.18	15.86	24.87	29.80	19.68	40.15
	D-dimer (ng/mL)					
	Control Group			**Treated Group**		
Time-point	**Median**	**Q1**	**Q3**	**Median**	**Q1**	**Q3**
M0	0	0	0	0	0	0
M1	32000	1000	32000	8000	8000	16000
M2	8000	8000	8000	4000	2000	32000
M3	8000	4000	16000	2000	1000	8000
M4	4000	4000	8000	2000	2000	8000
M5	2000	1000	4000	500	0	1000
M6	4000	500	4000	500	500	1000
M7	500	500	2000	0	0	2000

There was no significant difference between groups. Q1 = first quartile; Q3 = third quartile.
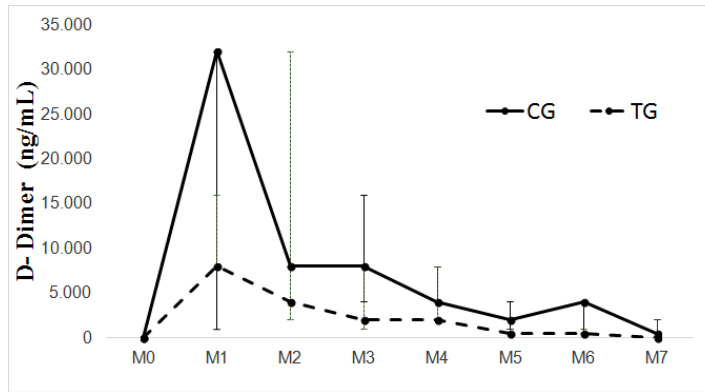
Fig. 1. Comparison of the median peritoneal D-dimer concentrations between groups at different time-points. The error bars represent the first and third quartiles.

Table 2

Comparison of the peritoneal concentrations of tPA, PAI-1 and D-dimer between the CG and TG in the combined assessment of all time-points.

Groups	tPA (IU/mL)*			PAI-1 (IU/mL)			D-dimer (ng/mL)		
	Median	Q1	Q3	Median	Q1	Q3	Median	Q1	Q3
CG	2.59	1.65	2.81	18.91	16.66	25.28	4000	500	8000
TG	2.03	0.92	2.63	18.83	16.50	30.90	1500	0	6000

* Significant difference between groups ($p < 0.005$) Q1 = first quartile; Q3 = third quartile.

Although it was expected that the surgical stimulus would trigger mesothelial injury and a consequent reduction in tPA, the present study found an increase in tPA in both groups after surgery. Although this increase was not significant, the intensity of the trauma caused by the surgical procedure failed to result in the depletion of tPA and an increase in PAI-1, which would translate into a stimulus for the reduction of fibrinolysis (Holmdahl, 1997; Shimomura, 2012). Thus, it is noteworthy that although the surgical stimulation resulted in inflammation, it was not enough to trigger the formation of adhesions, as suggested by the unaltered PAI-1 level.

Although there was no increase in PAI-1 in either group, it was observed, according to the difference in tPA concentration between groups, that heparin effectively decreased fibrin formation due to reduced tPA activation. This finding is consistent with the observed difference in the rate of formation of D-dimer (higher in the CG), demonstrating that heparin acted by inhibiting the conversion of fibrinogen into fibrin.

The latex agglutination test was used for determining the D-dimer concentration because of its advantages over ELISA and immunofluorescence tests, including its greater market availability, ease of use and rapid results (Stokol et al., 2005). The concentration of D-dimer is directly related to the diagnosis and prognosis of equines with colic, with higher concentrations associated with a worse prognosis (Stokol et al., 2005; Delgado, 2009; Cesarini, 2010).

Although there was no significant difference in the D-dimer concentration between groups, higher values tended to be observed for animals in the CG, which is consistent with the notion that abdominal surgery results in a state of hyper-fibrogenesis and hyperfibrinolysis (Delgado et al., 2009). Because the D-dimer fragment is released exclusively during fibrin lysis by plasmin, it was inferred that the animals in the heparin-treated group, despite undergoing the same surgical procedure, had lower D-dimer concentrations due to reduced formation and deposition of fibrin and consequently lower cleavage rates of fibrin.

Given that the TG exhibited lower tPA concentration and lower D-dimer formation, it is possible that heparin treatment decreased the formation of fibrin and therefore also the fibrinolytic activity. Because high D-dimer

values are directly related to poor prognosis and high mortality, the indication for the use of heparin in hypercoagulable states and after abdominal surgery in equines was strengthened by the results of our study.

Although the animals in the present study did not show abdominal adhesions, our results highlight the potential use of heparin for prophylaxis in cases of abdominal adhesions. However, further clinical studies should evaluate the use of heparin in equines suffering from colic syndrome and those subjected to laparotomy.

Acknowledgements

Conflict of interest

There are none conflict of interest.
1. Penicillin G Potassium - Ariston Indústrias Químicas e Farmacêuticas Ltda.
2. Gentatec - Chemitec, Agro-veterinária Ltda.
3. Flunixin (injectable) - Chemitec, Agro-veterinária, Ltd.
4. Dormium V - Agener União, Ltd.
5. Torbugesic - Fort Dodge, Ltd.
6. Xylestesin - Cristália, produtos químicos e farmacêuticos Ltda.
7. Riohex - Rioquímica, Ltd.
8. Brasuture - Brasuture suturas cirúrgicas [Brasuture surgical sutures].
9. Vicryl 2 - Johnson & Johnson, Ltd.
10. Dourado - Nylon monofilament, 0.60 mm.
11. Mogipen - Bimeda, Mogivet Farmacêutica S.A.
12. Vencosat - Vencofarma Laboratories of Brazil, Ltd.
13. D-dimer test - Horiba medical, Ltd.
14. Human tPA activity assay - Molecular Innovations SPSS Inc, Chicago, IL.
15. Human PAI-1 activity assay - Molecular Innovations SPSS Inc, Chicago, IL.

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