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MMP-2 plays an important role during the early acute developmental phase of oligofructose-induced equine laminitis

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Abstract

The study was conducted on 24 Mongolian horses, with oligofructose-induced equine laminitis (10 g/kg b.w.). The objective of the study was to investigate the relationships among matrix metalloproteinase 2 (MMP-2), P38 mitogen-activated protein kinases (P38 MAPK), tissue inhibitor of metalloproteinase 2 (TIMP-2), lipopolysaccharides (LPS), and tumour necrosis factor-α (TNF-α) during acute developmental phase of laminitis, and to determine whether there are any characteristic tendencies. Moreover, plasma concentrations of LPS and TNF-α were measured in order to determine the time of leukocytes' activation. Eleven of the 12 horses showed clinical signs of laminitis. The contents of MMP-2 and P38 MAPK increased significantly from 8 h to 64 h, and the content of TIMP-2 decreased significantly at the same time. Plasma LPS concentrations increased significantly between 8 h and 20 h and reached a peak of 0.024 ± 0.009 EU/mL (equivalent to 3.04 ± 1.19 pg/mL) at 12 h. TNF-α concentration increased between 20 h and 36 h. This data indicates that MMP-2 plays an important role during the early acute developmental phase of oligofructose-induced equine laminitis.

Keywords: horses, laminitis, oligofructose, MMP-2, P38 MAPK.

Introduction

Laminitis affects the structural integrity of the digital lamellar interface and the soft tissue in the equine axial skeleton. Pathological changes induced within the suspensory apparatus of the distal phalanx (SADP) results in foot pain that alters the characteristics of stance and gait (19). If the degradation of SADP is minimal, the animal may recover from the condition of acute laminitis (acute phase of laminitis). However, SADP failure can progress resulting in chronic anatomic changes altering the pattern of hoof horn production (chronic phase of laminitis) (4). For naturally-occurring laminitis, during the early stages of acute phase, the "rotation" of the distal phalanx is in fact a reverse rotation of the hoof in relation to the distal phalanx resulting from compensatory hyperplasia of the epidermal lamellae in an attempt to obtain more nutriment from the basement membrane (8).

Typical experimental models of laminitis include alimentary overload of carbohydrates (starch or oligofructose), black walnut heartwood extract, and intravenous infusion of insulin (2, 7, 27). These models have all been shown to replicate closely the natural causes of laminitis, especially the cause of unusual hind gut fermentation resulting from consumption of excessive grain or lush pasture grasses (11).

In carbohydrates overload models, starch (28) or oligofructose (24) is administered to horses *via* nasogastric intubation. Fructans are rapidly fermented to lactic acid by Gram-negative streptococcal species as they pass into the hindgut (15), causing the caecal pH to decrease precipitously, from approximately 7 to 4, which in turn triggers extensive damage to the epithelium and also increases the permeability of the mucosal barrier (20). Subsequently, death of the Gram-negative bacteria ensues, causing lipopolysaccharides (LPS) concentrations to increase dramatically in the caecal fluid (17). In addition, LPS have been found in the blood of horses following the administration of carbohydrates (1), and it is hypothesised that LPS and other bacterial products translocate into the circulation and initiate systemic inflammation. A number of

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factors have been stated to contribute to the loss of integrity of the hoof-lamellar attachment interface during laminitis, including a loss of hemidesmosomes, inflammation associated with systemic leukocyte activation, neutrophil lamellar infiltration, and upregulation of proteases (24).

Inflammation plays a primary role in the pathogenesis of equine laminitis, which is the result of leukocyte activation and infiltration (6, 24). During the developmental phase of carbohydrate laminitis models, the activation of peripheral leukocytes can increase the expression of hepatic and pulmonary cytokines (23), resulting in the development of laminitis. Inflammation also contributes to peripheral insulin resistance (26), which is a risk factor for laminitis. Interestingly, low levels of LPS are sufficient to activate equine platelets (which are considerably more sensitive to LPS); however, these concentrations may be unlikely to activate equine leukocytes directly (1). Nonetheless, one of the main markers for activated leukocyte is tumour necrosis factor-α (TNF-α) (3) and platelets activate the signalling molecule P38 mitogen-activated protein kinases (P38 MAPK) by the influence of LPS (1).

P38 MAPK is also known to enhance the expression of matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinase 2 (TIMP-2) (9, 21). MMP-2 has a major impact on a number of physiological properties, including angiogenesis and elevated levels of microvascular basement membrane permeability, which contribute to the compensatory hyperplasia of the epidermal lamellae (22).

Previous reports (7, 14) have measured MMP-2 expression at one point-in-time, and could not determine the effects of MMP-2 during the whole acute developmental phase of equine laminitis. In this study circulating MMP-2 was measured continuously, in order to determine the role of MMP-2 in the whole acute developmental phase of oligofructose-induced equine laminitis. We also investigated the impact of P38 MAPK and TIMP-2 on plasma level of MMP-2. Moreover, plasma concentrations of LPS and TNF-α were measured in order to determine the time of leukocyte activation. The aim was to investigate the relationships among MMP-2, P38 MAPK, TIMP-2, LPS, and TNF-α during the acute developmental phase of equine laminitis, and determine whether there are any characteristic tendencies.

Material and Methods

Animals. Twenty-four adult Mongolian horses (12 mares and 12 geldings) with a median body weight of 411 kg (325 kg to 498 kg) and a median age of five years (three to seven years old) were used. Each horse was determined to be healthy (body condition scores of 4-5) (10) and free of digital pathologies, determined by physical and lameness examinations and radiographic evaluation of the distal phalanx. Horses were randomly divided into two groups: control group (n = 12) and experimental group (n = 12). Prior to the experiment, horses were quarantined for two weeks at the Northeast Agricultural University, housed in stalls and fed a regular ration of free choice hay. For 3 d prior to the induction of laminitis, 1 g/kg b.w. of oligofructose (ORAFTI Active Food Ingredients) was added to the basal ration in order to gradually adjust the hindgut microflora to this carbohydrate (12). All experiments were performed in accordance with the Ethical Committee for Animal Experiments (Northeast Agricultural University, Harbin, China).

Oligofructose-induced laminitis. For the experimental group, 10 g/kg b.w. of oligofructose was dissolved in approximately 6 L of water and administered *via* nasogastric tube. Alternatively, 6 L of water was administered *via* nasogastric tube to each horse in the control group. All horses were examined physically (rectal temperature, heart rate, respiratory rate, faecal consistency, and faecal pH), and the grading of lameness was evaluated (horses were walked and trotted in straight lines and walked in circles in each direction on a flat concrete floor). A catheter was fixed in the jugular vein immediately prior to nasogastric tube passage and blood samples were collected. Lameness was assessed according to the Obel lameness grading (25).

Sample collection. Jugular venous blood samples were collected every 4 h into heparinised tubes (Sigma Chemical Company) from 0 h to 72 h, and following each collection, immediately centrifuged at 300 × g for 10 min at 4°C, and the plasma was stored at −80°C until assayed. Each horse was physically examined and lameness evaluation was performed following each of blood collections.

Plasma LPS measurements. Blood was collected at 4 h intervals into heparinised LPS-free glass tubes and centrifuged at 300 × g for 10 min at 4°C, and the plasma was stored at −80°C until assayed. A kinetic limulus amoebocyte lysate assay (Charles River Endosafe) was used to measure LPS in plasma, as previously described (16).

ELISA. Concentrations of MMP-2, TIMP-2, P38 MAPK, and TNF-α were measured by ELISA (Shsaimo), according to the protocol provided by the manufacturer. The ELISA was a double antibody sandwich enzyme-linked immunosorbent specific for horse antibodies. Briefly, each plasma sample was added in duplicate to the microplate, followed by 50 μL of HRP-conjugate and 10 μL of Ab diluent. The microplates were incubated for 1 h at 37°C, washed 5 × in PBS to remove unbound antibodies, proteins, and unbound conjugate. The presence of the immune complexes was revealed by adding 100 μL of chromogen solution to each well and incubating in the dark for 10 min at 37°C. The enzymatic reaction was stopped by adding a solution of 50 μL of H_2SO_4 and subsequently read bi-chromatically at 450 nm. The

conditions of validation described by the manufacturer were complied with the interpretation of the results obtained for different samples. As described by the manufacturer, a standard curve was established by using the optical density (OD) values obtained for each quantification standard (S1–S6). The concentrations were obtained after a direct reading of the standard curve and were expressed as equivalent units per millilitre.

Data analysis. Plasma concentrations of MMP-2, TIMP-2, P38 MAPK, LPS, and TNF-α were analysed using GraphPad Prism 5.1 (GraphPad Software Inc., USA). All data were analysed using one-way ANOVA followed by Tukey's *post hoc* test at every time point. The values were considered to be statistically significant when $P < 0.05$. Data were presented as mean ± standard deviation, unless otherwise noted.

Results

Clinical signs. None of the control horses showed any clinical signs of laminitis throughout the observation period. Alternatively, out of 12 horses administered oligofructose, clinical signs of laminitis were detected in 11 animals. Onset of Obel grade 1 lameness occurred between 8 h and 16 h post-induction and Obel grade 2 lameness was observed between 20 and 60 h. In addition, rectal temperature increased from 37.6 ± 0.2°C at 0 h to a maximum of 39.3 ± 0.8°C at 40 h, mean respiratory rate increased from 16.4 ± 1.6 to 28 ± 2.2 breaths/min, and heart rate increased from 45 ± 2.5 to 65 ± 4.3 bpm at the onset of Obel grade 2 lameness. Faecal pH decreased from 6.49 ± 0.09 to a nadir of 4.37 ± 0.21 at 20 h and all 12 horses in the experimental group developed profuse watery diarrhoea between 12 h and 24 h. All the clinical symptoms improved after 64 h.

The change of indexes in plasma. An increase in concentrations of P38 MAPK (Fig. 1A), MMP-2 (Fig. 1B), LPS (Fig. 2A), and TNF-α (Fig. 2B) was demonstrated and a decrease in TIMP-2 concentration (Fig. 1C) throughout most of the trial was found. More specifically, following Obel grade 1 lameness, the content of MMP-2 ($P < 0.001$), and P38 MAPK ($P < 0.01$) increased significantly at 8 h, levels of TIMP-2 became significantly down-regulated ($P < 0.01$) from 8 to 16 h. Subsequently, following Obel grade 2 lameness, the content of MMP-2 and P38 MAPK increased significantly from the onset until it finally began to decrease towards control levels at 68 h. Interestingly, plasma LPS concentrations increased significantly between 8 h and 20 h, reached a peak of 0.024 ± 0.009 EU/mL (equivalent to 3.04 ± 1.19 pg/mL) at 12 h, while TNF-α (Fig. 2A) increased between 20 and 36 h, reaching its maximum value at 24 h. Reduced concentrations of TIMP-2 were also detected, rebounding to non-significant levels at 20, 32, 36, and 44 h following oligofructose administration.

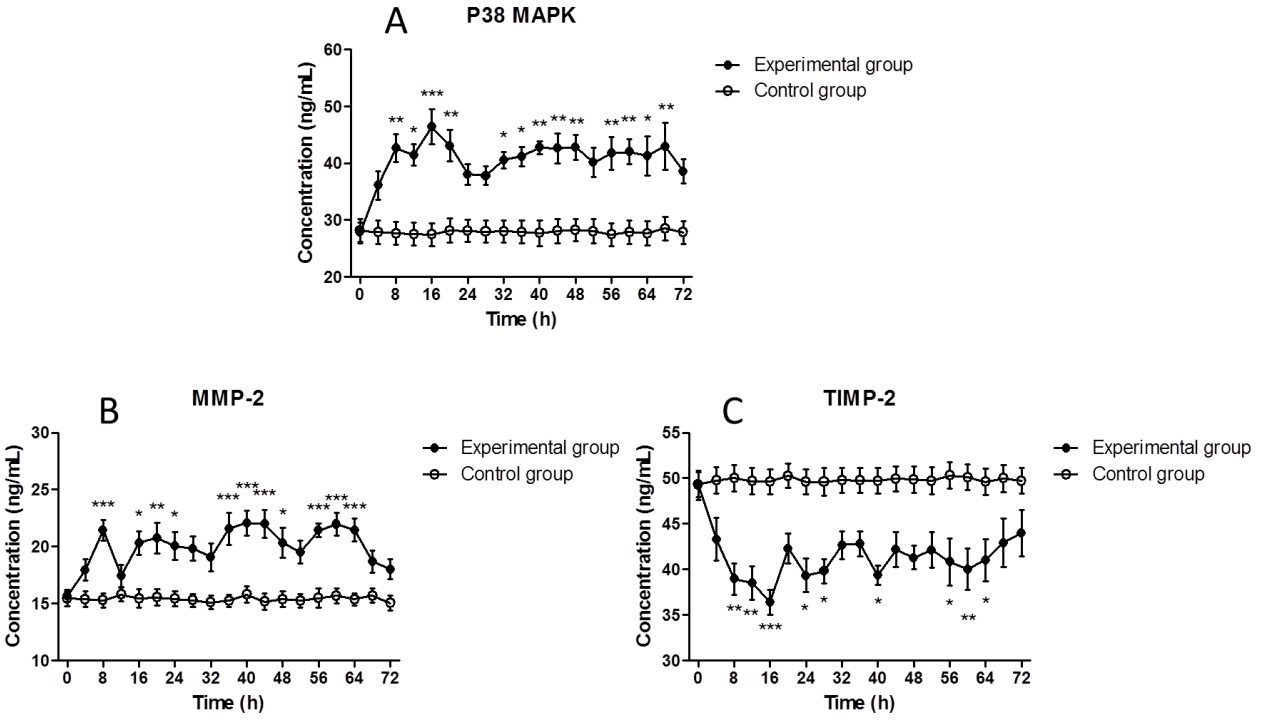


Fig. 1. Change in plasma of P38 MAPK (A), MMP-2 (B), and TIMP-2 (C) in horses following administration of 10 g/kg OF from 0 to 72 h. The data is presented as mean ± standard deviation. * refer to significant time effects on all graphs. * $P < 0.05$ *versus* control group, ** $P < 0.01$ *versus* control group, *** $P < 0.001$ *versus* control group at each time point

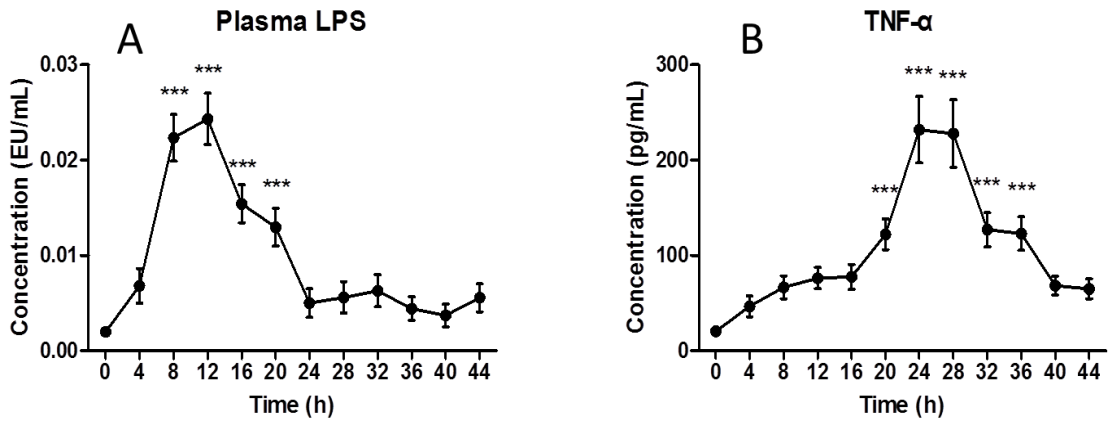


Fig. 2. Change in plasma of TNF-α and LPS in horses following administration of 10 g/kg OF from 0 to 44 h. The content of TNF-α and LPS decrease towards control levels from 44 to 72 h. The data are presented as mean ± standard deviation. * significant time effects on all graphs. * $P < 0.05$ *versus* control group, ** $P < 0.01$ *versus* control group, *** $P < 0.001$ *versus* control group at each time point

Discussion

Results presented in this study show that fluctuating levels of MMP-2 and P38 MAPK are very similar (correlation index is 0.69) and that the activated-MMP inhibitor, TIMP-2, is down-regulated (correlation index is -0.58) during acute laminitis, likely through the well-established mechanism in which pro-MMP-2 and MMP-2 in turn influences the function of TIMP-2 (9, 14). The influence of LPS on platelets includes the activation of the signalling molecule P38 MAPK, and the release of serotonin and thromboxane A_2 (3). Furthermore, P38 MAPK facilitates the activation of MMP-2 (21). At the same time, the 140 kDa fragment of thrombospondin-1 is dependent on P38 MAPK activation and is a potent stimulator of TIMP-2 (9). In the study, it was observed that TIMP-2 rebounded at 20, 32, 36, and 44 h, overlapping quite well with the observed increases in P38 MAPK levels. These results further indicate that the activation process of (pro) MMP-2 is accomplished through the formation of a ternary compound among P38 MAPK, MMP-2, and TIMP-2 (5), and enables to explain the critical role of MMP-2 in the development of laminitis.

Previous reports have demonstrated that MMP-2 regulates vessel formation (22), indicating that MMP-2 plays a significant role in angiogenesis. Furthermore, activation of MMP-2 destroys the extracellular matrix and induces degradation of type IV collagen by collagenase, resulting in elevated levels of microvascular basement membrane permeability and deleterious structural changes (30) resulting from fluid leaking into the spaces created between the dermal and epidermal lamina. On the other hand, MMP-2 is the key protease in fibroblast-mediated collagen contraction (29) and MMP-2 is up-regulated as fibroblasts transition away from a myofibroblast phenotype (13).

We saw an initial peak in MMP-2 level at 8 h, matching with the absorption time of LPS into the circulation. *In vitro* studies suggest that low levels of LPS are unlikely to have a direct role in activating equine leukocytes; however, these concentrations may be sufficient to activate equine platelets (3). In the present study, LPS was detected at much lower concentrations (3.04 ± 1.19 pg/mL) and the threshold for leukocyte activation by LPS using this measure is approximately 10 ng/mL (3), several orders of magnitude greater than that observed in oligofructose-induced laminitis.

TNF-α is produced mainly by leukocytes and is one of the earliest cytokines to become elevated in the plasma during endotoxaemia (18). We found that the onset of Obel grade 1 lameness occurred between 8 and 16 h post induction. Nevertheless, TNF-α only became detectable in the plasma from 20 to 36 h (1). We assumed that LPS may primarily activate platelets, leading indirectly to the activation of leukocytes, and leukocytes were not activated until the 20^{th} h when the content of TNF-α increased significantly; however, all these assumptions need to be further investigated in the future.

Moreover, even though we saw some peak values of MMP-2 at 20, 36, and 44 h; we did not observe a concurrent decrease in TIMP-2 levels. It can be hypothesis that there is a more complex relationship between MMP-2, P38 MAPK, and TIMP-2, than that previously evaluated. Therefore, additional studies on the mechanisms involved in the regulation of TIMP-2 and the effects of P38 MAPK are needed.

In conclusion, MMP-2 plays an important role during the early acute developmental phase of oligofructose-induced laminitis. Moreover, we found that P38 MAPK has a powerful stimulatory effect on laminitis. Finally, this research points towards the potential use of MMP-2 inhibition in the treatment of acute laminitis in horses.

Conflict of Interest Statement: The authors declare that they have no conflicts of interest.

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Animal Rights Statement: In research studies using animal subjects, the trial's design, conduct and reporting of results conform to Good Clinical Practice in Food and Drug Administration (FDA)-Regulated Clinical Trials (USA).

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