David Argüelles Capilla

ROLE OF TRANSFORMING GROWTH FACTOR β_1 AND D-DIMER IN HORSES WITH SEVERE GASTROINTESTINAL DISEASE

PhD Thesis

(TESIS DOCTORAL)

Directed by

Marta Prades Robles

Department of Animal Medicine and Surgery

Faculty of Veterinary Medicine

Universitat Autònoma de Barcelona

Codirected by

Jorge U. Carmona

Department of Animal Health

School of Veterinary Medicine and Animal Science

Universidad de Caldas, Colombia

Bellaterra, 2014

ROLE OF TRANSFORMING GROWTH FACTOR β_1 AND D-DIMER IN HORSES WITH SEVERE GASTROINTESTINAL DISEASE

Academic dissertation approved for the partial fulfillment of the requirements for the degree of doctor of philosophy by the Universitat Autònoma de Barcelona, Spain

Aknowledgments

The author thanks Antoni Iborra, Paz Martínez, Laia Viñals and Lluis Monreal and Marta Prades. This research was partially supported by a grant of the European College of Veterinary Surgeons (ECVS). Also thanks to all the staff of the Equine Hospital at UAB, Frederic Climent, Lara Armengou, Silvia Alsonso and Carme Sastre. Special thanks to my friend and co-supervisor Jorge U. Carmona, for his help during the thesis.

CONTENTS

	Pag.
Introduction…….	1
Objectives……	5
References……	6
II. Literature review……	9
The mesothelial cell and transforming growth factors.....	10
.Mammalian transforming growth factor-βs.....	12
Coagulation and fibrinolysis ……..	14
Pathofisiology of adhesion formation	15
Tissue markers as predictors of postoperative adhesions…….	19
References.....	20
III. Study one: Peritoneal concentrations of transforming growth β1 in horses with colic……..……..	25
Summary.....	26
...Introduction.....	28
...Material and methods.....	29
...Results.....	34
...Discussion.....	37

IV. Study two: Plasma and peritoneal fluid concentrations of D-dimer and transforming growth factor ß1 in horses with colic……	
...Abstract.....	43
...Introduction.....	44
...Material and methods……..	46
...Results.....	53
...Discussion.....	60
General references……..	75
V. Concluding discussion……	64
..LIST OF.CONCLUSIONS.....	73
General summary.....	82
Resumen General.....	83

List of tables

	Pag.
Study one: Peritoneal concentrations of transforming growth factor ß in horses with colic	
Table 1. Plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations in the control and different colic groups.....	30
Table 2. Number and percentages of horses classified according to the diagnosis and the outcome.....	32
Table 3. Peritoneal concentrations of TGF-β_1 and TGF-β_3 according to the type of peritoneal fluid.....	33
Table 4. Number a nd percentages of hor ses c lassified a ccording t o t he t ype o f peritoneal fluid analysis and the outcome.....	34
Study two: Plasma and peritoneal fluid concentrations of D-dimer and transforming growth factor beta 1 in horses with colic	
Table 1. Number and percentages of horses classified according to the diagnosis and the outcome.....	52
Table 2 . Number a nd percentages of hor ses c lassified a ccording t o t he t ype o f peritoneal fluid analysis and the outcome.....	55
Table 3. Plasma and peritoneal D-dimer and TGF-β1 concentrations in the control and various colic groups.....	59

List of figures

	Pag.
Study one: Peritoneal concentrations of transforming growth factor beta in horses with colic	
Figure 1 . A) Box plots of the mean concentrations of plasma ßβ1 (ng/mL) in survivors and non-survivors. Whiskers represent the 95% confidence interval. B) Box plots of the mean concentrations of peritoneal TGF-β1 (ng/mL) in survivors and non-survivors. Whiskers represent the 95 % confidence interval.....	35

1. INTRODUCTION

Introduction

Mesothelial cells that line the serosal surfaces of the peritoneal cavity provide a natural protective barrier that prevents viscerae from adhering to adjacent apposing surfaces. Injury occurring after surgical procedures, infection or inflammation, compromises the integrity of these cells resulting in a local biological reparative response which releases various pro-, anti- and immunomodulatory mediators. These include chemokines, cytokines, growth factors and products of the coagulation and fibrinolysis systems, amongst others. It has been seen that, mesothelial cells may be involved in pro-coagulant activity, but may also show marked intrinsic fibrinolitic activity and anti-inflammatory properties in order to prevent abdominal adhesions (Delgado et al. 2009)

The pro-coagulant activity of mesothelial cells induces secretion of mesothelial plasminogen activator inhibitor type 1 (PAI-1) a fibrinolysis inhibitor that induces fibrin formation and deposition in an attempt to repair peritoneal injury. Fibrinolysis is simultaneously activated by the secretion of tissue-plasminogen activator (t-PA) that is essential to degrade the fibrin formed after different gastrointestinal injuries and to decrease subsequent risk of adhesion formation. Peritoneal fibrinolysis activation can be detected by measuring peritoneal D-dimer concentrations, which are fibrin degradation fragments released exclusively by plasmin-mediated lysis of cross-linked fibrin. Thus peritoneal D-dimer concentration measurement is a specific way of monitoring peritoneal fibrinolysis activity and subsequently of peritoneal fibrinogenesis. The test for measuring plasma and peritoneal D-dimer concentration has been previously used for assessing plasma hypercoagulation and hyperfibrinolysis in horses after endurance competition, in horses with gastrointestinal disorders, in those with laminitis, and in septic foals. Thus, plasma D-dimer concentration is considered a sensitive marker in assessing fibrinolytic activity and subsequently coagulation activity. Any increase in D-dimer concentration is strongly correlated to an increase in fibrin

destruction (hyperfibrinolysis) subsequent to an increase in f ibrin f ormation (hypercoagulation or hyperfibrinogenesis) (Chegini et al 2001).

When cellular o r tis sue in jury is e xtensive, it leads to ex cess m igration an d cel l proliferation that often results i n t he de velopment of pe ritoneal a dhesions. T hese adhesions are know n t o be t he m ajor c ause of bow el obstruction, pa in a nd hos pital readmission in humans and the major cause of repeated laparotomy in horses (Hobson et al. 2003).

There are clinical studies in humans and experimental studies in rats that demonstrate the imp lication o f th e peritoneal TGF-β series i n t he d evelopment of a bdominal a dhesions (Chegini e t a l. 2001) . Peritoneal f luid a nd a dhesion t issue analysis in hum ans suggest t hat alterations occur in peritoneal expression of c ytokines, g rowth f actors a nd pr oteases, which may b e a key f actor in the pathogenesis of adhesion f ormation. Increased s ecretion o f interleukins, t umor ne crosis f actor, t ransforming growth f actor β cytokine f amily that regulate t he i nflammatory and i mmune r esponses, e nhance t issue fibrosis a nd a nti-inflammatory a nd a nti-fibrotic act ivities r espectively (Barton et a l. 1999). T hese c ytokines also r egulate t he e xpression of e nd pr oteases, t hus c ontrolling f ibrin de position a nd degradation, c hemotactic mig ration o f in flammatory c ells a nd f ibroblasts, c ell g rowth a nd differentiation, angiogenesis and deposition of extra cellular matrix (Horohov et al. 2000).

Over-expression of TGF-β_1 has been implicated in fibrotic disorders at various sites of the bod y s uch a s f ibrosis, g lomerulonephritis, c irrhosis of t he l iver a nd de rmal s carring (Hobson et al. 200, Raaf et al., 2011; Ueha et al., 2012, Åkerberg et al., 2013). Evidence that implicates T GF-β in pe ritoneal a dhesion f ormation arises from experiments s howing that elevated TGF-β concentrations were obs erved in a dhesion t issue and peritoneal f luid o f patients with adhesions. Furthermore, post-operative peritoneal administration of TGF-β_1 has been s hown t o i ncrease the i ncidence of adhesion f ormation, w hile ne utralizing a ntibodies directed against TGF-β_1 reduced this incidence (Barton et al. 1999). On the other hand, TGF-

β_3 is a TGF-β isoform found in small concentrations in peritoneal fluid from patients with adhesions, in which TGF-β_1 and β_2 have been shown to be up-regulated and associated with adhesion formation (Falk et al., 2009). TGF-β_3 mainly counteracts profibrotic effects of TGF-β_1 and β_2 (Gorvy et al., 2005) and this is specially recognised during fetal wound healing, since this protein is implicated in a complex regenerative wound healing mechanism with no scarring To note, TGF-β_1 profibrotic mechanisms are activated once the animal is born (Namazi et al. 2011).

Adhesions express significantly more TGF-β_1 than parietal peritoneum (Chegini et al. 2001). TGF-β has been detected in equine chronic pneumonia, indicating that the stimulus of TGF-β produces and increase in collagen deposits. The key role of TGF-β in peritoneal healing and adhesion-genesis is based on the facts that the TGF-ßs are chemotactic for fibroblasts and inflammatory cells and promote cell proliferation, differentiation and angiogenesis (Rodríguez et al. 1996).

A study performed recently by our group showed that horses with severe inflammatory and ischemic gastrointestinal disorders had a significant increase in peritoneal TGF-β concentration (Argüelles et al. 2009). Additionally, in another study performed by our group (Delgado et al. 2009), peritoneal D-dimer concentrations were observed to be significantly higher in horses with peritoneal fluid alterations that were suffering peritonitis, and inflammatory and ischemic gastrointestinal problems when compared with horses with a normal peritoneal fluid analysis. These results confirmed that horses with the most severe forms of gastrointestinal disorders presented higher peritoneal fibrinolytic activity (hyperfibrinolysis) most likely as a consequence of increased peritoneal fibrin formation and deposition (hyperfibrinogenesis).

To our knowledge there are no studies in horses that demonstrate a relationship between peritoneal fibrinolysis activity and peritoneal TGF-β concentration in horses with

colic. Thus, the aims of this study were 1) to assess and compare the TGF-β1 and D-dimer concentrations in peritoneal fluid of horses with different types of colic, 2) to demonstrate the relationship between peritoneal TGF-β and D-dimer concentrations and, finally, 3) to assess the relationship between the peritoneal fibrinolytic activity, coagulation and these inflammatory regulators in peritoneal fluid of horses with colic by the type of peritoneal fluid (trasudate, modified trasudate and exudate) and outcome (survivors, and non-survivors).

Objetives:

1. To determine both plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations in horses with different types of colic.
2. To compare these concentrations according to the type of peritoneal fluid: transudate, modified transudate and exudates.
3. To compare and correlate plasma and peritoneal concentrations of TGF-β_1 and TGF--β_3 and the types of peritoneal fluid according to the colic group and outcome.
4. To compare and correlate the peritoneal concentrations of TGF-β_1 and D-dimer in horses with different types of colic.

References

Argüelles, D., Carmona, J.U., Pastor, J., Iborra, A., Viñals, L., Martínez, P., Bach, E. and Prades, M. (2006) Evaluation of single and double centrifugation tube method for concentrating equine platelets. *Res. Vet. Sci.* **81**, 237-245.

Åkerberg, D., Posaric-Bauden, M., Isaksson, K., Andersson, R. and Tingstedt, B. (2013) Prevention of pleural adhesions by bioactive polypeptides - A pilot study. Int J of Med Sci **10**, 1720-1726.

Barton, M.H. and Collates, C. (1999) Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J. Vet. Intern. Med.* **13**, 457-464.

Carmona, J.U., Argüelles, D. and Prades, M. (2008) Transforming growth factor beta-3 and nitric oxide levels in four autologous platelet concentrates and plasma derived from equine blood. *Arch. Med. Vet.* **40**, 155-160.

Davidson, A.J., Edwards, G.B., Proudman, C.J., Cripps, P.J. and Matthews, J.B. (2002) Cytokine mRNA expression pattern in horses with large intestinal disease. *Res. Vet. Sci.* **72**, 177-185.

Delgado, M.A., Monreal, L., Armengou, L., Ríos, J. and Segura, D. (2009) Peritoneal D-dimer concentration for assessing peritoneal fibrinolytic activity in horses with colic. *J. Vet. Intern. Med.* **23**, 882-889.

Chegini, N., Kotseos, K., Zhao, Bennett, B., McLean, F.W., Diamond, M.P., Holmdahl, L.and Burns J. (2001) Differential expression of TGF-beta1 and TGF-beta3 in serosal tissues of human intraperitoneal organs and peritoneal adhesions. *Hum. Reprod.* **16**, 1291-1300.

Falk, P., Bergström, M., Palmgren, I., Holmdahl, L., Breimer, M.E. and Ivarsson, M.L. (2009) Studies of TGF-beta(1-3) in serosal fluid during abdominal surgery and their effect on in

vitro human mesothelial cell proliferation. *J. Surg. Res.* 154, 312-316.

Ghellai, A.M., Stucchi, A.F., Chegini, N., Ma, C., Andry, C.D., Kaseta, J.M., Burns, J.W., Skinner, K.C. and Becker, J,M. (2000) Role of transforming growth factor beta-1 in peritonitis-induced adhesions. *J. Gastrointest. Surg.* **4**, 316-323.

Gorvy, D.A., Herrick, S.E., Shah, M. and Ferguson, M.W. (2005) Experimental manipulation of transforming growth factor-beta isoforms significantly affects adhesion formation in a murine surgical model. *Am. J. Pathol.* **167**, 1005-1019.

Gorvy, D.A., Edwards, G.B. and Proudman, C.J. (2008) Intra-abdominal adhesions in horses: a retrospective evaluation of repeat laparotomy in 99 horses with acute gastrointestinal disease. *Vet. J.* **175**, 194-201.

Holmdahl, L., Kotseos, K., Bergström, M., Falk, P., Ivarsson, M.L. and Chegini, N. (2001) Overproduction of transforming growth factor-beta1 (TGF-beta1) is associated with adhesion formation and peritoneal fibrinolysis impairment. *Surgery* **129**, 626-632.

Javelaud, D. and Mauviel, A. (2004) Mammalian transforming growth factor-betas: Smad signaling and physio-pathological roles. *Int. J. Biochem. Cell Biol.* **36**, 1161-1165.

Laun, R.A., Schröder, O., Schoppnies, M., Röher, H.D., Ekkernkamp, A. and Schulte, K.M. (2003) Transforming growth factor-beta1 and major trauma: time-dependent association with hepatic and renal insufficiency. *Shock* **19**, 16-23.

Milne, E.M., Doxey, D.L., Gilmour, J.S. (1990) Analysis of peritoneal fluid as a diagnostic aid in grass sickness (equine dysautonomia). *Vet. Rec.* **127**, 162-165.

Mirshafiey, A. and Mohsenzadegan, M. (2009). TGF-beta as a promising option in the treatment of multiple sclerosis. *Neuropharmacology* **56**, 929-936.

Namazi MR, Fallahzadeh MK, Schwartz RA (2011). Strategies for prevention of scars: what can we learn from fetal skin? *Int J Dermatol* **50**, 85-93.

Pachot, A., Monneret, G., Voirin, N., Leissner, P., Venet, F., Bohé, J., Payen, D., Bienvenu,

J., Mougin, B. and Lepape, A. (2005) Longitudinal study of cytokine and immune transcription factor mRNA expression in septic shock. *Clin. Immunol.* **114**, 61-69.

Penha-Goncalves, M.N., Onions, D.E. and Nicolson, L. (1997) Cloning and sequencing of equine transforming growth factor beta 1 (TGF-b1) cDNA. *DNA Seq.* **7**, 375–378.

Raaf, L., Noll, C., Cherifi, M.E.H., Samuel, J.L., Delcayre, C., Delabar, J.M., Benazzoug, Y. and Janel, N. (2011) Myocardial fibrosis and TGFB expression in hyperhomocysteinemic rats. *MolCel Biochem* **347**, 63-70

Rougier, J.P., Guia, S., Hagege, J., Nguyen, G. and Ronco, P.M. (1998) PAI-1 secretion and matrix deposition in human peritoneal mesothelial cell cultures: Transcriptional regulation by TGF-$ß_1$. *Kidney Inter.* **54**, 87-98.

Sánchez-Muñoz, F., Domínguez-López, A. and Yamamoto-Furusho, J.K. (2008) Role of cytokines in inflammatory bowel disease. *World J. Gastroenterol.* **14**, 4280-4288.

Shah, M., Foreman, D.M. and Ferguson, M.W. (1995) Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J. Cell Sci*. **108**, 985-1002.

Ueha, S., Shand, F.H.W. and Matsushima, K. (2012) Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. *Front Immunol* **3**.

Ward, N.S., Casserly, B., Ayala, A. (2008) The Compensatory Anti-inflammatory response syndrome (CARS) in critically III patients. *Clin. Chest Med.* **29**, 617–625.

Werners, A.H., Bull, S. and Fink-Gremmels, J. (2005) Endotoxaemia: a review with implications for the horse. *Equine Vet. J.* **37**, 371-383.

II. Literature review

The mesothelial cell

The mesothelial cells form a monolayer of a specialised pavement-like structure that lines the body's serous cavities and internal organs. The primary function of this layer, termed the mesothelium, is to provide a slippery, non-adhesive and protective surface. However, mesothelial cells play other pivotal roles involving fluid and cell transport across the serosal cavities, immunological and anti-inflammatory properties, tissue repair, antigen presentation, inflammation and tissue repair, coagulation, fibrinolysis and tumor cell adhesion.

Mammalian mesothelium is considered essentially similar regardless of species or anatomical site. Embryologically, it develops from the mesodermal tissue between 8 and 18 days of gestation depending on the species. The mesothelium is a slowly renewing tissue with 0.16-0.5% of cells undergoing mitosis at any one time. However, if appropriately stimulated, its mitotic activity can be greatly increased. Soluble mediators released from inflammatory and injured cells are potent stimulators for this rapid increase in proliferation.

Mesothelial cells are unique in that not only are they derived from the mesoderm and express the mesenchymal intermediate filaments vimentin and desmin, but they also present cytokeratins which are intermediate filaments from epithelial cells. Mesothelial cells have the ability to change their phenotype comparable to changes seen in epithelial-to mesenchymal transition. When a peritoneal injury is produced or peritonitis is present and induction of epithelial-to-mesenchymal transition (EMT) in mesothelial cells is unchained. This process is characterized by the loss of cell–cell contact, reorganization of the actin cytoskeleton, basement membrane degradation, and acquisition of a migratory and invasive phenotype. EMT contributes to peritoneal fibrosis and adhesion formation (Aoki *et al.* 2014, Yung *et al.* 2013).

An *in vitro* study demonstrated that the incubation of mesothelial cells with higher concentrations TGF-$ß_1$ induced epithelial-to mesenchymal transition and up-regulated smooth

muscle actin and type I collagen expression, consistent with a myofibroblast phenotype, and this is a basic step in peritoneal fibrosis. This could imply that the mesothelium is a likely source of fibrinogenic cells during serosal inflammation and wound healing and may play important roles in early serosal fibrosis and adhesion formation (Falk et al., 2013).

Historically, the main roles attributed to the mesothelium have been to act as a protective barrier against physical damage and invading organisms and provide a frictionless interface for the free movement of apposing organs and tissues (Ksiazek, 2013, Jiang et al., 2013). However, more recent studies have begun to elucidate many other different roles for mesothelial cells. The mesothelial cells are tightly bound together by tight junctions, and secrete surface glycosaminoglycans, predominantly hyaluronan, which is assembled into hyaluronan-containing peri-cellular matrix coated around microvilli, protecting the cells from abrasive damage and infective agents (Jung and Chan, 2012). Hyaluronan may also be important in cell differentiation and preventing adhesion formation and tumor cell dissemination. Mesothelial cells also secrete phosphatidylcholine, the major constituent of lamellar bodies and pulmonary surfactant, acting as a lubricant to reduce friction between serosal surfaces (Bodega et al 2012).

Mesothelial cells participate in initiating and resolving serosal inflammation and repair by secreting various pro-, anti- and immunomodulatory mediators. These include products of the coagulation cascade, chemokines, cytokines and growth factors, prostaglandins and prostacyclin, reactive nitrogen and oxygen species, antioxidant enzymes and extracellular matrix molecules (Jiang et al., 2013).

Secretion of chemokines by mesothelial cells promotes directed trans-mesothelial migration of neutrophils and monocytes leading to a massive influx of leukocytes from the vascular compartment into the serosal space. Mesothelial cells also participate in regulating efflux of inflammatory cells and release mediators in response to injury which initiate cell

proliferation, migration and extracellular matrix synthesis (Ksiazandek, 2013, Jiang et al., 2013). Growth factors, including TGF-β, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor and members of the epidermal growth factor families are likely to regulate theses processes. Mesothelial cells synthesise extracellular matrix molecules including collagen types I, III and IV, elastin, fibronectin and laminin and are able to organise these components into complex structures that resemble extracellular matrix components (Falk et al., 2013, Jiang et al., 2013, Aoki et al., 2014). They can also regulate extracellular matrix turnover by secreting matrix metalloproteinases and their respective tissue inhibitors (Ksiazandek, 2013).

Regulation of the pro-, anti-, and immuno-modulator mediator balance by mesothelial cells in serosal tissues is key for a good outcome following surgical insult and infection (Zeillemaker et al., 1999). The correct balance will result in tissue regeneration and re-establishment of normal serosal function, however, loss of this balance can result in enhanced permeability, oedema, formation of protein rich exudates and ultimately adhesion formation and fibrosis (Brochhausen et al., 2013).

Mammalian transforming growth factor-βs

The TGF-β superfamily consists of a diverse range of proteins that regulate many different physiological processes, including embryonic development, homeostasis, chemotaxis and cell cycle control (Bielefeld et al., 2013, Andrianifahanana et al., 2013). TGF-beta is a regulatory cytokine with several effects on hematopoietic cells and extra-cellular matrix (ECM) metabolism. TGF beta-1 increases ECM production and TGF beta-3 inhibits it (Gorvy et al., 2005). Their balanced expression of both growth factors is crucial for adequate tissue repair (Kamato et al., 2013). They are widely expressed in all cells, including macrophages, lymphocytes, endothelial cells, fibroblasts, astrocytes, osteoclasts and platelets

(Bielefeld et al., 2013). TGF-βs are present in 3 isoforms, which are released as inactive precursor peptides (latent inactivated form), and require proteolytic cleavage by furins and other convertases such as plasmin, calpain and matrix metalloproteinases to form active signaling molecules (Bielefeld et al., 2013, Kamato et al., 2013). Other mechanisms involved in latent TGF-β activation include the presence of extreme pH, temperature changes, radiation or contact with reactive oxygen species and drugs such as glucocorticoids.

Transforming growth factor β is one of the main immune-suppressive factor secreted by cells. TGF-β substantially suppresses interleukin 12 and inhibits inter leukin 2 and interleukin-2- induced proliferation in T cells. In CD8+ cytotoxic T lymphocytes and NK cells, TGFβ is a strong antagonist of interferon-γ production. TGFβ has a negative effect on B-cell proliferation and differentiation. TGFβ is needed for the differentiation of both T-helper-17 (Th17) and induced regulatory T (Treg) cells (Lippitz 2013).

TGF-β signaling

Signaling by TGF-βs starts at the cell surface by binding to the type II TGF receptor. This triggers recruitment and activates phosphorylation of the type I receptor, which in turn phosphorylates the receptor-regulated transcription coregulators (R-Smads) Smad2 and Smad3. Heteromerization of R-Smads with the common mediator, Smad4, and nuclear translocation subsequently ensue, culminating in modulation (induction or repression) of Smad-regulated genes. Furthermore, non-Smad signaling pathways are also equally important in mediating TGFβ's effects and possibly these two pathways show some interaction (Bielefeld et al., 2013, Andrianifahanana et al., 2013).

Profibrotic effects of TGF-β

TGF-β exerts its profibrotic effects by inducing fibroblast proliferation, myofibroblast

differentiation, and extracellular matrix remodeling. These events not only are mediated by this protein, but other cytokines are also pivotal for Smad activation and the subsequent expression of fibrogenic intermediate effectors, such as platelet-derived growth factor (PDGF) or connective tissue growth factor (CTGF) (Bielefeld et al., 2013). Furthermore, non-Smad pathways, such as the p21-activated kinase 2 (PAK2)/cAbl Akt/mTOR, the mitogen-activated protein kinase (MAPK) family, also exhibit potent profibrotic activities) as well as epidermal growth factor receptor (EGFR) (Andrianifahanana et al., 2013).

The main consequence derived from the activation of these molecular pathways is the induction of fibroblast to myofibroblast differentiation, however, the presence of other cytokines and growth factors, such as PDGF and CTGF, are pivotal for this cell phenomenon to occur Bielefeld et al., 2013). In addition, TGF-β remains as the master-key for inducing epithelial to mesenchymal transition (EMT). During EMT epithelial cells downregulate epithelial marker expression, upregulate mesenchymal markers expression and gain functional characteristics of mesenchymal cells. Other cytokines such as TNFα and Il-1β are able to drive EMT only under the influx of TGF-β1 (Borthwick et al., 2013).

Coagulation and fibrinolysis

Mesothelial cells play an important role in local fibrin deposition and clearance within serosal cavities. Their fibrinolytic activity is a key factor in the prevention and removal of fibrin deposits that form following mechanical injury, hemoperitoneum and infection. If the fibrinolytic capacity is insufficient and fibrin accumulation is not resolved, fibrous adhesions form between opposing serosal surfaces (Imudia et al. 2008).

Mesothelial cells have both procoagulant and fibrinolytic activity. The procoagulant activity is due to tissue factor, the main cellular initiator of the extrinsic coagulation cascade. Fibrin deposition is also aided by the secretion of plasminogen activator inhibitors (PAI),

PAI-1 and PAI-2. Fibrinolytic activity is mediated through secretion of tissue plasminogen activator (tPA) and urokinase (uPA) (Brochhausen et al., 2013).

The PAs convert the inactive zymogen plasminogen into active plasmin which in turn enzymatically breaks down fibrin. Mesothelial cells are the main source of tPA in serosal cavities but secrete lower levels of uPA. The levels of these mediators are regulated by inflammatory factors including lipopolysaccharide, tumor necrosis factor-α and IL-1 and fibrinogenic mediators such as TGF-β and thrombin (Arung et al., 2011).

Since impaired mesothelial regeneration is a likely cause of post-operative adhesion formation clinical impact is obvious. Adhesions develop following direct insult to the mesothelium. During normal repair, fibrin, which is deposited between closely apposed injured surfaces, is removed by the action of PAs secreted by regenerating mesothelial cells. Within days the fibrin is reabsorbed and by day 7 there is complete regeneration of the mesothelial layer. However, if the normal healing process is impaired, fibrin subserosal fibroblasts migrate into the remaining fibrin matrix, deposit collagen and hence permanent adhesions between opposing organs form (Jeong et al., 1998).

Pathophysiology of adhesion formation

Peritoneal adhesions are one of the most frequent complications after colic surgery in horses. Adhesions occur more frequently after surgery for treatment of lesions involving the small intestine. Ischemia/reperfusion and intra-luminal distension and decompression have been shown to cause severe changes in the sero-muscular layer of the small intestine, such as serosal edema, leukocyte infiltration, and erythrocyte leakage with fibrin accumulation, whereas a similar insult to the ascending colon does not result in comparable seromuscular lesions (Eggleston and Mueller, 2003).

Adhesions are the second most common reason for repeat laparotomy in horses with GI

disease (Kelmer, 2009). Adhesion formation then can be viewed as a variant of the normal physiologic healing process. Ischemia, distension, drying, or abrasion of the peritoneum during manipulation and decompression of the intestine, as well as hemorrhage, the introduction of foreign material, or infection in the peritoneal cavity, can all result in peritoneal inflammation initiating adhesion formation (Imudia et al. 2008, Arung et al., 2011).

An intact mesothelial cell layer is critical for the prevention of adhesion formation. Once this layer is disrupted, the underlying connective tissue containing blood vessels, collagen, lymphocytes, fibroblasts, mast cells, macrophages, and plasma cells become exposed (Brochhausen et al., 2013). The release of vasoactive substances such as PGE2, serotonin, bradykinin and histamine from the exposed sub-mesothelial tissue mediates increased vascular permeability with extravasation of fibrinogen-rich inflammatory exudates (Ho-Dac-Pannekeet, 1998). The release of thromboplastin and exposure of subendotheilal collagen activate the intrinsic and extrinsic clotting cascade, leading to thrombin-mediated conversion of fibrinogen to fibrin, with the fibrin adhering to sites of injury (Imudia et al. 2008, Arung et al., 2011).

In the normal healing process, the fibrin is lysed by plasmin, and the peritoneal injury is covered within 2 to 5 days by a single layer of mesothelial cells originating from metaplasia of underlying mesenchymal cells, attachment of free-floating mesothelial cells, or transformation of macrophages (Imudia et al. 2008, Arung et al., 2011). But in the case where fibrinolysis is inadequate, fibroblasts migrate over the fibrin deposit with neovascularization and produce collagen by day 4, initiating fibrous adhesion formation (Brochhausen et al., 2013).

Inactive plasminogen is converted to plasmin by tissue plasminogen activators that are proteases released by the mesothelial cells. After a peritoneal injury due to ischemia, infection or inflammation, plasminogen activator is decreased. This is due an increased production of

plasminogen activator inhibitor, which reduces the level of plasminogen activator activity. An extensive damage to either the parietal or visceral peritoneal surface tips the balance in favor of adhesion formation, by increasing the extent of fibrin formation and deposition and by decreasing fibrinolytic activity (Ksiaz and ek, 2013).

Role of TGF-β in peritoneal adhesion formation

Peritoneal adhesions are in fact a fibrotic disorder (Borthwick et al., 2013). As it was mentioned, TGF-β, especially the 1 isoform plays a crucial role in the genesis of this abdominal pathologic alteration (Chegini et al., 2013) via secondary cytokine interaction and EMT (Falk et al., 2013). Many situations may alter the peritoneal homeostasis in horses with colic; these conditions may include intestinal ischemia, distension, enteritis, peritonitis, surgical trauma and hemorrhage, amongst others (Argüelles et al., 2010). Under a peritoneal inflammatory environment such as happens in horses with colic it is very likely that the proteolitic cleavage of latent forms of TGF-β1 are massively released to the peritoneal fluid and consequently the profibrotic mechanisms of this growth factor are activated. It is known that horses with colic present alterations in the peritoneal fibrinolytic system. Necessarily, higher plasmin peritoneal concentrations will be present in these cases with subsequent TGF-β_1´s fibrotic way activation (Delgado et al., 2009). Even if the plasmin system is important for the activation of TGF-β but the knowledge of its role in regulation of release in the equine abdominal cavity is limited.

Experimental studies blocking the TGF-β isoforms with neutralizing antibodies injected into the peritoneal cavity have been demonstrated to reduce peritoneal adhesion formation, while an increase in TGF-β1 concentrations in peritoneal tissue is associated with adhesion development (Gorvy et al., 2005). However intraoperative release and activation profiles of TGF-β1 and 3 in response to surgery in the equine abdomen are not fully

understood. The presence of TGF-β 1 and 3 isoforms in serosal and adhesion tissue has been reported *in vivo*, together with a synergistic effect between the TGF-β 1 and 2 isoforms that could be important in adhesion formation by generating connective tissue (Chegini et al., 2013).

TGF-β as a biomarker for fibrosis and cancer

Currently, there is great interest to know about the central role of TGF-β for inducing EMT and consequently organ fibrosis and cancer. Thus, TGF-β monitoring has become frequently evaluated in animal models and patients with fibrotic problems and cancer (Carew et al., 2012, Lippitz 2013). However, even though it is recognized as very important, this cytokine is only a part in the biological puzzle of these pathologic conditions.

TGF-β is one of the cytokines produced by tumor cells with immunosuppressive activity and a potent inhibitory effect on several other cytokines. With an essential role in maintenance of homoeostasis, TGF-β suppresses anti-tumour immune responses and creates a local environment of immune tolerance. Increased serum concentrations of the immunosuppressive cytokine TGF-β are a frequent finding in patients with cancer, to the extent that it could potentially represent a general cancer-associated feature (Lippitz 2013).

Increased TGF-β_1 serum concentrations have been found in patients with lung cancer, breast cancer, glioblastoma multiforme, colorectal carcinoma, hepatocellular carcinoma, bladder carcinomas, renal cell carcinoma, and gastric carcinoma and were associated with poor prognosis in patients with gastric carcinoma, adenocarcinoma of the lung, and breast cancer. Additionally, TGF-β was associated with metastases in patients with breast cancer, gastric cancer, colorectal cancer, non-small-cell lung cancer, malignant melanoma, and renal-cell cancer (Lippitz 2013).

TGF-β1has also been used for evaluating patients with renal (Carew et al., 2012) and

pulmonary fibrosis (Fernandez and Eickelberg 2012). Increased plasma and urinary TGF-β1 concentrations might contribute to the development of chronic tubule-interstitial disease (Carew et al., 2012). Furthermore, recently, it has proposed the monitoring of this growth factor in patients receiving allotransplantation, since TGF- β has a potent ability to alter immune responses. Maybe the modulation of the TGF-β pathway for treatment of transplantation patients could be effective if carried out in a target selective manner (Regateiro et al., 2011).

Tissue markers as predictors of postoperative adhesions

There is reduced fibrinolytic capacity in peritoneal tissue in patients with a greater propensity for development of adhesions. This suggests that components of the fibrinolytic system may be used as markers of an increased risk of adhesion development (Imudia et al. 2008, Arung et al., 2011).

Components of the plasminogen system, particularly PAI-1, could be used as tissue markers to document the likelihood of adhesion development after surgery and may have potential for identification of high risk patients who would benefit from adjuvant therapy. Theoretically, it may be possible to reduce adhesion formation, by locally inhibiting the plasminogen inhibitor PAI-1 (Fortenberry, 2013).

Tissue plasminogen activator is the main physiological plasminogen activator in peritoneal tissue and the reduction in functional fibrinolytic activity seen in inflammation is mediated by plasminogen activator-inhibitor (Imudia et al. 2008, Arung et al., 2011).

References

Andrianifahanana, M., Wilkes, M.C., Gupta, S.K., Rahimi, R.A., Repellin, C.E., Edens, M., Wittenberger, J., Yin, X., Maidl, E., Becker, J. and Leof, E.B. (2013) Profibrotic TGFβ responses require the cooperative action of PDGF and ErbB receptor tyrosine kinases. *FASEB Journal* **27**, 4444-4454.

Aoki, S., Takezawa, T., Oshikata-Miyazaki, A., Ikeda, S., Kuroyama, H., Chimuro, T., Oguchi, Y., Noguchi, M., Narisawa, Y. and Toda, S. (2014) Epithelial-to-mesenchymal transition and slit function of mesothelial cells are regulated by the cross talk between mesothelial cells and endothelial cells. *Am J Physiol - Renal Physiol* **306**, F116-F122.

Arung, W., Meurisse, M. and Detry, O. (2011) Pathophysiology and prevention of postoperative peritoneal adhesions. *World J Gastroent* **17**, 4545-4553.

Argüelles, D., Carmona, J.U., Pastor, J., Iborra, A., Viñals, L., Martínez, P., Bach, E. and Prades, M. (2006) Evaluation of single and double centrifugation tube method for concentrating equine platelets. *Res. Vet. Sci.* **81**, 237-245.

Barton, M.H. and Collates, C. (1999) Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J. Vet. Intern. Med.* **13**, 457-464.

Bielefeld, K.A., Amini-Nik, S. and Alman, B.A. (2013) Cutaneous wound healing: Recruiting developmental pathways for regeneration. *Cellular and Molecular Life Sciences* **70**, 2059-2081.

Bodega, F., Pecchiari, M., Sironi, C., Porta, C., Arnaboldi, F., Barajon, I. and Agostoni, E. (2012) Lubricating effect of sialomucin and hyaluronan on pleural mesothelium. *Resp Physiol Neurobiol* **180**, 34-39.

Borthwick, L.A., Wynn, T.A. and Fisher, A.J. (2013) Cytokine mediated tissue fibrosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1832**, 1049-1060.

Brochhausen, C., Schmitt, V.H., Planck, C.N.E., Rajab, T.K., Hollemann, D., Tapprich, C., Krämer, B., Wallwiener, C., Hierlemann, H., Zehbe, R., Planck, H. and Kirkpatrick, C.J. (2012) Current Strategies and Future Perspectives for Intraperitoneal Adhesion Prevention. *J Gastroint Surg* **16**, 1256-1274.

Carew, R., Wang, B. and Kantharidis, P. (2012) The role of EMT in renal fibrosis. *Cell Tissue Res* 347, 103-116.

Carmona, J.U., Argüelles, D. and Prades, M. (2008) Transforming growth factor beta-3 and nitric oxide levels in four autologous platelet concentrates and plasma derived from equine blood. *Arch. Med. Vet.* **40**, 155-160.

Davidson, A.J., Edwards, G.B., Proudman, C.J., Cripps, P.J. and Matthews, J.B. (2002) Cytokine mRNA expression pattern in horses with large intestinal disease. *Res. Vet. Sci.* **72**, 177-185.

Delgado, M.A., Monreal, L., Armengou, L., Ríos, J. and Segura, D. (2009) Peritoneal D-dimer concentration for assessing peritoneal fibrinolytic activity in horses with colic. *J. Vet. Intern. Med.* **23**, 882-889.

Eggleston, R.B. and Mueller, P.O.E. (2003) Prevention and treatment of gastrointestinal adhesions. *Veterinary Clinics of North America - Equine Practice* **19**, 741-763.

Fernandez, I.E. and Eickelberg, O. (2012) The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. *Proc Am Thorac Soc* **9**, 111-116.

Chegini, N., Kotseos, K., Zhao, Bennett, B., McLean, F.W., Diamond, M.P., Holmdahl, L.and Burns J. (2001) Differential expression of TGF-beta1 and TGF-beta3 in serosal tissues of human intraperitoneal organs and peritoneal adhesions. *Hum. Reprod.* **16**, 1291-1300.

Falk, P., Bergström, M., Palmgren, I., Holmdahl, L., Breimer, M.E. and Ivarsson, M.L. (2009) Studies of TGF-beta(1-3) in serosal fluid during abdominal surgery and their effect on in vitro human mesothelial cell proliferation. *J. Surg. Res.* 154, 312-316.

Falk, P., Angenete, E., Bergström, M. and Ivarsson, M.L. (2013) TGF-β1 promotes transition of mesothelial cells into fibroblast phenotype in response to peritoneal injury in a cell culture model. *Int J Surgery* **11**, 977-982.

Fortenberry, Y.M. (2013) Plasminogen activator inhibitor-1 inhibitors: A patent review (2006-present). *Expert Opinion on Therapeutic Patents* **23**, 801-815.

Ghellai, A.M., Stucchi, A.F., Chegini, N., Ma, C., Andry, C.D., Kaseta, J.M., Burns, J.W., Skinner, K.C. and Becker, J,M. (2000) Role of transforming growth factor beta-1 in peritonitis-induced adhesions. *J. Gastrointest. Surg.* **4**, 316-323.

Gorvy, D.A., Herrick, S.E., Shah, M. and Ferguson, M.W. (2005) Experimental manipulation of transforming growth factor-beta isoforms significantly affects adhesion formation in a murine surgical model. *Am. J. Pathol.* **167**, 1005-1019.

Gorvy, D.A., Edwards, G.B. and Proudman, C.J. (2008) Intra-abdominal adhesions in horses: a retrospective evaluation of repeat laparotomy in 99 horses with acute gastrointestinal disease. *Vet. J.* **175**, 194-201.

Ho-Dac-Pannekeet, M.M. (1998) Peritoneal fluid markers of mesothelial cells and function. *Advances in Renal Replacement Therapy* **5**, 205-211.

Holmdahl, L., Kotseos, K., Bergström, M., Falk, P., Ivarsson, M.L. and Chegini, N. (2001) Overproduction of transforming growth factor-beta1 (TGF-beta1) is associated with adhesion formation and peritoneal fibrinolysis impairment. *Surgery* **129**, 626-632.

Imudia, A.N., Kumar, S., Saed, G.M. and Diamond, M.P. (2008) Pathogenesis of intra-abdominal and pelvic adhesion development. *Seminars in Reproductive Medicine* **26**, 289-297.

Javelaud, D. and Mauviel, A. (2004) Mammalian transforming growth factor-betas: Smad signaling and physio-pathological roles. *Int. J. Biochem. Cell Biol.* **36**, 1161-1165.

Jeong Seon, R. and Hong Lyeol, L. (1998) Coagulation and fibrinolysis in exudative pleural

effusions. *Tuberculosis and Respiratory Diseases* **45**, 1214-1222.

Jiang, C.G., Lv, L., Liu, F.R., Wang, Z.N., Na, D., Li, F., Li, J.B., Sun, Z. and Xu, H.M. (2013) Connective tissue growth factor is a positive regulator of epithelial-mesenchymal transition and promotes the adhesion with gastric cancer cells in human peritoneal mesothelial cells. *Cytokine* **61**, 173-180.

Kamato, D., Burch, M.L., Piva, T.J., Rezaei, H.B., Rostam, M.A., Xu, S., Zheng, W., Little, P.J. and Osman, N. (2013) Transforming growth factor-β signalling: Role and consequences of Smad linker region phosphorylation. *Cellular Signalling* **25**, 2017-2024.

Ksiazek, K. (2013) Mesothelial cell: A multifaceted model of aging. *Age Res Rev* **12**, 595-604.

Laun, R.A., Schröder, O., Schoppnies, M., Röher, H.D., Ekkernkamp, A. and Schulte, K.M. (2003) Transforming growth factor-beta1 and major trauma: time-dependent association with hepatic and renal insufficiency. *Shock* **19**, 16-23.

Lippitz, B.E. (2013) Cytokine patterns in patients with cancer: a systematic review. *The Lancet Oncology* **14**, e218-e228.

Milne, E.M., Doxey, D.L., Gilmour, J.S. (1990) Analysis of peritoneal fluid as a diagnostic aid in grass sickness (equine dysautonomia). *Vet. Rec.* **127**, 162-165.

Mirshafiey, A. and Mohsenzadegan, M. (2009). TGF-beta as a promising option in the treatment of multiple sclerosis. *Neuropharmacology* **56**, 929-936.

Oh, S.A. and Li, M.O. (2013) TGF-β: Guardian of T cell function. *Journal of Immunology* **191**, 3973-3979.

Pachot, A., Monneret, G., Voirin, N., Leissner, P., Venet, F., Bohé, J., Payen, D., Bienvenu, J., Mougin, B. and Lepape, A. (2005) Longitudinal study of cytokine and immune transcription factor mRNA expression in septic shock. *Clin. Immunol.* **114**, 61-69.

Penha-Goncalves, M.N., Onions, D.E. and Nicolson, L. (1997) Cloning and sequencing of equine transforming growth factor beta 1 (TGF-b1) cDNA. *DNA Seq.* **7**, 375–378.

Regateiro, F.S., Howie, D., Cobbold, S.P. and Waldmann, H. (2011) TGF-beta in transplantation tolerance. *Curr Opin Immunol* **23**, 660-669.

Rougier, J.P., Guia, S., Hagege, J., Nguyen, G. and Ronco, P.M. (1998) PAI-1 secretion and matrix deposition in human peritoneal mesothelial cell cultures: Transcriptional regulation by TGF-beta 1. *Kidney Inter.* **54**, 87-98.

Sánchez-Muñoz, F., Domínguez-López, A. and Yamamoto-Furusho, J.K. (2008) Role of cytokines in inflammatory bowel disease. *World J. Gastroenterol.* **14**, 4280-4288.

Shah, M., Foreman, D.M. and Ferguson, M.W. (1995) Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J. Cell Sci.* **108**, 985-1002.

Ward, N.S., Casserly, B., Ayala, A. (2008) The Compensatory Anti-inflammatory response syndrome (CARS) in critically III patients. *Clin. Chest Med.* **29**, 617–625.

Werners, A.H., Bull, S. and Fink-Gremmels, J. (2005) Endotoxaemia: a review with implications for the horse. *Equine Vet. J.* **37**, 371-383.

Yung, S. and Chan, T.M. (2012) Pathophysiological changes to the peritoneal membrane during PD-related peritonitis: The role of mesothelial cells. *Med Inflamm* **2012**.

Yung, S., Jiang, N. and Chan, T.M. (2013) Peritoneal fibrosis and the putative role of decorin. *Hong Kong J Nephrol* **15**, 55-61.

Zeillemaker, A.M., Diepersloot, R.J.A. and Leguit, P. (1999) Mesothelial cell activation by micro-organisms. *Sepsis* **3**, 285-291.

III. STUDY ONE

Peritoneal concentrations of TGF-β in horses with colic.

Summary

Reasons for performing the study: In humans, peritoneal transforming growth factor beta (TGF-β) is associated with peritoneal diseases and subsequent adhesion formation. No studies on plasma and peritoneal TGF-β concentrations in horses with colic are available.

Objectives: 1) To determine both plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations in horses with different types of colic (not previously subjected to abdominal surgery), 2) to compare these concentrations according to the type of peritoneal fluid (transudate, modified transudate and exudate), and 3) to compare and correlate plasma and peritoneal concentrations of TGF-β_1 and TGF-β_3 and the types of peritoneal fluid according to the colic group and outcome.

Methods: Peritoneal fluid and plasma samples from 78 horses with colic and 8 healthy horses were obtained. Patients were classified according to diagnosis (obstructions, enteritis, ischemic disorders and peritonitis), peritoneal fluid analysis (transudate, modified transudate and exudate), and outcome (survivors and non-survivors). Plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations were determined by ELISA. Data were analyzed by parametric and non parametric tests. A $P \leq 0.05$ was considered as statistical significant.

Results: Concentrations of peritoneal fluid TGF-β_1 were significantly (P=0.01) higher in horses with peritonitis in comparison with all other colic groups and controls. Horses with ischemic lesions had significantly (P=0.01) higher concentrations of peritoneal TGF-β_1 in comparison with controls and the group of horses with obstructions. Peritoneal TGF-β_1 concentration also was significantly (P=0.01) higher in exudates in comparison with transudates. Peritoneal TGF-β_1 and TGF-β_3 concentrations and plasma TGF-β_1 concentration were significantly increased in non-survivors compared to survivors (P=0.001, P=0.004 and P=0.05, respectively).

Conclusions: Peritoneal TGF-β_1 concentration was higher in horses with severe gastrointestinal diseases (ischemic intestinal lesions and peritonitis), in horses with an altered peritoneal fluid (exudate), and in non-survivors.

Potential relevance: Peritoneal TGF-β concentration increases in horses with severe gastrointestinal disease as an anti-inflammatory response.

Keywords: equine colic; peritoneal transforming growth factor beta1 and beta3; peritoneal fluid analysis; outcome.

Introduction

The transforming growth factors beta 1, 2 and 3 (TGF-β_{1-3}) are proteins produced by macrophages, lymphocytes, endothelial cells, fibroblasts, astrocytes, osteoblasts, osteoclasts and platelets (Javelaud and Mauviel 2004). These peptides may stimulate or inhibit cellular proliferation, cellular differentiation, cellular motility, cellular adhesion or cellular death. These mechanisms can occur depending on the type and stage of development of every cell (Javelaud and Mauviel 2004; Mirshafiey and Mohsenzadegan 2009). TGF-β is a regulatory cytokine with several effects on haemopoietic cells. The pivotal function of this peptide in the immune system is to maintain tolerance via the regulation of lymphocyte proliferation, differentiation, and survival. Among T cells, $CD4^+$, $CD25^+$, $FOXP3^+$ and T regs contain the main source of TGF-β that suppresses immune responses in inflammatory sites (Mirshafiey and Mohsenzadegan 2009). These proteins also regulate the extra-cellular matrix (ECM) metabolism. Particularly, TGF-β_1 increases the ECM synthesis and TGF-β_3 inhibits it. The balanced expression of both growth factors is crucial for adequate tissue repair (Shah *et al.* 2005).

In horses with colic, several pathophysiological events (such as intestinal ischemia, distension, enteritis and peritonitis) could produce a release of precursor peptides of TGF-βs (either from devitalized viscera or inflamed peritoneum) to the peritoneal fluid. An impaired abdominal environment, such happens in colic patients, could induce activation of these peptides to their active forms (Ghellai *et al.* 2000; Falk *et al*. 2009).

No reports are available on TGF-β_1 and TGF-β_3 in horses with colic. The knowledge of variations of these growth factors on plasma and peritoneal fluid in clinically healthy horses

and in horses with colic would allow to know how they change according to different types of colic. Thus, the objectives of this study were 1) to measure TGF-β_1 and TGF-β_3 concentrations in both plasma and peritoneal fluid of horses with different types of colic (not previously subjected to abdominal surgery); 2) to compare peritoneal concentrations of these growth factors according to the type of peritoneal fluid (transudate, modified transudate and exudate); and 3) to compare and correlate plasma and peritoneal concentrations of TGF-β_1 and TGF-β_3 and the types of peritoneal fluid according to the outcome.

Materials and methods

The study was approved by the Ethical Committee of the authors' institution in Barcelona. The clinically healthy horses (free of abdominal disease) were used as controls after obtaining the owner's consent.

Inclusion criteria and study design

This prospective observational clinical study was undertaken during one year with those horses admitted at the Equine Teaching Hospital of Barcelona for abdominal pain. Patients included in the study were those not previously subjected to abdominal surgery, which blood and peritoneal fluid samples could be taken on admission, and their outcome could be established. A group of 8 clinically healthy horses in regular training (control group) also was included.

Horses with colic were allotted to 4 groups according to the diagnosis, based on clinical history, physical examination and results of complementary diagnostic tests (CBC, plasma biochemistry, blood gas analysis, peritoneal fluid analysis, abdominal ultrasonography and sometimes radiology).

Table 1. Plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations in the control and different colic groups.

Variable	Controls *(n= 8)*	Obstructive group *(n= 28)*	Enteritis group *(n= 20)*	Ischemic group *(n=22)*	Peritonitis group *(n= 8)*
Plasma					
TGF-β_1 (ng/mL)*	4.2 (1.9)	4.0 (1.8)	4.3 (1.7)	4.8 (2.6)	6.4 (2.8)
TGF-β_3 (pg/mL)**	<30 (0)	<30 (54)	<30 (1.5)	<30 (0)	61.8 (223)
Peritoneal fluid					
TGF-β_1 (ng/mL)*	0.8 (0.2)	1.3 (0.7)	1.8 (1.4)	1.9 (0.8)[a,b]	4.8 (2.3)[a,b,c,d]
TGF-β_3 (pg/mL)**	<30	<30 (0)	<30 (0)	<30 (0)	62.6 (104) [a,b,c,d]

* Data are presented as mean (standard deviation). ** Data are presented as median (interquartile range).

[a] Significantly different from the control group

[b] Significantly different from the obstructive group.

[c] Significantly different from the enteritis group.

[d] Significantly different from the ischemic group.

Findings of laparotomy or postmortem examination also were used for this classification when they were performed. Therefore, horses with colic were grouped as follows: *1) Obstructive group:* horses with acute abdomen associated with large colon obstructions or displacements. *2) Enteritis group:* horses with clinical signs of acute abdomen due to inflammatory disorders, such as duodenojejunitis or typhlocolitis. *3) Ischemic group:* horses with acute abdomen caused by strangulating lesions of the small or large intestine. *4) Peritonitis group:* horses with clinical signs of acute abdomen caused by peritonitis due to gastric or intestinal ruptures, bowel devitalisation, or intra-abdominal abscesses.

Horses also were grouped according to the results of peritoneal fluid analysis as previously reported (Milne *et al.* 1990; Delgado *et al.* 2009): *1) Transudate:* when nucleated cell count (NCC) was =5000 cells/μL and total protein (TP) =2.5 g/dL. *2) Modified transudate:* when NCC was =5000 cells/μL and TP >2.5 g/dL, or NCC >5000 cells/μL and TP = 2.5 g/dL, with a normal peritoneal fluid cytology. *3) Exudate:* when NCC was >5000 cells/μL and TP >2.5 g/dL, or NCC >5000 cells/μL and TP =2.5 g/dL with inflammatory peritoneal fluid cytology.

Finally, horses were grouped according to the outcome as survivors (horses that were discharged from the hospital) and non-survivors (horses that died during hospitalization or were euthanized due to poor prognosis). Patients that were discharged without the clinicians' consent or euthanized due to financial constraints were not included in this analysis.

Blood and peritoneal fluid sampling procedure

Blood samples were collected into evacuated tubes with sodium citrate (3.8%) to measure plasma concentration of TGF-β_1 and TGF-β_3. Peritoneal fluid was collected by abdominocentesis, using both EDTA and citrated tubes for routine peritoneal fluid analysis and measurement of peritoneal concentrations of TGF-β_1 and TGF-β_3, respectively.

Citrated tubes with blood and peritoneal fluid samples were centrifuged at 1000 x *g* for 15

minutes. The supernatant was aliquoted and frozen at -70°C for subsequent determination of growth factors.

Table 2. Number and percentages of horses classified according to the diagnosis and the outcome.

Outcome	Obstructive group *(n= 28)*	Enteritis group *(n= 20)*	Ischemic group *(n=22)*	Peritonitis group *(n= 8)*
Survivors	27 (96.4)	15 (75)	13 (59)	0 (0)
Nonsurvivors	1 (3.6)	5 (5)	9 (41)	8 (100)

Data expressed as n (%)

Measurement of TGF-β_1 and TGF-β_3

Concentrations of TGF-β_1 and TGF-β_3 in plasma and peritoneal fluid samples were determined by triplicate using a sandwich ELISA test developed with commercial antibodies against both human TGF-β_1[1] and TGF-β_3[2], and previously standardized and validated for use in horses (Argüelles *et al.* 2007; Carmona *et al*. 2008).

Murine anti-human capture antibodies were used for TGF-β_1 (part 840116) and TGF-β_3 (part 840417) each one in a concentration of 2 µg/mL. The detection antibodies were chicken anti-human TGF-β_1 (part 840117) and biotinilated goat anti-human TGF-β_3 (part 840418) in concentrations of 300 and 100 ng/mL, respectively. Standard concentrations were done with both human recombinant TGF-β_1 (part 840118) and TGF-β_3 using 2-fold serial dilutions in reagent diluent. The lower value for each growth factor was 30 pg/mL and the higher value was 2,000 pg/mL. Both growth factors were activated by an acetic acid 2.5 N/urea 10M solution, and samples were diluted 12 to 24 times. ELISA tests were revealed with a conjugation of streptavidine to peroxidase (part 840419) and the colorimetric substrate

Fast OPD[3]. The final reading was performed by a plate reader at 492 nanometers for TGF-β_1 and 540 nanometers for TGF-β_3. The detection threshold established for both proteins was 30 pg/mL.

Table 3. Peritoneal oncentrations of TGF-β_1 and TGF-β_3 according to the type of peritoneal fluid.

Peptide	Transudate	Modified transudate	Exudate
TGF-β_1 (ng/mL)*	1.32 (1.1)	1.7 (0.76)	3 (2)[a]
TGF-β_3 (pg/mL)**	<30 (0)	<30 (0)	<30 (37.8)

Data expressed as n (%)

[a] Significantly different from transudate

Statistical analysis

Data were analyzed using the statistical program SPPS 17.0[4]. Both plasma and peritoneal concentrations of TGF-β_1 were statistically analyzed using the ANOVA test after log transformation. A Games-Howell's test was used for *post-hoc* comparisons, since these variables did not present homogeneity of variance (Levene's test, P=0.05). The tests were used to compare concentrations of this protein among the different colic groups and types of peritoneal fluid. These variables are presented as mean (standard deviation).

Because TGF-β_3 concentrations presented a high proportion (73%) of left-censored data, these values were equalled to the detection threshold for this protein (30 pg/mL) and analyzed by a Kruskal-Wallis test. A Mann-Whitney U test was used for *post-hoc* comparisons of non-paired samples. These variables were presented as median (interquartile range). These tests were used to compare concentrations of this protein among the colic

groups and types of peritoneal fluid. Comparisons between the concentration of plasma and peritoneal fluid of TGF-β_3 with peritoneal NCC according to survival were performed using a Mann-Whitney U test for non-parametric non paired samples.

Table 4. Number and percentages of horses classified according to the type of peritoneal fluid analysis and the outcome.

Outcome	Transudate *(n= 38)*	Modified transudate *(n= 15)*	Exudate *(n= 22)*
Survivors	31 (81.6)	12 (80)	11 (50)
Nonsurvivors	7 (18.4)	3 (20)	11 (50)

Data expressed as n (%)

Comparisons between the concentration of plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations with survivors and non-survivors were evaluated by T test and Mann-Whitney U test, respectively. Kendall tau rank (τ) correlations were used to compare plasma and peritoneal concentrations of TGF-β_1 and TGF-β_3 between the type of peritoneal fluid and outcome. A value of P =0.05 was stated as statistically significant for all tests.

Results

Horses

Seventy eight horses of different breeds, mainly Andalusians (~80%), with an average age of 7 years old (IR= 7 years), with different types of colic, and 8 clinically healthy crossbreed

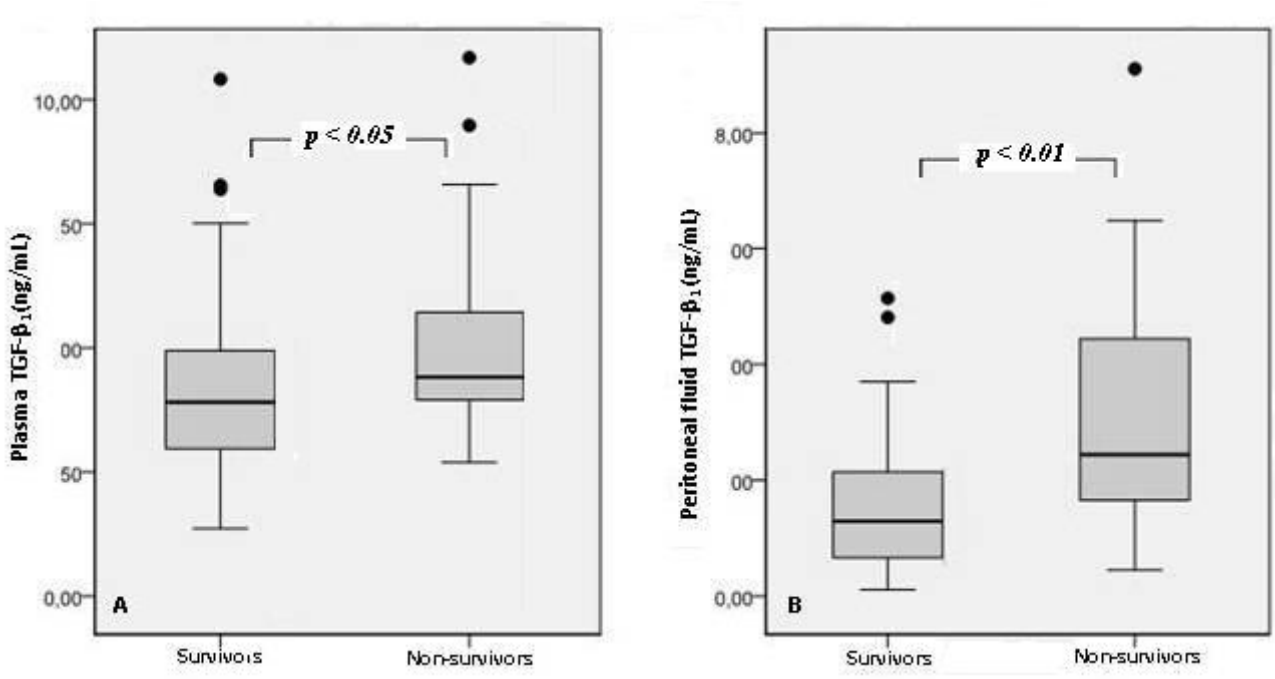

Figure 1. A) Box plots of the mean concentrations of plasma TGF-β1 (ng/mL) in survivors and non-survivors. Whiskers represent the 95% confidence interval. B) Box plots of the mean concentrations of peritoneal TGF-β1 (ng/mL) in survivors and non-survivors. Whiskers represent the 95% confidence interval.

horses with an average age of 5.5 years old (IR: 5.4 years) were used in this study. Of the 78 colic horses, 28 animals were included in the obstructive group (13 cases were resolved by medical treatment and 15 were surgically resolved), 20 animals were included in the enteritis group, 22 animals were included in the ischemic group (12 with small intestine strangulation lesions and 10 with colon volvulus), and 8 were included in the peritonitis group. The average age for these groups was similar.

Plasma concentration of TGF-β_1 and TGF-β_3

No significant differences were obtained between plasma TGF-β_1 and TGF-β_3 concentrations

according to the diagnosis (Table 1). Plasma concentration of TGF-β_3 was above 30 pg/mL in only 23/86 horses (28%), and, of those 23, 10 horses belonged to the obstructive group, 5 to the enteritis group, 2 to the ischemic group, 4 to the peritonitis group and 2 belonged to the control group. Plasma concentrations of TGF-β_1 were significantly higher (T test, P =0.05) in non-survivors compared with survivors (Fig 1a). Plasma concentrations of TGF-β_3 were not statistically different between survivors and non-survivors.

Peritoneal concentrations of TGF-β_1 and TGF-β_3

Peritoneal TGF-β_1 concentration was significantly (P =0.01) higher in horses with peritonitis when compared with all other colic groups and controls (Table 1). The ischemic group also presented significantly (P =0.01) higher concentrations of peritoneal TGF-β_1 when compared with the obstructive and control groups. No statistical differences for this peritoneal peptide were obtained between the other colic and control groups (Table 1). On the other hand, peritoneal TGF-β_3 concentrations showed significant differences between the peritonitis and the other groups (Table 1). Peritoneal concentrations of TGF-β_3 were higher than 30 pg/mL only in 15/86 horses (17%). Of these 15 horses, 3 animals belonged to the obstructive group, 2 horses to the enteritis group, 1 to the ischemic group, and 5 horses to peritonitis group. Results of horses according to diagnosis and outcome are shown in Table 2.

Results of the peritoneal fluid analysis were available for 75 horses and were classified as follows: 38/75 transudates (51%), 15/75 modified transudates (20%), and 22/75 exudates (29%). When peritoneal TGF-β_1 and TGF-β_3 concentrations were compared according to the type of peritoneal fluid analysis, TGF-β_1 concentration was significantly (P =0.01) higher in exudates compared to transudates, whereas this concentration in modified transudates did not differ in exudates and transudates (Table 3). In contrast, no statistically significant differences were seen between any type of peritoneal fluid for TGF-β_3 concentrations.

According to the outcome, 55 out of 78 colic horses (70.5%) were included in the survival group and 23/ 78 (29.5%) in the non-survival group. Results of peritoneal fluid analysis according to the outcome are shown in Table 4. Peritoneal concentrations of TGF-β_1 were significantly (T test, P =0.001) increased in non-survival horses (Fig 1b). This finding was similar for peritoneal concentrations of TGF-β_3 between survivors and non-survivors (Wilcoxon test, P =0.004).

Correlations

Statistically significant correlations although weak were obtained between diagnosis and peritoneal TGF-β_3 concentration (τ: 0.236, P =0.019), type of peritoneal fluid and outcome (τ: 0.237, P =0.041), type of peritoneal fluid and peritoneal TGF-β_1 concentration (τ: 0.379, P =0.001), outcome and peritoneal TGF-β_1 and TGF-β_3 concentrations (τ: 0.310, P =0.001; τ: 0.301, P =0.006, respectively).

Discussion

To the authors' knowledge, this is the first clinical study in which concentrations of both plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations are reported and compared among horses with different types of colic, different types of peritoneal fluids and between survivors and non-survivors. Human ELISA kits were used to measure plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations because all proteins of the TGF-β family have a high homology among mammals (Penha-Goncalves *et al.* 1997; Javelaud and Mauviel 2004).

In this study, it was observed that plasma concentrations of TGF-β_1 and TGF-β_3 were not different among colic and healthy groups. Failure to detect a significant difference among

horses with different type of colic may have been caused to sample size. On the other hand, plasma TGF-β_1 concentration was statistically different between survival and non survival horses. This finding also has been reported in human beings with severe trauma (Laun *et al.* 2003) and sepsis (Pachot *et al.* 2005). In horses with colic, mortality is closely related to the degree of endotoxaemia (Barton and Collates 1999). Endotoxins produce activation of the inflammatory cascade, including the up-regulation of pro-inflammatory cytokines (TNF-α and IL-6), and release of acute phase proteins and eicosanoids (systemic inflammatory response syndrome (SIRS)) (Werners *et al.* 2005). In many instances, SIRS is preceded by a compensatory anti-inflammatory response syndrome (CARS), which leads the patient to immunosuppression and finally to death (Ward *et al.* 2008). IL-10 and TGF-β_1 are the most important anti-inflammatory growth factors up-regulated during CARS to produce control of the inflammatory response (Laun *et al.* 2003, Pachot *et al.* 2005).

Concentrations of TGF-β_1 in peritoneal fluid of horses with colic should be analyzed in function of the pathophysiological picture of each colic group and of the proportion of the active form of this peptide in peritoneal fluid (Ghellai *et al.* 2000). However, one limitation of this study was that peritoneal concentration of the latent and active forms of TGF-β_1 were not measured. Horses with peritonitis had a poor prognosis and usually were destroyed because most cases were intestinal ruptures. Barton and Collates (1999) described higher peritoneal fluid concentrations of endotoxin, TNF-α and IL-6 in horses with peritonitis produced by intestinal rupture. Our findings suggest that the higher peritoneal TGF-β_1 concentration in horses with peritonitis could be related with an activation of a CARS state to diminish the peritoneal inflammatory effect caused by endotoxins (Pachot *et al.* 2005, Ward *et al.* 2008, Mirshafiey and Mohsenzadegan 2009).

TGF-β_1 also plays a crucial role in intra-abdominal adhesion formation in septic peritonitis (Ghellai *et al.* 2000). Sepsis has been shown to facilitate activation of the latent TGF-β_1 (Ghellai *et al.* 2000). Only bioactive TGF-β_1 is able to bind to the TGF-β_1 receptor to exert biologic effects, including the synthesis and deposition of extracellular matrix (Ghellai *et al.* 2000). On the other hand, TGF-β_3 has an antagonistic effect respect to the profibrotic effects of TGF-β_1 in skin wound repair (Shah *et al.* 1995) and peritoneal adhesion formation (Gorvy *et al.* 2005). Peritoneal fluid concentrations of TGF-β_3 were significantly higher in the peritonitis group in comparison with the other colic groups and healthy horses. This could be related with an up-regulatory effect of active form of TGF-β_1 released to the peritoneal fluid. Although, Zaed *et al.* (2000) demonstrated *in vitro* (using hypoxic human mesothelial cells) that TGF-β_1 down-regulates the expression of TGF-β_3; Gorvy *et al.* (2005) observed in an experimental murine model of adhesions that immune-staining for TGF-β_3 was moderately increased in concordance with the staining for TGF-β_1 during first 24-48 hours.

Strangulating lesions of the small intestine present higher rates of adhesions in comparison with ischemic lesions of the colon and non-strangulating lesions of the intestine (Gorvy *et al.* 2008). In the present study, no significant differences were found on peritoneal TGF-β_1 and TGF-β_3 concentrations between small and large intestine ischemic lesions. Therefore, horses with ischemic lesions of the small and large intestine were maintained in the same group. No significant differences were found between the enteritis and ischemic groups. However, as the active fractions of these growth factors were not measured, it is possible that horses with ischemic lesions presented a higher proportion of the active form of TGF-β_1 than horses with enteritis. Further studies are warranted to elucidate whether these differences may exist.

Horses with non-parasitic inflammatory bowel disease (IBD) express TNF-α and other pro-inflammatory cytokines and TGF-β_1 (Davidson *et al.* 2002). On the other hand, in human beings reduced TGF-β_1 activity is considered to be responsible for the development of IBD. TGF-β_1 promotes a potent immunosuppressive effect and acts in concert with other growth factors to protect host tissue from luminal challenges and facilitate repair of mucosal injury in IBD (Sánchez-Muñoz *et al.* 2008). Horses with enteritis presented higher peritoneal concentrations of TGF-β_1, although no statistically different respect to the control, obstructive and ischemic groups. It is possible that this growth factor plays a different pathophysiological role in horses with enteritis respect to the other colic groups, and their higher peritoneal concentration could be related with a positive resolution of the bowel inflammation (Sánchez-Muñoz *et al.* 2008).

Acute abdominal disease may produce changes in the peritoneal fluid analysis that, depending on severity of the illness and sampling time, vary between modified transudates and exudates (Milne *et al.* 1990). In the present study, a statistically higher peritoneal TGF-β_1 concentration was observed in exudates when compared with transudates, although not statistically different compared with modified transudates. This growth factor may be produced by activated leukocytes in peritoneal fluid, which would be higher in exudates. In contrast, this phenomenon was not seen for peritoneal TGF-β_3, although this peptide was weakly correlated with TGF-β_1 concentration on peritoneal fluid. In this sense, it is possible that peritoneal fluid concentration of TGF-β_3 could be up-regulated by peritoneal TGF-β_1 in horses with colic (Gorvy *et al.* 2005). On the other hand, in this study a very weak correlation was found between the type of peritoneal fluid and outcome. Maybe the low number of horses included in the study or the fact that normal peritoneal fluid was collected in some horses with severe intestinal lesions could have interfered with these results.

Peritoneal concentrations of TGF-β_1 and TGF-β_3 were statistically higher in non-survival horses compared to survivors. The higher concentration of these growth factors in non-survivors may be related with a local CARS response to endotoxin insult (Ward *et al.* 2008). As previously reported, horses with peritonitis presented detectable concentrations on endotoxin in their peritoneal fluid (Barton and Collates 1999), and peritoneal TGF-βs include the anti-inflammatory repertory to counterest the potent pro-inflammatory effect of the TNF-α during septic peritonitis (Werners *et al.* 2005; Ward *et al.* 2008).

In conclusion, this study reports new knowledge on changes of plasma and peritoneal TGF-β concentrations in horses with colic. Peritoneal TGF-β_1 concentration was higher in horses with peritonitis and ischemic lesions, maybe as a consequence of peritoneal inflammation or damaged intestine. Further studies should be performed to prove the association between peritoneal TGF-β concentrations and the development of abdominal adhesions.

Manufacturers' addresses

[1] Human TGF-β1. R&D Systems, Abingdon, UK

[2] Human TGF-β_3. R&D Systems, Abingdon, UK

[3] Sigma, St Louis, MO, USA

[4] SPSS inc., Chicago, IL, USA

IV STUDY TWO

Plasma and peritoneal fluid concentrations of D-dimer and TGF-β_1 in horses with colic.

Abstract

Horses with severe colic or with altered peritoneal fluid at admission, as well as horses classified as non survivors of acute abdominal crisis, will have greater increases in plasma and peritoneal D-dimer and peritoneal transforming growth factor beta-1 (TGF-β_1) concentrations. The aims of this study were to describe and to assess the correlation between these molecules. The animals studied were 124 horses with colic and 12 control horses. The horses with colic were grouped according to diagnosis (obstructions, enteritis, ischemic problems, peritonitis and other mixed problems), results of the peritoneal fluid analysis (transudate, modified transudate and exudate) and outcome (survivors and non-survivors). Plasma and peritoneal D-dimer concentrations and peritoneal TGF-β_1 concentrations were significantly higher in horses with enteritis, peritonitis and ischemic disorders, when compared to horses with large intestinal obstructions. Peritoneal D-dimer and TGF-β_1 concentrations were significantly higher in horses with altered peritoneal fluid (modified transudate and exudate) compared to horses with normal peritoneal fluid analysis. The concentration of these proteins was also significantly higher in the peritonitis group. Peritoneal and plasma D-dimer concentrations and TGF-β_1 were also significantly higher in non-survivors. There were significant correlations, from weak to strong, between the evaluated proteins and the diagnosis, the outcome and the type of peritoneal fluid.

Key words: equine, gastrointestinal disease, D-dimer, transforming growth factor beta-1

1. Introduction

Peritoneal D-dimer is a specific marker of peritoneal fibrinolysis activity and, subsequently, of peritoneal fibrinogenesis. Peritoneal injury is repaired in part due to the pro-coagulant activity of mesothelial cells that produce fibrin deposition by the release of mesothelial plasminogen activator inhibitor type 1 (PAI-1), a fibrinolysis inhibitor that aids fibrin formation, whereas the fibrinolytic activity is concurrently activated by the secretion of tissue-plasminogen activator (t-PA) that is essential to destroy the fibrin formed after peritoneal insult to decrease subsequent risk of adhesion formation (Brokelman et al., 2009; Goedde et al., 1997). The activation of the peritoneal fibrinolysis activity produces an increase in peritoneal D-dimer concentration, which is a degradation fragment released exclusively by the plasmin-mediated lysis of cross-linked fibrin (Delgado et al., 2009).

. The test for measuring plasma and peritoneal D-dimer concentrations has been used for plasma hypercoagulation and hyperfibrinolysis assessment in horses (Armengou et al., 2008; Cesarini et al., 2010). Plasma D-dimer concentration is a sensitive marker in assessing fibrinolytic activity and thus coagulation activity. Any increase in D-dimer concentration is related to an increase in fibrin destruction (hyperfibrinolysis), subsequent to an increase in fibrin formation (hypercoagulation or hyperfibrinogenesis) (Delgado et al., 2009).

Transforming growth factor beta 1 (TGF-β_1) is a pleiotropic protein involved in cellular proliferation induction, connective tissue deposition and cellular death (Javelaud and Mauviel, 2004). However, the most important function of this protein is controlling several immune-tolerance mechanisms by modulating the proliferation, differentiation and survival of several subsets of lymphocytes (Mirshafiey and Mohsenzadegan, 2009). Thus, in conjunction with other proteins such as interleukin 10, TGF-β_1 is considered to be one the most powerful anti-inflammatory proteins in mammals. Both proteins are up-regulated when systemic

inflammatory response syndrome (SIRS) is unchained in patients with diseases that are severe or which have a poor prognosis, such as severe trauma, sepsis or cancer, amongst others (Laun et al., 2003).

Recently, a study demonstrated that horses with colic presenting severe gastrointestinal disorders also had a significant increase in the concentration of peritoneal TGF-β_1 (Argüelles et al., 2010). In addition, other studies showed that peritoneal D-dimer concentrations were significantly higher in horses with altered peritoneal fluid when compared to horses with a normal peritoneal fluid analysis. These results confirmed that those horses with inflammatory and ischemic problems, as well as those with peritonitis, had higher peritoneal fibrinolytic activity (hyperfibrinolysis) probably as a response to the higher peritoneal fibrin formation and deposition (hyperfibrinogenesis) (Cesarini et al., 2010; Delgado et al., 2009).

To our knowledge, no relationship between the peritoneal fibrinolysis activity (using D-dimer measurements) and the peritoneal TGF-β_1 concentration has been previously shown in horses with GI abdominal insult. More knowledge about the biological interaction between the fibrinolysis system and the TGF-β_1 mediated compensatory anti-inflammatory response could be useful for understanding the adaptive mechanisms during the development of gastrointestinal (GI) disease in horses.

The aims of this study were 1) to measure and compare the plasma D-dimer concentrations in normal horses and in horses with different types of colic, 2) to assess and compare the D-dimer and TGF-β_1 concentrations in the peritoneal fluid of normal horses and of horses with different types of colic, 3) to investigate the possible relationship between peritoneal TGF-β_1 and D-dimer concentrations by outcome (survivors and non-survivors) and by peritoneal fluid type (transudate, modified transudate and exudate).

2. Materials and methods

This study was approved by the Ethical Committee of the authors' institution. The clinically healthy horses were used as controls after obtaining the owner's consent.

2.1 Study design

In this prospective clinical study, horses (at least one year of age or older) with colic admitted between September 2004 and September 2010 were included. Peritoneal fluid samples were collected upon admission for diagnostic purposes with the owner's permission. In addition, samples from healthy horses, without GI disorders, were collected via identical techniques and became part of the control group.

Horses with colic were grouped subsequently into one of 5 groups according to the diagnosis: *Obstructive group,* which included non-inflammatory, non-strangulating disorders such as impactions and large colon displacements without signs of intestinal devitalisation resolving with medical therapy. *Enteritis group,* including horses with duodeno-jejunitis and typhlocolitis. *Ischemic group,* including horses with strangulating disorders, such as volvulus, torsion, inguinal hernias, and epiploic foramen entrapment. *Peritonitis group,* including horses with gastric or intestinal ruptures, as well as those with septic peritonitis caused by bowel devitalisation without rupture, and *mixed/other processes group,* including horses with two or more disorders of similar severity and horses with other diseases, such as malignancy. The diagnoses were made based on clinical history, complete physical examination, and the results of complementary diagnostic tests (CBC, plasma biochemistry, blood gas analysis, abdominal ultrasonography and peritoneal fluid analysis). Abdominal radiology, laparoscopy, or post-mortem examination findings aided in group classification whenever performed.

To assess the prognostic value of D-dimer and TGF-β_1 peritoneal concentrations of horses upon admission, patients were also grouped according to the outcome: *survivors* (horses that were discharged from the hospital) and *non-survivors* (horses that died during the hospitalisation). An additional *economic constraint* group was also considered as an outcome, because some horses were euthanised for economic reasons and not due to pathological alterations.

Horses were also grouped according to peritoneal fluid analysis, namely in to *transudate* (nucleated cell count (NCC) $\leq$ 5000 cells/µL and total protein $\leq$ 2.5 g/dL), *modified transudate* (NCC $\leq$ 5000 cells/µL and total protein > 2.5 g/dL or NCC > 5000 cells/µL and total protein $\leq$ 2.5 g/dL, with a normal peritoneal fluid cytology) and *exudate* (NCC > 5000 cells/µL and total protein > 2.5 g/dL with inflammatory peritoneal fluid cytology) (Delgado et al., 2009).

2.2 Sample collection

Blood was collected in 4.5 mL tubes containing 3.8% sodium citrate (w/v) (BD Vacutainer, Franklin Lakes, NJ, USA). Peritoneal fluid was collected aseptically, using a sterile, blunt teat cannula, 2 cm to the right of the middle of the most dependent area of the ventral abdomen, according to the standard technique. Peritoneal fluid was collected in tubes containing sodium citrate for D-dimer and TGF-β_1 analysis and in 1 mL tubes containing K_3EDTA (Greiner Vacuette Minicollect K3 EDTA tube, Greiner Bio-one GmbH, Kremsmünster, Austria) for total nucleated cell counts, total peritoneal protein concentration (TP) measurements and cytologic evaluation. Blood and peritoneal citrated samples were immediately centrifuged at 1000 x g for 15 minutes, separated from the sediment and frozen at -84ºC until assayed.

2.3 Peritoneal fluid analysis

A routine analysis was performed on EDTA samples by a specialised laboratory within 12 hours after collection. An automated blood cell counter was used for peritoneal NCC determination. Peritoneal TP was measured by refractometry. Cyto-spin preparations and Diff-Quick stain (Microscopy Hemacolor, Merck Egaa, Darmstadt, Germany) were used for the microscopic evaluation of all peritoneal cytologies. The morphological appearance of the cells was used to determine the inflammatory changes in the peritoneal fluid. Smears were analysed in order to assess the proportion of lymphocytes to neutrophils, for cell morphology and for the presence of bacteria. The peritoneal fluid cytology was considered to be normal when the proportion of lymphocytes to neutrophils was near to 1:1 and when both degenerate leukocytes and bacteria were absent. Inflammatory peritoneal fluid was considered when the proportion of lymphocytes to neutrophils was altered, with predominance of neutrophils in acute peritonitis or predominance of mononuclear cells in chronic peritonitis, and the presence of both degenerative leukocytes with cytolysis, karyorrhexis, or karyolysis and bacteria (de Heer et al., 2002)

2.4 Plasma and peritoneal D-Dimer and TGF-β_1 concentration measurements

The D-dimer concentration in plasma and peritoneal fluid was determined in duplicate using a quantitative immunoturbidimetric latex agglutination assay (Miniquant, Biopool, Trinity Biotech, Wicklow, Ireland) (Armengou et al., 2008; Delgado et al., 2009). Concentrations of TGF-β_1 in peritoneal fluid samples were determined in duplicate, using an ELISA Development Kit (Human TGF-beta 1 DuoSet, DY240E, R&D System, Abingdon,

Table 1: Number and percentages of horses classified according to the diagnosis and the outcome.

Outcome	**Obstructive group** ***(n= 39)***	**Enteritis group** ***(n= 32)***	**Ischemic group** ***(n=24)***	**Peritonitis group** ***(n= 15)***	**Mixed group** ***(n=14)***	**Total outcome** ***(n=124)***
Survivors	34 (87.2)	22 (68.8)	13 (54.2)	1 (6.7)	5 (35.7)	83 (62.9)
Non-survivors	2 (5.1)	8 (25)	8 (33.3)	13 (86.7)	8 (57.1)	39 (29.5)
Economic constraints	3 (7.7)	2 (6.3)	3 (12.5)	1 (6.7)	1 (7.1)	10 (7.6)
Total diagnosis	39 (31.4)	32 (25.8)	24 (19.4)	15 (12.1)	14 (11.3)	124 (100)

Data expressed as n (%)

Oxfordshire, UK) with antibodies against human TGF-β_1 (Argüelles et al., 2006; Argüelles et al., 2010). This protein was only assayed in peritoneal fluid.

2.5 Statistical analysis

Data were analysed using the statistical software SPPS 18.0 (SPSS inc., Chicago, Illinois, USA). Both plasma and peritoneal D-dimer concentrations were processed by means of Kruskal-Wallis test. A Mann-Whitney U test was used for *post hoc* comparisons of non-paired samples between the colic and the outcome groups and the types of peritoneal fluid. These variables were presented as median (range (R)), since they did not show parametric distribution (Shapiro-Wilk test, $P \leq 0.05$) after several transformations. These tests were used to compare concentrations of this protein amongst the colic groups and types of peritoneal fluid.

TGF-β_1 peritoneal concentrations were analysed using an ANOVA test after log transformation. The Games-Howell's or Tukey's *post-hoc* tests were used to compare the concentrations of these variables among the different colic groups, the outcomes and the types of peritoneal fluid. These variables are presented as mean and standard error. Both Kendall's tau rank (τ) and Spearman's (ρ) general (including all the colic groups) and specific (by colic group) correlations were used to compare plasma and peritoneal concentrations of D-dimer and peritoneal concentrations of TGF-β_1 according to the diagnosis, the outcome and the type of peritoneal fluid.

3. Results

3.1 Horses

One hundred and twenty-four horses with colic, including 46 (36.8%) Andalusians, 25 (20%) crossbred horses, 15 (12%) Western European Warmbloods, 5 (4%) Anglo-Arabians, 7 (5.6%) ponies, 3 (2.4%) Thoroughbreds, 3 (2.4%) Quarter Horses, 3 (2.4%) Trotters and 15 (12%) horses representing other breeds were evaluated. These patients presented a median age of 10 years (range (R) = 25 years). Furthermore, 12 clinically healthy, crossbred horses with an average age of 2.5 years (R=15.34 years) were included as controls. Thirty-four of the 132 animals included were stallions (25.8%), 50/132 were geldings (37.9%) and 48/132 were mares (36.4%).

Of the 124 horses with colic, 39 animals were included in the obstructive group, 32 animals were included in the enteritis group, 24 animals were included in the ischemic group, 15 were included in the peritonitis group and 14 were included in the mixed group. Results from the horses according to diagnosis and outcome are shown in table1, while those grouped according to the outcome and the type of peritoneal fluid are summarised in table 2.

3.2 Plasma D-dimer concentrations according to the diagnosis

No significant differences were observed in plasma D-dimer concentration between the control group and most of the colic groups, with the exception of the peritonitis group. Plasma D-dimer concentration in the peritonitis group was also different from that of the obstructive, the enteritis and the mixed groups. A statistically significant difference was found between the enteritis and the ischemic groups (Table 3).

3.3 Plasma concentration of D-Dimer according to the outcome

Table 2: Number and percentages of horses classified according to the type of peritoneal fluid analysis and the outcome[#].

Outcome	**Transudate** ***(n= 57)***	**Modified transudate** ***(n= 29)***	**Exudate** ***(n= 32)***	**Total outcome** ***(n= 118)***
Survivors	48 (82.5)	20 (69)	9 (28.1)	76 (64.4)
Non-survivors	8 (14)	7 (24.1)	19 (59.4)	34 (28.8)
Economic constraints	2 (3.5)	2 (6.9)	4 (12.5)	8 (6.8)
Total type of peritoneal fluid	57 (48.3)	29 (24.6)	32 (27.1)	118 (100)

Data expressed as n (%). [#]Data from six horses have been lost.

Plasma concentrations of D-dimer were significantly higher (U-Mann-Whitney (M-W) test, P =0.001) in non-survivors (*n=39*) compared to survivors (*n=83*). Those horses that were euthanized due to economic constraints (*n=10*) did not present differences respect survivors; however, this group was statistically different (M-W, P=0.043) from those horses that did not survive (Table 4).

3.4 Plasma concentration of D-Dimer according to the type of peritoneal fluid

When plasma D-dimer concentrations were compared according to the type of peritoneal fluid analysis, D-dimer concentration was significantly different (P =0.05) for each type of peritoneal fluid (Table 5).

3.5 Peritoneal concentration of D-dimer according to the diagnosis

Peritoneal D-dimer concentration was significantly (P=0.01) lower in control horses than in the other colic groups. The enteritis, ischemic, peritonitis and mixed groups also presented significantly (P=0.01) higher peritoneal D-dimer concentrations when compared to the obstructive group. However, the enteritis group presented significantly lower (P=0.01) D-dimer concentrations when compared to the ischemic, peritonitis and mixed groups (Table 3).

3.6 Peritoneal concentration of D-dimer according to the outcome

Peritoneal fluid concentrations of D-dimer were significantly higher (U-Mann-Whitney (M-W) test, P =0.001) in non-survivors (*n=39*) compared to survivors (*n=83*) and horses in the economic constraint group (n=10). The latter did not present differences (M-W test, P =0.9) with respect to the horses that did not survive (Table 4).

3.7 Peritoneal concentration of D-dimer according to the type of peritoneal fluid

When peritoneal D-dimer concentrations were compared according to the type of peritoneal fluid analysis, D-dimer concentration was significantly different (P=0.01) for each type of peritoneal fluid (Table 5).

3.8 Peritoneal concentration of TGF-β_1 according to the diagnosis

There were no statistical differences between any of the colic groups and the control group in terms of peritoneal fluid TGF-β_1 concentration. However, the enteritis, ischemic, peritonitis and mixed groups presented higher peritoneal concentrations of this protein than did the

obstructive group.

3.9 Peritoneal concentration of TGF-β_1 according to the outcome

Peritoneal concentrations of TGF-β_1 were significantly (Tukey test, P=0.001) increased in non-surviving horses, in comparison to the concentrations in horses that survived. However, no differences for this protein were found between the group of horses that were euthanized due to economic constraints and the surviving and non-surviving groups (Table 4).

3.10 Peritoneal concentration of TGF-β_1 according to the type of peritoneal fluid

When peritoneal TGF-β_1 concentrations were compared according to the type of peritoneal fluid analysis, the TGF-β_1 concentration was significantly higher in exudates (P=0.00) and in modified transudates (P =0.00) when compared to transudates, whereas the peritoneal TGF-β_1 concentration did not differ between exudates and modified transudates (Table 5).

3. 11 General correlations

Weak, statistically significant correlations were obtained between diagnosis and plasma D-dimer concentration (τ: 0.25, P=0.003), diagnosis and peritoneal D-dimer concentration (τ: 0.41, P=0.000), diagnosis and peritoneal TGF-β_1 concentration (τ: 0.33, P =0.000), outcome and peritoneal D-dimer concentration (τ: 0.25, P =0.000), outcome and peritoneal TGF-β_1 concentration (τ: 0.24, P =0.001), type of peritoneal fluid and peritoneal D-dimer concentration (τ: 0.45, P=0.000), type of peritoneal fluid and peritoneal TGF-β_1 concentration (τ: 0.40, P =0.000), type of peritoneal fluid and outcome (τ: 0.41, P=0.000), type of peritoneal fluid and peritoneal TGF-β_1 concentration (τ: 0.38, P=0.001), plasma D-dimer concentration

and peritoneal D-dimer concentration (ρ: 0.47, P=0.000), plasma D-dimer concentration and peritoneal TGF-β_1 concentration (ρ: 0.55, P=0.000) and peritoneal D-dimer concentration and peritoneal TGF-β_1 concentration (ρ: 0.52, P=0.000).

3.12 Specific correlations

Plasma D-dimer concentration and peritoneal TGF-β_1 concentration were significantly correlated in the enteritis (67%) and mixed (82%) colic groups. Other weak, specific correlations are presented in table 6.

Table 3: Plasma and peritoneal D-dimer and TGF-β1 concentrations in the control and various colic groups.

Variable	**Controls** ***(n= 12)***	**Obstructive group** ***(n= 39)***	**Enteritis group** ***(n= 32)***	**Ischemic group** ***(n=26)***	**Peritonitis group** ***(n= 14)***	**Mixed group** ***(n=15)***
Plasma						
D-dimer (ng/mL)*	161 (920)	473 (18041)	1363.5 (4797)[b]	462 (4510)[c]	7514 (22613)[a,b,c]	2256.5(4977)[e]
Peritoneal fluid						
D-dimer (ng/mL)*	166.25 (829)	1846.5 (21492)[a]	6534 (103038)[a,b]	18113 (81296)[a,b,c]	73776 (479724)[a,b,c]	22400 (199587)[a,b,c]
TGF-β_1 (ng/mL)**	0.8 (0.19)	0.55 (0.053)	1.09 (0.13)[b]	0.78 (0.055)[b]	1.48 (0.159)[b,d]	3.6 (1.2)[b]

* Data are presented as median (range). **Data are presented as mean (standard error).

[a] Significantly different from the control group.

[b] Significantly different from the obstructive group.

[c] Significantly different from the enteritis group.

[d] Significantly different from the ischemic group.

[e] Significantly different from the peritonitis group.

4. Discussion

The main objective of this study was to evaluate the interaction (or relationship) of at least one component related to the fibrinolytic systems (D-dimer) with another component of the anti-inflammatory systemic response system (TGF-β_1). This investigation was conducted because the isolated results obtained in some studies pointed to the the role and prognostic value of D-dimer concentration detection (Cesarini et al., 2010; Delgado et al., 2009; Dunkel et al., 2010) and TGF-β_1 (Argüelles et al., 2010) in horses with colic. The present study investigates the plasma and peritoneal correlational relationship of these molecules in horses with different types of colic.

During a gastrointestinal insult (colic), several systemic and local (visceral and peritoneal) pathophysiological mechanisms are activated to counteract the effect of endotoxins released into both the blood stream (Nieto et al., 2009) and into the peritoneal cavity, and to promote the repair of the compromised viscera (Barton and Collatos, 1999; Delgado et al., 2009). Some of these mechanisms include the activation of both the coagulation cascade (Cesarini et al., 2010; Dunkel et al., 2010) and of systemic anti-inflammatory mechanisms mediated by TGF-β_1 (Argüelles et al., 2010), IL-10 and other regulatory cytokines (Lopes et al., 2010).

Plasma and peritoneal concentrations of D-dimer observed in horses with colic in this study presented similar behaviour to that found in previously published studies (Cesarini et al., 2010; Delgado et al., 2009). In this sense, plasma and peritoneal D-dimer concentrations were greatly increased in horses with peritonitis or with severe ischemic gastrointestinal insults, as well as in non-surviving horses and in peritoneal fluids classified as exudates (Cesarini et al., 2010; Delgado et al., 2009). On the other hand, peritoneal fluid TGF-β_1 concentrations for the control horses were similar, but were lower in the colic groups than those measured in previous studies (Argüelles et al., 2010). However, peritoneal fluid TGF-

β_1 concentrations were mainly increased in the inflammatory groups, particularly in horses in the peritonitis and mixed groups. The same tendency was noticed when the outcome and the type of peritoneal fluid were considered. However, peritoneal fluid TGF-β_1 concentrations were slightly lower for these classificatory variables than they were in comparison to previous results (Argüelles et al., 2010). A possible explanation for the discrepancy in the peritoneal fluid growth factor concentration of these studies could be related to the size of each colic group, or could be because the horses in both studies were different. It is possible that the horses in the present research presented less severe clinical-pathological conditions in comparison to those in the aforementioned study (Argüelles et al., 2010)

The general analysis of the Kendall tau rank correlations between the classificatory factors diagnosis (group of colic), the outcome and the type of peritoneal fluid and the response variables (plasma and peritoneal D-dimer concentration and peritoneal TGF-β_1 concentration) showed significant, but weak correlations. This situation could be explained by the tremendous variation observed in the response variables, due in part to the wide variation in the duration of the episode of colic of each patient. However, this situation may not be controlled in a clinical study of this nature and it is possible that many horses allotted to a similar colic group presented different classificatory alterations, which could be re-allotted to subgroups inside the general groups arbitrarily evaluated in this study. This fact is considered to be a common cause of possible misinterpretations and pitfalls in other colic studies of the same nature (Argüelles et al., 2010; Cesarini et al., 2010; Collatos et al., 1995; Delgado et al., 2009; Dunkel et al., 2010; Watts et al., 2011) and, of course, it is also a pitfall in the present study.

In the present study, weak general correlations were found between plasma and peritoneal D-dimer concentration (ρ: 0.47, P =0.000). This situation was different from the results of previous studies, where no correlations were found between these molecules in

plasma and in peritoneal fluid (Collatos et al., 1995; Delgado et al., 2009). In addition, other important, although weak, correlations were noticed between peritoneal TGF-β_1 concentration and plasma (ρ: 0.55, P=0.000) and peritoneal D-dimers concentration (ρ: 0.52, P=0.000).

However, the most important findings of the present study were possibly the moderate to strong specific correlations observed between D-dimer and TGF-β_1 peritoneal fluid concentrations in horses with severe inflammatory GI disease, particularly in horses with enteritis, ischemic intestinal disease or with mixed gastrointestinal disease. These findings could suggest that some horses with colic and secondary systemic inflammation display compensatory related mechanisms, such as hyper-fibrinolysis (systemic and peritoneal) and local production of pro-fibrotic and anti-inflammatory proteins, such as TGF-β_1.

The TGF-β peptide superfamily represents a very complex signalling cascade which seems to increase in complexity as research progresses. This is why simply neutralising TGF-β may be ineffective for the treatment of fibrosis. Besides that, neutralising TGF-β might have systemic consequences, added unspecific undesired effects or even lack of anti-fibrosis function if it is not appropriately administered at the right place and time, meaning cellular location and cell type phase, and at specific time point (like at the beginning or in the inflammatory phase of the healing cascade).

Due to the enormous complexity of the TGF-β related-signalling pathways, and that any dysregulation of any member of this family might elicit different effects, special caution should be taken and further in vitro and in vivo research is warranted. Although TGF-β has long been known to induce matrix synthesis and contraction by fibroblasts, the precise contribution of this protein to fibrotic disease is still unclear.

It is known that plasmin is the main enzyme related to fibrin degradation and the production of D-dimer (Song et al., 1996). This enzyme was also the first reported protease with the capacity to activate TGF-β_1 (Jenkins, 2008). This situation could explain the

correlations observed in the present study and could possibly indicate that peritoneal TGF-β_1 is released by the alteration of the fibrinolitic system in horses with severe GI disease. This situation could be particularly true in cases of severe inflammation like enteritis and in cases with a poor prognosis, as peritoneal malignancy, where an especial environment for the alteration of the fibrinolitic system and activation of the latent TGF-β_1 possibly exists (Jenkins, 2008). However, one limitation of the present study was that the original, active form of the TGF-β_1 was not measured. This could be an additional reason to explain that the peritoneal concentration of TGF-β_1 presents a weak correlation with the D-dimer activity.

Further studies are necessary to determine latent and active forms of this growth factor in horses with colic.

V. CONCLUDING DISCUSSION

This is the first clinical study in which concentrations of both plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations are reported and compared among horses with different types of colic, different types of peritoneal fluids and between survivors and non-survivors. Human ELISA kits were used to measure plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations because all proteins of the TGF-β family have a high homology among mammals (Penha-Goncalves *et al.* 1997; Javelaud and Mauviel 2004).

In this study, no TGF-β_1 and TGF-β_3 plasma concentration differences were observed between colic and healthy groups. Failure to detect a significant difference among horses with different types of colic may have been due to a small sample size. On the other hand, plasma TGF-β_1 concentration was statistically different between survivors and non-survivor horses. This finding also has been reported in human beings with severe trauma (Laun *et al.* 2003) and sepsis (Pachot *et al.* 2005). In horses with colic, mortality is closely related to the degree of endotoxaemia (Barton and Collates 1999). Endotoxins produce activation of the inflammatory cascade, including the up-regulation of pro-inflammatory cytokines (TNF-α and IL-6), and release of acute phase proteins and eicosanoids (systemic inflammatory response syndrome (SIRS)) (Werners *et al.* 2005). In many instances, SIRS is preceded by a compensatory anti-inflammatory response syndrome (CARS), which leads the patient to immunosuppression and finally to death (Ward *et al.* 2008). IL-10 and TGF-β_1 are the most important anti-inflammatory growth factors up-regulated during CARS to produce control of the inflammatory response (Laun *et al.* 2003, Pachot *et al.* 2005).

Concentrations of TGF-β_1 in peritoneal fluid of horses with colic should be considered within the context of the pathophysiological picture of each colic group and the proportion of the active form of this peptide in peritoneal fluid (Ghellai *et al.* 2000) should be taken into account. However, one limitation of this study was that peritoneal concentration of the latent and active forms of TGF-β_1 were not measured. Horses with peritonitis had a poor prognosis

and usually were destroyed because most cases were intestinal ruptures. Barton and Collatoes(1999) described higher peritoneal fluid concentrations of endotoxin, TNF-α and IL-6 in horses with peritonitis produced by intestinal rupture. Our findings suggest that the higher peritoneal TGF-β_1 concentration in horses with peritonitis could be related with an activation of a CARS state to diminish the peritoneal inflammatory effect caused by endotoxins (Pachot *et al.* 2005, Ward *et al.* 2008, Mirshafiey and Mohsenzadegan 2009).

TGF-β_1 plays a crucial role in intra-abdominal adhesion formation in septic peritonitis (Ghellai *et al.* 2000). Sepsis has been shown to facilitate activation of the latent TGF-β_1 (Ghellai *et al.* 2000). Only bioactive TGF-β_1 is able to bind to the TGF-β_1 receptor to exert biologic effects, including the synthesis and deposition of extracellular matrix (Ghellai *et al.* 2000). On the other hand, TGF-β_3 has an antagonistic effect on the profibrotic effects of TGF-β_1 in skin wound repair (Shah *et al.* 1995) and peritoneal adhesion formation (Gorvy *et al.* 2005). Peritoneal fluid concentrations of TGF-β_3 were significantly higher in the peritonitis group in comparison with the other colic groups and healthy horses. This could be related with an up-regulatory effect of TGF-β_1 active form released to the peritoneal fluid. Although, Zaed *et al.* (2000) demonstrated *in vitro* (using hypoxic human mesothelial cells) that TGF-β_1 down-regulates the expression of TGF-β_3; Gorvy *et al.* (2005) observed in an experimental murine model of adhesions that immune-staining for TGF-β_3 was moderately increased in concordance with the staining for TGF-β_1 during first 24-48 hours.

Strangulating lesions of the small intestine present higher rates of adhesions in comparison with ischemic lesions of the colon and non-strangulating lesions of the intestine (Gorvy *et al.* 2008). In the present study, no significant differences were found in peritoneal TGF-β_1 and TGF-β_3 concentrations between small and large intestine ischemic lesions. Therefore, horses with ischemic lesions of the small and large intestine were maintained in the same group. Furthermore significant differences were found between the enteritis and

ischemic groups which was not expected. However, because the active fractions of these growth factors were not measured, it is possible that horses with ischemic lesions presented a higher proportion of the active form of TGF-β_1 than horses with enteritis. Further studies are warranted to elucidate whether these differences may exist.

Horses with non-parasitic inflammatory bowel disease (IBD) express TNF-α and other pro-inflammatory cytokines as well as TGF-β_1 (Davidson *et al.* 2002). On the other hand, in human beings reduced TGF-β_1 activity is considered to be responsible for the development of IBD. TGF-β_1 promotes a potent immunosuppressive effect and acts in concert with other growth factors to protect host tissue from luminal challenges and facilitates repair of mucosal injury in IBD (Sánchez-Muñoz *et al.* 2008). Horses with enteritis presented higher peritoneal concentrations of TGF-β_1, even if it wasn´t statistically different in respect to the control, obstructive and ischemic groups. This growth factor may play a different role in horses with enteritis respect to the other colic groups, and it is likely that higher peritoneal concentrations of this molecule in these instances could be related to the need of fighting inflammation and eventually of a positive resolution of bowel injury (Sánchez-Muñoz *et al.* 2008).

Acute abdominal disease may produce changes in the peritoneal fluid components that, depending on severity of the illness and sampling time, vary between modified transudates and exudates (Milne *et al.* 1990). In the present study, a statistically higher peritoneal TGF-β_1 concentration was observed in exudates when compared with transudates, although not statistically different compared with modified transudates. This growth factor may be produced by activated leukocytes in peritoneal fluid (Yiang *et al.* 2013), which would be higher in exudates. In contrast, this phenomenon was not seen for peritoneal TGF-β_3, although this peptide was weakly correlated with peritoneal fluid TGF-β_1 concentrations. In this sense, it is possible that peritoneal fluid concentration of TGF-β_3 could be up-regulated by peritoneal TGF-β_1 in horses with colic (Gorvy *et al.* 2005). On the other hand, in this study

a very weak correlation was found between the type of peritoneal fluid and outcome. Maybe the low number of horses included in the study or the fact that normal peritoneal fluid was collected in some horses with severe intestinal lesions could have interfered with these results.

Peritoneal concentrations of TGF-β_1 and TGF-β_3 were statistically higher in non-survival horses compared to survivors. The higher concentration of these growth factors in non-survivors may be related with a local CARS response to endotoxin insult (Ward *et al.* 2008). As previously reported, horses with peritonitis presented detectable concentrations of endotoxin in their peritoneal fluid (Barton and Collates 1999), and peritoneal TGF-βs form part of the anti-inflammatory repertoire to counteract the potent pro-inflammatory effect of the TNF-α during septic peritonitis (Werners *et al.* 2005; Ward *et al.* 2008).

In conclusion, this study reports new knowledge on changes of plasma and peritoneal TGF-β concentrations in horses with colic. Peritoneal TGF-β_1 concentration was higher in horses with peritonitis and ischemic lesions, maybe as a consequence of peritoneal inflammation or damaged intestine. Further studies should be performed to prove the association between peritoneal TGF-β concentrations and the development of abdominal adhesions.

The second part of the study was focused on the evaluation of the interactions (or relationship) of the fibrinolytic systems (D-dimer) and the anti-inflammatory systemic response system (TGF-β_1). The main reason to study this likely relationship based on the results obtained in some studies indicating the role and prognostic value of D-dimer as a biomarker (Cesarini et al., 2010; Delgado et al., 2009; Dunkel et al., 2010) and TGF-β_1 (Argüelles et al., 2010) in horses with colic.

During a gastrointestinal insult (colic), several systemic and local (visceral and peritoneal) pathophysiological mechanisms are activated to counteract the effect of endotoxins released into both the blood stream (Nieto et al., 2009) and into the peritoneal

cavity, and to promote the repair of the compromised viscera (Barton and Collatos, 1999; Delgado et al., 2009). Some of these mechanisms include the activation of both the coagulation cascade (Cesarini et al., 2010; Dunkel et al., 2010) and of systemic anti-inflammatory mechanisms mediated by TGF-β_1 (Argüelles et al., 2010), IL-10 and other regulatory cytokines (Lopes et al., 2010).

Plasma and peritoneal D-dimer concentrations observed in horses with colic in the study presented similar behaviour to that found in previously published studies (Cesarini et al., 2010; Delgado et al., 2009). In this sense, plasma and peritoneal D-dimer concentrations were greatly increased in horses with peritonitis or with severe chemical gastrointestinal insults, as well as in non-surviving horses and in peritoneal fluids classified as exudates (Cesarini et al., 2010; Delgado et al., 2009).

The general analysis of the Kendall tau rank correlations between the classificatory factors diagnosis (group of colic), the outcome and the type of peritoneal fluid and the response variables (plasma and peritoneal D-dimer concentration and peritoneal TGF-β_1 concentration) showed significant, but weak correlations. This situation could be explained by the tremendous variation observed in the response variables, due in part to the wide variation in the duration of the episode of colic of each patient. However, this situation may not be controlled in a clinical study of this nature and it is possible that many horses allotted to a similar colic group presented different classificatory alterations, which could be re-allotted to subgroups inside the general groups arbitrarily evaluated in this study. This fact is considered to be a common cause of possible misinterpretations and pitfalls in other colic studies of the same nature (Argüelles et al., 2010; Cesarini et al., 2010; Collatos et al., 1995; Delgado et al., 2009; Dunkel et al., 2010; Watts et al., 2011) and, unfortunately remains a pitfall in the present study.

In the present study, weak general correlations were found between plasma and peritoneal D-

dimer concentration (ρ: 0.47, P =0.000). This situation was different from the results of previous studies, where no correlations were found between these molecules in plasma and in peritoneal fluid (Collatos et al., 1995; Delgado et al., 2009). In addition, other important, although weak, correlations were noticed between peritoneal TGF-β_1 concentration and plasma (ρ: 0.55, P=0.000) and peritoneal D-dimers concentration (ρ: 0.52, P=0.000).

However, the most important findings of the present study were possibly the moderate to strong specific correlations observed between D-dimer and TGF-β_1 peritoneal fluid concentrations in horses with severe inflammatory GI disease, particularly in horses with enteritis, ischemic intestinal disease or with mixed gastrointestinal disease (Table 6). These findings could suggest that some horses with colic and secondary systemic inflammation display compensatory related mechanisms, such as hyper-fibrinolysis (systemic and peritoneal) and local production of profibrotic and anti-inflammatory proteins, such as TGF-β_1.

Plasmin is the main enzyme related to fibrin degradation and the production of D-dimer (Song et al., 1996). This enzyme was also the first protease detected with the capacity to activate TGF-β_1 (Jenkins, 2008). This situation could explain the correlations observed in the present study and could possibly indicate that peritoneal TGF-β_1 release is triggered by alterations of fibrinolysis in horses with severe GI disease. This may be best observed in cases of severe inflammation such as enteritis and in cases of severe insult to the bowel carrying a poor prognosis, such as peritoneal malignancy, where fibrinolytic system alteration and thus latent TGF-β_1 activation possibly occur (Jenkins, 2008). However, as mentioned earlier one of the limitations of the present study was that the original, active form of the TGF-β_1 was not measured. This could be one reason to explain that the peritoneal concentration of TGF-β_1 presents a weak correlation with the D-dimer activity. Further studies are necessary to determine latent and active forms of this growth factor in horses with colic.

Plasmin and thrombospondin-1 have been studied individually as activators of TGF-β_1 in humans, and it was found that TSP1 (matrix protein thromboposdin-1) is an important activator of TGF -β1. It is also known that when levels of TSP1 are low, then PLS (plasmin) becomes an activator of TGF -β1.

The relationship between inflammatory and fibrinolytic biomarkers is used in human medicine very often. D-dimers and TGF-β are widely used as reliable biomarkers for the ADD (acute aortic dissection) in humans (Ranashinge, 2010). Relationship between inflammatory and thrombotic fibrinolytic genotypes has been studied in relation of diagnosis of stroke and other human illness like thrombosis. On that studies impaired fibrinolytic function secondary to elevated plasminogen activator inhibitor-1(PAI-1) has been implicated in ischemic stroke and also high levels of plasminogen activator inhibitor Type 1 (PAI-1) has been implicated as a high risk factor for cardiovascular disease (Arno,2014). To date immnuno-inflamatory and haemostatic biomarkers could represent very useful markers for possible diagnosis of many illness, like acute ischemic stroke (Arno, 2014) and to many other pathologies in the future, and could be extrapolated to equine medicine also.

This thesis describes the participation of two key molecules involved in the complex pathophysiology related with severe gastrointestinal disease in horses. Although incomplete, the results from this study represent a small step to be continued in future research in this complex field. We believe it will be necessary to study a vast range of cytokines and molecules involved in the coagulation process during equine colic. For example, with the help of molecular biology we could study the expression of these substances in mesothelial cells from healthy horses and horses with severe gastrointestinal disease. It is very important to understand that TGF-β1 is not an undesirable molecule in a normal physiological environment, but rather it may be the imbalance between this and other regulator factors that may produce a negative or positive effects in the regulation of some of the pathological

processes happening in the body.

VI. LIST OF CONCLUSIONS

1. TGF-β_1 peritoneal fluid concentrations are increased in horses with ischemic lesions and septic peritonitis.

2. Peritoneal D-dimer and TGF-β_1 concentrations were significantly higher in horses with altered peritoneal fluid (modified transudate and exudate) compared with horses with normal peritoneal fluid analysis

3. Peritoneal and plasma D-dimer concentrations and TGF-β_1 were also significantly higher in non-survivors.

4. There is a weak correlation between the concentration of TGF-β_1 and D-dimer in horses with colic. More studies are necessary for determining the exact relationship between plasma and peritoneal D-dimer and active TGF-β_1 concentrations.

5. Results from the second study permit to conclude that during equine colic activation of the coagulation pathway occurs with a concomitant alteration of the fibrinolytic system mainly manifested by changes in the D-dimer concentrations.

6. The main pitfall of both studies related to TGF-β_1 assessment was that the active form of this protein was not determined. Instead of that all the active/inactive TGF-β_1 was activated for its determination.

7. Both clinical studies can be critiqued for technical problems such as a more strict patient classification, because factors like admission at different times of colic episodes and the intensity of the lesions could increase variability in the results. However, these methodological problems were also common in other studies in horses with colic.

8. Results from this thesis lead to establish that horses with colic develop adaptive changes to attempt to control peritoneal damage. These changes include activation of the fybrinolytic system and imnune response, amongst others.

9. Randomized controlled clinical studies will be necessary in order to know the actual association between D-dimer and active TGF-β_1 in the pathophysiology of adhesions in horses.. In addition, the measurement of cytokines as IL-1, TNF-α and connective tissue growth factors will be very helpful in solving this ongoing biological puzzle.

10. Finally, mesothelial cells recovered from peritoneal fluid should be evaluated by flow cytometry or PCR for expression of surface molecules or genes implicated in the phenotypic transition from epithelial cell to mesenchymal cell. Maybe, the key of adhesion formation in horses and human beings lies in the control of this cellular transition.

REFERENCES

Andrianifahanana, M., Wilkes, M.C., Gupta, S.K., Rahimi, R.A., Repellin, C.E., Edens, M., Wittenberger, J., Yin, X., Maidl, E., Becker, J. and Leof, E.B. (2013) Profibrotic TGFβ responses require the cooperative action of PDGF and ErbB receptor tyrosine kinases. *FASEB Journal* **27**, 4444-4454.

Aoki, S., Takezawa, T., Oshikata-Miyazaki, A., Ikeda, S., Kuroyama, H., Chimuro, T., Oguchi, Y., Noguchi, M., Narisawa, Y. and Toda, S. (2014) Epithelial-to-mesenchymal transition and slit function of mesothelial cells are regulated by the cross talk between mesothelial cells and endothelial cells. *Am J Physiol - Renal Physiol* **306**, F116-F122.

Åkerberg, D., Posaric-Bauden, M., Isaksson, K., Andersson, R. and Tingstedt, B. (2013) Prevention of pleural adhesions by bioactive polypeptides - A pilot study. Int J of Med Sci **10**, 1720-1726.

Argüelles, D., Carmona, J.U., Pastor, J., Iborra, A., Viñals, L., Martínez, P., Bach, E. and Prades, M. (2006) Evaluation of single and double centrifugation tube method for concentrating equine platelets. *Res. Vet. Sci.* **81**, 237-245.

Argüelles, D., Casteljins, G., Carmona, J.U., Armengou, L., Climent, F., Prades, M., 2010. Peritoneal concentrations of transforming growth factor beta in horses with colic. Equine Vet. J. 42, 451-455.

Arno, A., Ganglitz, G., Barret, J (2014) New molecular medicine-based scar management strategies. *Science Direct (in press).*

Armengou, L., Monreal, L., Tarancón, I., Navarro, M., Ríos, J., Segura, D., 2008. Plasma D-dimer concentration in sick newborn foals. J. Vet. Intern. Med. 22, 411-417.

Arung, W., Meurisse, M. and Dety, O. (2011) Pathophysiology and prevention of

postoperative peritoneal adhesions. *World J Gastroent* **17**, 4545-4553.

Barton, M.H. and Collates, C. (1999) Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J. Vet. Intern. Med.* **13**, 457-464.

Bielefeld, K.A., Amini-Nik, S. and Alman, B.A. (2013) Cutaneous wound healing: Recruiting developmental pathways for regeneration. *Cellular and Molecular Life Sciences* **70**, 2059-2081.

Bodega, F., Pecchiari, M., Sironi, C., Porta, C., Arnaboldi, F., Barajon, I. and Agostoni, E. (2012) Lubricating effect of sialomucin and hyaluronan on pleural mesothelium. *Resp Physiol Neurobiol* **180**, 34-39.

Borthwick, L.A., Wynn, T.A. and Fisher, A.J. (2013) Cytokine mediated tissue fibrosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1832**, 1049-1060.

Brochhausen, C., Schmitt, V.H., Planck, C.N.E., Rajab, T.K., Hollemann, D., Tapprich, C., Krämer, B., Wallwiener, C., Hierlemann, H., Zehbe, R., Planck, H. and Kirkpatrick, C.J. (2012) Current Strategies and Future Perspectives for Intraperitoneal Adhesion Prevention. *J Gastroint Surg* **16**, 1256-1274.

Brokelman, W.J.A., Holmdahl, L., Janssen, I.M.C., Falk, P., Bergström, M., Klinkenbijl, J.H.G., Reijnen, M.M.P.J., 2009. Decreased peritoneal tissue plasminogen activator during Prolonged Laparoscopic Surgery. J. Surg. Res. **151**, 89-93.

Carew, R., Wang, B. and Kantharidis, P. (2012) The role of EMT in renal fibrosis. *Cell Tissue Res* 347, 103-116.

Carmona, J.U., Argüelles, D. and Prades, M. (2008) Transforming growth factor beta-3 and nitric oxide levels in four autologous platelet concentrates and plasma derived from equine blood. *Arch. Med. Vet.* **40**, 155-160.

Cesarini, C., Monreal, L., Armengou, L., Delgado, M.A., Rios, J., Jose-Cunilleras, E., 2010.

Association of admission plasma D-Dimer concentration with diagnosis and outcome in Horses with Colic. J. Vet. Intern. Med. 24, 1490-1497.

Collatos, C., Barton, M.H., Prasse, K.W., Moore, J.N., 1995. Intravascular and peritoneal coagulation and fibrinolysis in horses with acute gastrointestinal tract diseases. J. Am. Vet. Med. Assoc. **207,** 465-470.

Davidson, A.J., Edwards, G.B., Proudman, C.J., Cripps, P.J. and Matthews, J.B. (2002) Cytokine mRNA expression pattern in horses with large intestinal disease. *Res. Vet. Sci.* **72**, 177-185.

Delgado, M.A., Monreal, L., Armengou, L., Ríos, J. and Segura, D. (2009) Peritoneal D-dimer concentration for assessing peritoneal fibrinolytic activity in horses with colic. *J. Vet. Intern. Med.* **23**, 882-889.

De Heer, L., Parry, D., Grindem, C., 2002. Peritoneal fluid, In: Diagnostic Cytology & Hematology of the Horse, Second edition ed. Mosby, Inc, St. Louis, pp. 127-162.

Dunkel, B., Chan, D.L., Boston, R., Monreal, L., 2010. Association between hypercoagulability and decreased survival in horses with ischemic or inflammatory gastrointestinal disease. J. Am. Vet. Med. Assoc. **24,** 1467-1474.

Eggleston, R.B. and Mueller, P.O.E. (2003) Prevention and treatment of gastrointestinal adhesions. *Veterinary Clinics of North America - Equine Practice* **19**, 741-763.

Fernandez, I.E. and Eickelberg, O. (2012) The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. *Proc Am Thorac Soc* **9**, 111-116.

Chegini, N., Kotseos, K., Zhao, Bennett, B., McLean, F.W., Diamond, M.P., Holmdahl, L.and Burns J. (2001) Differential expression of TGF-beta1 and TGF-beta3 in serosal tissues of human intraperitoneal organs and peritoneal adhesions. *Hum. Reprod.* **16**, 1291-1300.

Falk, P., Bergström, M., Palmgren, I., Holmdahl, L., Breimer, M.E. and Ivarsson, M.L. (2009) Studies of TGF-beta(1-3) in serosal fluid during abdominal surgery and their effect on in

vitro human mesothelial cell proliferation. *J. Surg. Res.* 154, 312-316.

Falk, P., Angenete, E., Bergström, M. and Ivarsson, M.L. (2013) TGF-β1 promotes transition of mesothelial cells into fibroblast phenotype in response to peritoneal injury in a cell culture model. *Int J Surgery* **11**, 977-982.

Fortenberry, Y.M. (2013) Plasminogen activator inhibitor-1 inhibitors: A patent review (2006-present). *Expert Opinion on Therapeutic Patents* **23**, 801-815.

Ghellai, A.M., Stucchi, A.F., Chegini, N., Ma, C., Andry, C.D., Kaseta, J.M., Burns, J.W., Skinner, K.C. and Becker, J,M. (2000) Role of transforming growth factor beta-1 in peritonitis-induced adhesions. *J. Gastrointest. Surg.* **4**, 316-323.

Goedde, M., Sitter, T., Schiffl, H., Bechtel, U., Schramm, W., Spannagl, M., (1997). Coagulation and fibrinolysis-related antigens in plasma and dialysate of CAPD patients. Perit. Dial. Int. 17, 162-166.

Gorvy, D.A., Herrick, S.E., Shah, M. and Ferguson, M.W. (2005) Experimental manipulation of transforming growth factor-beta isoforms significantly affects adhesion formation in a murine surgical model. *Am. J. Pathol.* **167**, 1005-1019.

Gorvy, D.A., Edwards, G.B. and Proudman, C.J. (2008) Intra-abdominal adhesions in horses: a retrospective evaluation of repeat laparotomy in 99 horses with acute gastrointestinal disease. *Vet. J.* **175**, 194-201.

Ho-Dac-Pannekeet, M.M. (1998) Peritoneal fluid markers of mesothelial cells and function. *Advances in Renal Replacement Therapy* **5**, 205-211.

Holmdahl, L., Kotseos, K., Bergström, M., Falk, P., Ivarsson, M.L. and Chegini, N. (2001) Overproduction of transforming growth factor-beta1 (TGF-beta1) is associated with adhesion formation and peritoneal fibrinolysis impairment. *Surgery* **129**, 626-632.

Imudia, A.N., Kumar, S., Saed, G.M. and Diamond, M.P. (2008) Pathogenesis of intra-abdominal and pelvic adhesion development. *Seminars in Reproductive Medicine* **26**,

289-297.

Javelaud, D. and Mauviel, A. (2004) Mammalian transforming growth factor-betas: Smad signaling and physio-pathological roles. *Int. J. Biochem. Cell Biol.* **36**, 1161-1165.

Jenkins, G., 2008. The role of proteases in transforming growth factor-β activation. The International Journal of Biochemistry & Cell Biology **40,** 1068-1078.

Jeong Seon, R. and Hong Lyeol, L. (1998) Coagulation and fibrinolysis in exudative pleural effusions. *Tuberculosis and Respiratory Diseases* **45**, 1214-1222.

Jiang, C.G., Lv, L., Liu, F.R., Wang, Z.N., Na, D., Li, F., Li, J.B., Sun, Z. and Xu, H.M. (2013) Connective tissue growth factor is a positive regulator of epithelial-mesenchymal transition and promotes the adhesion with gastric cancer cells in human peritoneal mesothelial cells. *Cytokine* **61**, 173-180.

Kamato, D., Burch, M.L., Piva, T.J., Rezaei, H.B., Rostam, M.A., Xu, S., Zheng, W., Little, P.J. and Osman, N. (2013) Transforming growth factor-β signalling: Role and consequences of Smad linker region phosphorylation. *Cellular Signalling* **25**, 2017-2024.

Ksiazandek, K. (2013) Mesothelial cell: A multifaceted model of aging. *Age Res Rev* **12**, 595-604.

Laun, R.A., Schröder, O., Schoppnies, M., Röher, H.D., Ekkernkamp, A. and Schulte, K.M. (2003) Transforming growth factor-beta1 and major trauma: time-dependent association with hepatic and renal insufficiency. *Shock* **19**, 16-23.

Lippitz, B.E. (2013) Cytokine patterns in patients with cancer: a systematic review. *The Lancet Oncology* **14**, e218-e228.

Lopes, M.A.F., Salter, C.E., Vandenplas, M.L., Berghaus, R., Hurley, D.J., Moore, J.N., 2010. Expression of genes associated with inflammation induced by ex vivo exposure to lipopolysaccharide in peripheral blood leukocytes from horses with gastrointestinal

disease. *Am. J. Vet. Res.* **71**, 1162-1169.

Milne, E.M., Doxey, D.L., Gilmour, J.S. (1990) Analysis of peritoneal fluid as a diagnostic aid in grass sickness (equine dysautonomia). *Vet. Rec.* **127**, 162-165.

Mirshafiey, A. and Mohsenzadegan, M. (2009). TGF-beta as a promising option in the treatment of multiple sclerosis. *Neuropharmacology* **56**, 929-936.

Oh, S.A. and Li, M.O. (2013) TGF-β: Guardian of T cell function. *Journal of Immunology* **191**, 3973-3979.

Nieto, J.E., Macdonald, M.H., Braim, A.E.P., Aleman, M., 2009. Effect of lipopolysaccharide infusion on gene expression of inflammatory cytokines in normal horses in vivo. Equine Vet. J. **41**, 717-719.

Pachot, A., Monneret, G., Voirin, N., Leissner, P., Venet, F., Bohé, J., Payen, D., Bienvenu, J., Mougin, B. and Lepape, A. (2005) Longitudinal study of cytokine and immune transcription factor mRNA expression in septic shock. *Clin. Immunol.* **114**, 61-69.

Penha-Goncalves, M.N., Onions, D.E. and Nicolson, L. (1997) Cloning and sequencing of equine transforming growth factor beta 1 (TGF-b1) cDNA. *DNA Seq.* **7**, 375–378.

Ranashinge AM, Bonsers RS (2010) Biomarkers in acute aortic dissection and other aortic syndromes *J Am Coll Cardiol 2; 56 (19)*

Regateiro, F.S., Howie, D., Cobbold, S.P. and Waldmann, H. (2011) TGF-beta in transplantation tolerance. *Curr Opin Immunol* **23**, 660-669.

Rougier, J.P., Guia, S., Hagege, J., Nguyen, G. and Ronco, P.M. (1998) PAI-1 secretion and matrix deposition in human peritoneal mesothelial cell cultures: Transcriptional regulation by TGF-beta 1. *Kidney Inter.* **54**, 87-98.

Sánchez-Muñoz, F., Domínguez-López, A. and Yamamoto-Furusho, J.K. (2008) Role of cytokines in inflammatory bowel disease. *World J. Gastroenterol.* **14**, 4280-4288.

Shah, M., Foreman, D.M. and Ferguson, M.W. (1995) Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J. Cell Sci*. **108**, 985-1002.

Song, K.S., Lee, A., Park, Q.E., Lee, S.M., Kwon, O.H., 1996. The relationship between cytokine concentrations and hemostatic abnormalities in patients with liver cirrhosis of postviral or cryptogenic origin. Fibrinolysis 10, 249-254

Ward, N.S., Casserly, B., Ayala, A. (2008) The Compensatory Anti-inflammatory response syndrome (CARS) in critically III patients. *Clin. Chest Med.* **29**, 617–625.

Watts, A.E., Fubini, S.L., Todhunter, R.J., Brooks, M.B., 2011. Comparison of plasma and peritoneal indices of fibrinolysis between foals and adult horses with and without colic. Am. J. Vet. Res. 72, 1535-1540

Werners, A.H., Bull, S. and Fink-Gremmels, J. (2005) Endotoxaemia: a review with implications for the horse. *Equine Vet. J.* **37**, 371-383.

Yung, S. and Chan, T.M. (2012) Pathophysiological changes to the peritoneal membrane during PD-related peritonitis: The role of mesothelial cells. *Med Inflamm* **2012**.

Yung, S., Jiang, N. and Chan, T.M. (2013) Peritoneal fibrosis and the putative role of decorin. *Hong Kong J Nephrol* **15**, 55-61.

Zeillemaker, A.M., Diepersloot, R.J.A. and Leguit, P. (1999) Mesothelial cell activation by micro-organisms. *Sepsis* **3**, 285-291.

General summary

Background: In humans, peritoneal transforming growth factor beta (TGF-β) is associated with peritoneal diseases and subsequent adhesion formation. No studies on plasma and peritoneal TGF-β concentrations in horses with colic are available. On the other hand horses with severe colic or with altered peritoneal fluid at admission, as well as non-survivors, will have greater increases in plasma and peritoneal D-dimer. *Aims:* The aims of this thesis were 1) To determine both plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations in horses with different types of colic (not previously subjected to abdominal surgery), 2) to compare these concentrations according to the type of peritoneal fluid (transudate, modified transudate and exudate), 3) to compare and correlate plasma and peritoneal concentrations of TGF-β_1 and TGF-β_3 and the types of peritoneal fluid according to the colic group and outcome and 4) to describe and to assess the correlation between TGF-β_1 and D-dimer. *Methods:* Two clinical studies were performed for developing these objectives. *Study 1:* Peritoneal fluid and plasma samples from 78 horses with colic and 8 healthy horses were obtained. Patients were classified according to diagnosis (obstructions, enteritis, ischemic disorders and peritonitis), peritoneal fluid analysis (transudate, modified transudate and exudate), and outcome (survivors and non-survivors). Plasma and peritoneal TGF-β_1 and TGF-β_3 concentrations were determined by ELISA. Data were analyzed by parametric an on parametric tests. A $P \leq 0.05$ was considered as statistical significant. *Results:* Concentrations of peritoneal fluid TGF-β_1 were significantly (P=0.01) higher in horses with peritonitis in comparison with all other colic groups and controls. Horses with ischemic lesions had significantly (P=0.01) higher concentrations of peritoneal TGF-β_1 in comparison with controls and the group of horses with obstructions. Peritoneal TGF-β_1 concentration also was significantly (P=0.01) higher in exudates in comparison with transudates. Peritoneal TGF-β_1 and TGF-β_3

concentrations and plasma TGF-β_1 concentration were significantly increased in non-survivors compared to survivors (P=0.001, P=0.004 and P=0.05, respectively). *Study two:* 124 horses with colic and 12 control horses were included. The horses with colic were grouped according to diagnosis (obstructions, enteritis, ischemic problems, peritonitis and other mixed problems), results of the peritoneal fluid analysis (transudate, modified transudate and exudate) and outcome (survivors and non-survivors). Plasma and peritoneal D-dimer concentrations and peritoneal TGF-β_1 concentrations were significantly higher in horses with enteritis, peritonitis and ischemic disorders, when compared to horses with large intestinal obstructions. Peritoneal D-dimer and TGF-β_1 concentrations were significantly higher in horses with altered peritoneal fluid (modified transudate and exudate) compared to horses with normal peritoneal fluid analysis. The concentration of these proteins was also significantly higher in the peritonitis group and in horses with altered peritoneal fluid. Peritoneal and plasma D-dimer concentrations and TGF-β_1 were also significantly higher in non-survivors. There were significant correlations, from weak to strong, between the evaluated proteins and the diagnosis, the outcome and the type of peritoneal fluid. *Conclusions:* Peritoneal TGF-β_1 and D-dimer concentration was higher in horses with severe gastrointestinal diseases (ischemic intestinal lesions and peritonitis), in horses with an altered peritoneal fluid (exudate), and in non-survivors. Peritoneal TGF-β_1 concentration increases in horses with severe gastrointestinal disease as an anti-inflammatory response and could be correlated with alterations in the fibrinolytic system mainly manifested by increasing in the plasma and peritoneal D-dimer concentrations.

Key words: equine, gastrointestinal disease, D-dimer, transforming growth factor beta-1

Resumen general

Antecedentes: En los seres humanos, el factor de crecimiento transformante beta peritoneal (TGF-β) se asocia con enfermedades peritoneales y la posterior formación de adherencias. No hay estudios sobre las concentraciones de TGF-β peritoneal en caballos con cólico plasma. Por otra parte, caballos con cólicos graves o con líquido peritoneal alterado al ingreso, así como los no sobrevivientes, tendrán mayores aumentos en el plasma y peritoneal de D-dímero. *Objetivos:* Los objetivos de esta tesis fueron: 1) Determinar las concentraciones en plasma y líquido peritoneal de TGF-β1 y TGF-β 3 en caballos con diferentes tipos de cólicos (no sometidas previamente a cirugía abdominal), 2) comparar estas concentraciones de acuerdo con el tipo de fluido peritoneal (trasudado, trasudado modificado y exudado), 3) comparar y correlacionar las concentraciones plasmáticas y peritoneales de TGF-β 1 y TGF-β3 y los tipos de líquido peritoneal según el grupo de los cólicos y la evolución clínica y 4) describir y evaluar la correlación entre TGF-β 1 y D- dímero. *Métodos:* Dos estudios clínicos se llevaron a cabo para el desarrollo de estos objetivos. *Estudio 1:* se obtuvo líquido peritoneal y plasma de 78 caballos con cólico y 8 caballos sanos. Los pacientes fueron clasificados de acuerdo al diagnóstico (obstrucciones, enteritis, trastornos isquémicos y peritonitis), el análisis del líquido peritoneal (trasudado, trasudado modificado y exudado), y el resultado (sobrevivientes y no sobrevivientes). Las concentraciones plasmáticas y peritoneales de TGF-β1 y TGF-β3 se determinaron por ELISA. Los datos se analizaron por pruebas paramétricas y no paramétricas. Un valor de $P \leq 0,05$ fue considerado estadísticamente significativo. *Resultados:* Las concentraciones de líquido peritoneal de TGF-β1 fueron significativamente (P= 0,01) más altas en caballos con peritonitis en comparación con todos los otros grupos de cólico y controles. Los caballos con lesiones isquémicas tuvieron significativamente (P= 0,01) mayores concentraciones peritoneales de TGF-β1 en comparación con los controles y el grupo de caballos con obstrucciones. La concentración

peritoneal de TGF-β1 también fue significativamente (P= 0,01) más alta en exudados en comparación con trasudados. Las concentraciones peritoneales de TGF -β1 y TGF-β3 y la concentración plasmática de TGF-β1 aumentaron significativamente en los caballos no sobrevivientes en comparación con los sobrevivientes (p= 0,001, P = 0,004 y P = 0,05, respectivamente). *Estudio dos :* se incluyeron 124 caballos con cólico y 12 caballos como grupo control. Los caballos con cólico fueron agrupados según el diagnóstico (obstrucciones, enteritis, problemas isquémicos, peritonitis y otros problemas mixtos), los resultados del análisis del líquido peritoneal (trasudado, trasudado modificado y exudado) y la evolución clínica (sobrevivientes y no sobrevivientes). Las concentraciones plasmáticas y peritoneales de D -dímero y las concentraciones peritoneales de TGF–β1 fueron significativamente mayores en los caballos con la enteritis, peritonitis y trastornos isquémicos en comparación con los caballos con obstrucciones intestinales. Las concentraciones peritoneales de dímero D y TGF-β1 fueron significativamente mayores en los caballos con líquido peritoneal alterado (trasudado modificado y exudado) en comparación con los caballos con el análisis normal de líquido peritoneal. La concentración de estas proteínas también fue significativamente mayor en el grupo con peritonitis y en caballos con líquido peritoneal alterado. Las concentraciones peritoneales y plasmáticas de dímero D en y TGF-β1 fueron mayores en los caballos no sobrevivientes. Se encontraron correlaciones significativas, entre débiles y fuertes, entre las proteínas evaluadas y el diagnóstico, la evolución clínica y el tipo de líquido peritoneal. *Conclusiones:* La concentración peritoneal de TGF-β1 y de dímero D fue mayor en los caballos con enfermedades gastrointestinales graves (lesiones isquémicas intestinales y peritonitis), en caballos con un fluido peritoneal alterado (exudado) y en los no sobrevivientes. La concentración peritoneal de TGF-β1 aumenta en caballos con enfermedad gastrointestinal grave como una respuesta anti-inflamatoria y podría estar correlacionado con alteraciones en el sistema fibrinolítico, manifestado principalmente por el aumento en las

concentraciones plasmáticas y peritoneales de D-dímero.

Palabras c lave: enfermedad ga strointestinal, e quino, dí mero D , f actor de c recimiento transformante beta- 1