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Expression of matrix metalloproteases-2 and -9 in horse hoof laminae after intestinal obstruction, with or without Hydrocortisone treatment

Expressão de metaloproteinases 2 e 9 no tecido laminar do casco de equinos após obstrução intestinal e tratamento com hidrocortisona

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ABSTRACT

Twenty horses were used in the experiment, for composed control group, (Cg) instrumented group, (Ig;without intestinal obstruction), treated group (Tg;submitted to intestinal obstruction and hydrocortisone treatment) and non-treated group (Ntg;submitted to intestinal obstruction without treatment). Immunohistochemistry and zymography techniques were used for researches on MMPs 2 and 9 in horse hoof laminae. There was an increase in the expression of MMP-2 in animals of Tg and Ntg. MMP-9 increased on animals from groups Ntg and Ig, however there was no rise of this MMP on the Tg when compared to the other groups in the immunohistochemistry analysis. Based on the results, it was observed that the intestinal injury caused by enterotomy and intestinal obstruction raise the quantities of MMPs in the hoof laminae.

Key words: *laminitis, metalloprotease, horse, zymography, immunohistochemistry.*

RESUMO

Vinte cavalos foram usados no experimento: para compor o grupo controle (Cg), grupo instrumentado, Ig (sem obstrução intestinal), grupo tratado, Tg (submetidos à obstrução intestinal e tratamento com hidrocortisona) e grupo não tratado, Ntg (submetidos à obstrução intestinal, sem tratamento. Técnicas de zimografia e imunoistoquímica foram utilizadas para pesquisa de MMP-2 e MMP-9 no tecido laminar do casco dos equinos. Houve um aumento na expressão de MMP-2 nos animais dos grupos Tg e Ntg. A MMP-9 aumentou nos animais dos grupos Ig e Ntg. Houve aumento desta MMP no Tg quando comparado aos demais grupos na análise por zimografia. Observou-se que a injúria intestinal, causada pela enterotomia e obstrução intestinal, eleva a quantidade de MMPs no tecido laminar do casco.

Palavras-chave: *laminite, metaloproteinases, equinos, zimografia, imunoistoquímica.*

INTRODUCTION

It is believed that the pathophisiology of laminitis is related to high degradation of the basement membrane (BM), caused by metalloproteases (MMPs), and/or loss of hemidesmosomes present in the secondary epidermal laminae. There are two distinct gelatinases of the MMPs family, MMP-2 and MMP-9, which are also found as proenzymes, produced by basal cells for physiologic control of hoof growth (FRENCH & POLLITT, 2004). It has been demonstrated that the concentration of these substances increases, substantially, 48 hours after laminitis induction through carbohydrate administration (KYAW-TANNER & POLLITT, 2004), and that MMP-9 seems to be better associated to inflammatory processes, since it is activated by proinflammatory cytokines and released by leukocytes, while MMP-2 is found in large quantities on normal tissues (KYAW-TANNER & POLLITT, 2004, LOFTUS et al., 2006).

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Inflammatory mediators involvement on laminitis development phase has been recently demonstrated (LOFTUS et al., 2007; NOSCHKA et al., 2009). Therefore, anti-inflammatory administration could effectively be a preventive treatment to avoid laminae injuries, once its use reduces or suppresses inflammatory activation during experimental endotoxemia (CAMPEBELL et al., 2007). The use of corticoids has initially been discouraged, since laminitis occurrence in animals needing high and/or repeated doses of these medicines is very often (POLLITT, 1999).

Considering that horses with gastrointestinal disorders have a higher tendency to develop laminitis (PARSONS, 2007), and that MMPs are positively related to the development of the disease, this work aimed at researching MMPs 2 and 9 concentrations on hoof laminae of horses submitted to experimental intestinal obstruction, and hydrocortisone treatment.

MATERIALS AND METHODS

Animals*:* Laminae fragments of twenty healthy, adult, no defined breed, male or female, horses with no locomotor deficiencies were used. Control group (*Cg*) was composed of seven animals coming from commercial slaughter (Abatedouro e Frigorífico Pomar - Pomar Slaughterhouse - Araguari, Minas Gerais, Brazil) and the other groups were composed of animals used in partnership with another experimental work. These experimental groups were randomly distributed: instrumented group (*Ig*), with five animals, whose surgical procedure involved bowels loops manipulation to locate the segment where jejunum incision was made to set the latex balloon, without distending it; treated group (*Tg*), composed of four animals that were submitted to enterotomy for latex balloon set and insufflation, producing jejunal obstruction, and single intravenous administration of hydrocortisone (Solu-Cortef, Rhodia Farma) in the dose of 4mg kg^{-1}; and finally, non-treated group (*Ntg*), composed of four animals, submitted to balloon jejunal distension, without any treatment.

Experimental design*:* Before surgical procedure, animals were submitted to hydrous and food fasting. They were sedated intravenously with xylazine 10% (Sedazine, Fort Dodge) and butorphanol (Torbugesic, Fort Dodge). Local anesthesia was given using the inverted “L” technique, with lidocaine 2% (Xilestesin, Cristália), in which the middle third of the jejunum was exposed through left paracostal fossa, in standing position, and submitted to enterotomy followed by intestinal wall compression, upon latex balloon implantation on the lumen (FALEIROS et al., 2002). The balloon was filled with air, until it reached 12mmHg in pressure, and removed after four hours of intestinal obstruction. After 18 hours of circulatory reperfusion, animal were submitted to anesthetic overdose euthanasia (Thiopentax, Cristália).

Hoof laminae extraction*:* After metacarpophalangeal dislocation of both forelimbs with a cutting instrument, the hoof was sectioned in median plane (POLLITT, 1996), removing laminae fragments using tweezers and scalpel blade. Fragments were put in formaldehyde at 10%, buffered with PBS pH 7.6, for 24 hours, dehydrated in alcohol and inserted in paraffin, and also frozen through immersion in liquid nitrogen and packed in a freezer at -70°C (-94°F).

Immunohistochemistry in Metalloproteases (MMPs) research*:* Laminae were cut 5µm thick, dew axed in oven at 60ºC (140°F) (1 hour) and hydrated with xylol and alcohol bathes, in decreasing concentrations. Citrate buffer (pH 6.0) was used for antigenic recovery, and endogenous peroxidase block was made with commercial blocker (TA-060-HP, Hydrogen Peroxidase Block, Lab Vision). Cuts were incubated at room temperature with primary antibody anti-MMP-2 (AB-19 – clone CA-4001, Lab Vision, Fremont – CA, USA), developed in a mouse for 1 hour, at 1:100 dilution. Primary antibody anti-MMP-9 (SC-6840, Santa Cruz Biotechnology, Santa Cruz – CA, USA) was developed in a goat, incubated within the same conditions, at 1:200 dilution. Streptavidin-biotin complex (LSAB,K0690, Dako) was the method used, having diaminobenzidine (K3468, Dako) as chromogen. Harris' Hematoxylin was used in the counterstaining. Canine mammal tissue was used as positive control for both antibodies, and in the negative control, the primary antibody was omitted on the tissue, incubating only the antibody diluents. Immunostaining was classified through scores of staining intensity (marking), varying from 0 (attenuated), 1 (light), 2 (moderate) and 3 (intense). Means of the results between groups were obtained afterwards.

Zymography*:* Frozen laminae fragments were crushed for protein extraction in buffer with 50mM Tris-HCl pH 7.4; 0.2M NaCl; 0.1% Triton; 10mM CaCl2 and 1% protease inhibitor cocktail (Sigma Chemical Company, Sant Louis – MO, USA). Protein quantification was carried out according to BRADFORD (1976), using standard curve of Bovine serum albumin (BSA). Zymography for MMP-2 and -9 activity analysis was carried out in polyacrylamide gel 10% with gelatin 0.1%. Approximately 20µg of protein per sample were applied on gel. After electrophoresis, gel was washed at room temperature with 2.5% Triton

X-100, incubated overnight (approximately 15 hours) in buffer with 50mM Tris-HCl, pH 7.4 with 0.1 M NaCl and 0.03% sodium azide, at 37ºC (98.6°F). Gel was stained with Coomassie Brilliant blue dye. Bands corresponding to gelatinolytic activity could be observed after washing with a solution containing methanol (30%) and acetic acid (10%). Gels were evaluated by bands densitometry through Scion Image software.

Statistical method*:* The Kruskal Wallis test was the statistical method used to evaluate MMP-2 and MMP-9 immunostaining, and Dunn's multiple comparison test (P<0.05) was used to determine the difference. Enzymes obtained through zymography were evaluated by analysis of variance in completely randomized design, and difference was detected by Tukey's test (P<0.05). Data comparison between groups was done by the means of results obtained on left and right forelimbs.

RESULTS

It was possible to observe, through immunohistochemistry, marking of both Metalloproteases, 2 and 9, which was found on the cytoplasm of basement cells (CB) that occurs on the secondary epidermal laminae (SEL). Therefore, observed marking was uniform, filling SEL extension. Most animals of all experimental groups showed positive marking of the hoof laminae, which presented differences related to marking intensity, varying from score 0 or attenuated, to score 3 or intense (Figure 1).

Regarding MMP-2 behavior, the enzyme showed a positive marking in most of stained laminae (Figure 1). Animals of control group (Cg) showed attenuated marking predominance when compared to animals of the obstructed groups treated and non-treated (Tg and Ntg), which had more intense scores. Nevertheless, the comparison with animals that were submitted to surgical procedure without intestinal obstruction (Ig) did not present increase, thus showing resemblance to the control group. MMP-9 also presented positive marking in all animals, however it did not show attenuated marking, varying from scores 1 to 3. Control group had many animals with light intensity, present in over 80% of this group's laminae. Such behavior was similar to MMP-2, noting positive marking of lower intensity in animals of control group (Cg) compared to obstructed non-treated group (Ntg). Nonetheless, control group also differed significantly from instrumented group (Ig), submitted to enterotomy, which had moderated to strong predominant marking. Treated group did not show any difference in immunostaining intensity when compared to either control group or the other experimental groups, showing several stained laminae with light and moderated intensity.

Zymography data showed the presence of MMP-2 and -9 activity in the laminae of all animals of the experimental groups. This enzymatic activity was particularly high on animals of instrumented group (Ig), treated group (Tg) and non-treated group (Ntg) compared to animals from control group (Cg). Although all three isoforms of MMP-2 could be identified, individualized qualification of latent and intermediate forms were not possible, having to be evaluated together (MMP-2 L+ I – latent + intermediate). Only active MMP-2 (MMP-2A) could be evaluated, with densitometry of 62kDa. MMP-9 activity was observed in the latent (92kDa – MMP-9L) and active forms (82kDa – MMP-9A). There was a significant increase of enzymatic activity in groups with experimental procedure (Ig, Tg and Ntg) for the variables MMP-2A, MMP-2L + I and MMP-9A (Table 1). MMP-9 latent form did not present difference between experimental groups.

DISCUSSION

Immunohistochemistry and zymography are the main techniques currently used in metalloproteases detection, in studies of various fields of research. Immunohistochemistry analysis detects MMPs tissue expressions, and zymography shows the proteolytic action, and not the antigenicity, of these enzymes. It provides semi-quantitative measures and distinguishes MMPs active and latent forms (proenzyme) (PEREIRA et al., 2006).

Through the results obtained in positively immunostained laminae battery, and those used as negative control, it was concluded that immunostaining was specific for MMPs, being diffuse in CB cytoplasm. Likewise, it was also satisfactory, once there was an increase of marking intensity in animals of the groups submitted to intestinal surgical procedures (Ig, Tg and Ntg), which was expected by the relation between laminitis and gastrointestinal disorders (EADES et al., 2002). These findings are also backed by the enzymatic response of these proteases obtained through zymography analysis.

There was an increase of immunostaining of MMP-2 latent and active form activity on animals of group Tg and Ntg, in which the instrumented group was composed by immunostained MMP-2 intermediate values when compared to control group. It is believed that this group (Ig), composed of animals submitted to

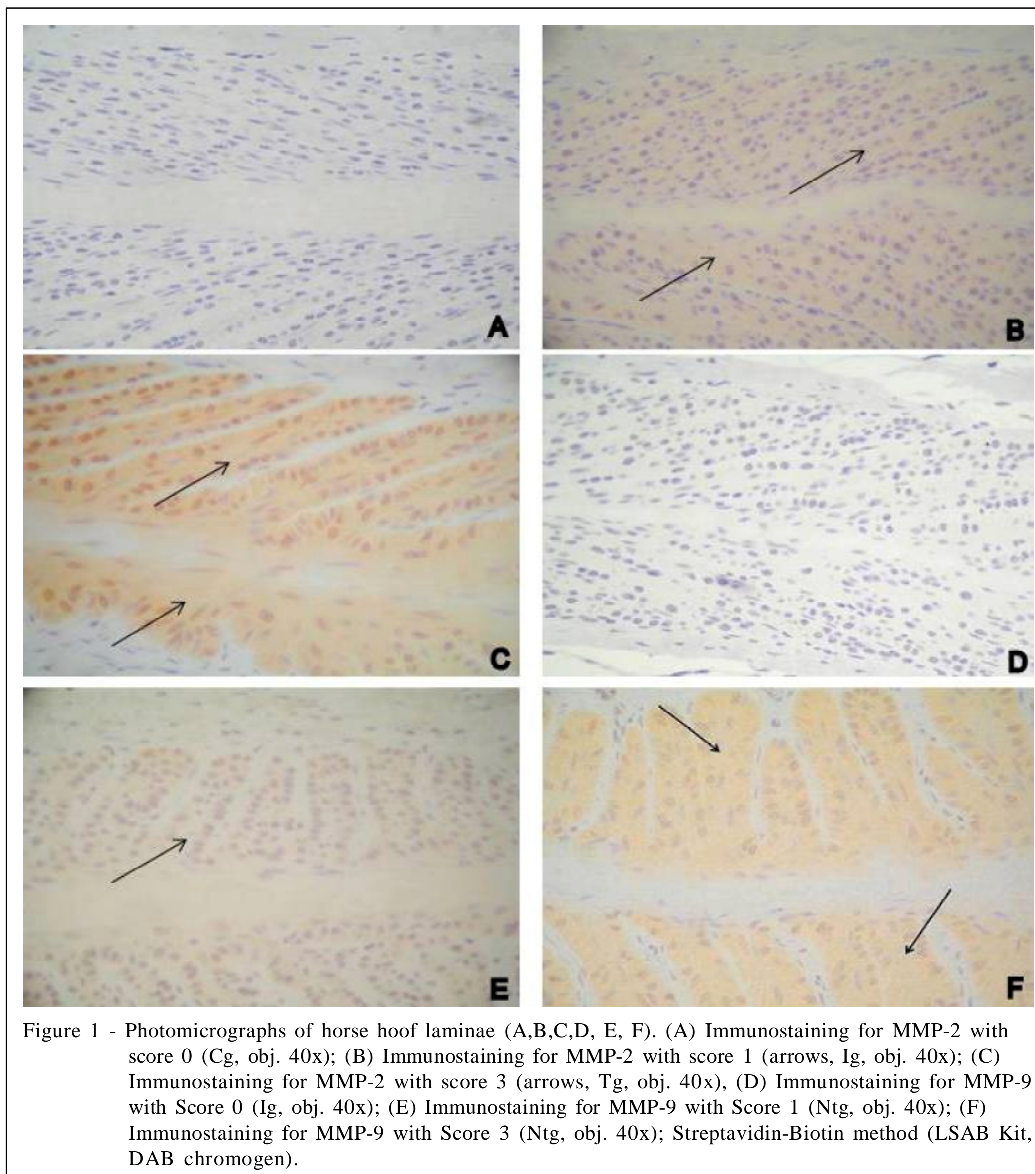

Figure 1 - Photomicrographs of horse hoof laminae (A,B,C,D, E, F). (A) Immunostaining for MMP-2 with score 0 (Cg, obj. 40x); (B) Immunostaining for MMP-2 with score 1 (arrows, Ig, obj. 40x); (C) Immunostaining for MMP-2 with score 3 (arrows, Tg, obj. 40x), (D) Immunostaining for MMP-9 with Score 0 (Ig, obj. 40x); (E) Immunostaining for MMP-9 with Score 1 (Ntg, obj. 40x); (F) Immunostaining for MMP-9 with Score 3 (Ntg, obj. 40x); Streptavidin-Biotin method (LSAB Kit, DAB chromogen).

enterotomy without injuries caused by obstruction in the jejunal wall, might have presented an increase of MMP-2 in virtue of bowel loops manipulation done to enable instrumental procedures, which triggered a systemic inflammatory response release, but in lower intensity compared to animals submitted to jejunal compression. This statement was demonstrated by FUKUDA et al. (2005), who confirmed the presence of inflammatory cytokines released in the laparotomy, specially the prostacyclin, after bowel loops manipulation with consequent reduction of intestinal motility.

It is described in muscular injuries, on the onset of inflammation, an increase of MMP-2 and MMP-9. Nevertheless, during the chronic period of the injury, when tissue regeneration initiates, there is only MMP-9 reduction on the injured tissue, and MMP-2 permanently elevated concentrations (ZIMOWSKA, et al., 2008). Thus, MMP-2 increase on tissue injuries might be related to the tissue regeneration and renewal phase, process that physiologically occurs on injured organic tissues, associating to the fact that this enzyme is present in expressive concentrations in the healthy tissue (VISSER & POLLITT, 2012).

Horses in the prodromal phase of laminitis, both induced and of natural occurrence, show MMP-9 increase on laminae (MUNGALL & POLLITT, 1999; LOFTUS et al., 2006; LOFTUS et al., 2007), found only on smaller quantities in normal animals. The animals of

Table 1 - Average and variability coefficient of MMP-2 and MMP-9, detected by zymograph, between experimental groups (p<0,05), in 10^3 pixels.

VARIATION	*Cg*[1]	*Ig*[1]	*Tg*[1]	*Ntg*[1]	*V.C.*
MMP-2ª*	13 ± 3a	141 ± 85b	89 ± 45b	98 ± 34B	61
MMP-2L+I*	77 ± 9a	635 ± 164b	515 ± 147b	601 ± 99B	27
MMP-9A*	10 ± 3a	67 ± 41b	62 ± 20b	61 ± 17B	50
MMP-9L*	13 ± 7a	71 ± 62a	63 ± 51a	80 ± 55A	92

*Values transformed in log(observation+1);
[1]Values not transformed. Average followed by the same letter in colunms not differ by Tukey test (P<0.05)

this study also presented this response, with MMP-9 elevation in the intestinal injured groups. Some studies describe the increase of MMP-9 latent form (proenzyme) during laminitis development phase (MUNGALL & POLLITT, 1999; LOFTUS et al., 2006; LOFTUS et al., 2009); however, no difference regarding latent MMP-9 in this model was observed. LOFTUS et al. (2006) induced laminitis in horses with black walnut extract and had a considerable increase only of MMP-9 inactive form, demonstrating that some models of laminitis induction probably do not have other components necessary for the activation of this MMP.

As observed by ANNANE & CAVAILLON (2003), glucorticoids raise the survival of human patients with severe sepsis, and the association of this information to ALJADA et al. (2001) results, in which the administration of hydrocortisone reduces MMP-2 and -9 activities, make it possible to believe that its use as laminitis preventive medication in horses might be important, once the several alterations triggered by sepsis are observed in laminitis induction, specially the release of inflammatory mediators. LASKOSKI et al. (2009) observed severe morphological alterations hoof laminar tissue of horses that died due to lethal colic, and the severity of the hoof laminar alterations was associated with the animal general medical condition, mainly leukopenia that probably indicates tissue leukocytes infiltration. FALEIROS et al (2008) described in horses submitted to experimental intestinal obstruction the presence of neutrophils in lung tissue besides hoof laminar tissue alterations, which indicates the occurrence of a systemic inflammatory response . Thus, the glicocorticoid use could have beneficial effects in laminitis prodromal phase, not aggravating the process, as researchers still believe. It was not possible to observe in this study the exact effect of hydrocortisone, because the samples of Tg submitted to immunohistochemistry for MMP-9 analysis did not differ from the other groups. LASKOSKI et al. (2010) found morphological alterations in horse hoof laminar tissue after ischemic and reperfusion syndrome during experimental bowel obstruction. However, the use of hydrocortisone did not permit the aggravation of the alterations in the laminar tissue. Similar results was observed by RIO TINTO et al. (2004), showing that the glicocorticoid use in a single dose could have beneficial effects when used in horses with colic.

Laminitis evolution, until clinical signs appearance, usually varies from 24 to 48 hours in animals with colic syndrome (WHITE, 1990). Thus, maybe the hydrocortisone effect reducing MMP-9 concentrations could be seen in a later phase, considering the fact that injuries could still evolve. Maybe another hydrocortisone administration is necessary, since its half-life is only 8 hours long, therefore smaller than the 18 hours of the circulatory reperfusion used in this study (BEHREND & KEMPPAINEN, 1997). Thereafter, an additional dose of the medication could effectively control the inflammatory reaction triggered by intestinal obstruction.

CONCLUSION

MMP-2 expression is possibly related to the gravity of the morphological alterations of the laminae, due to its increase in animals submitted to intestinal obstruction.

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