

Changes in the levels of MMP-9, MMP-2, and their inhibitors in the serum of dairy cows with laminitis*

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Received 15.02.2022

Accepted 13.03.2022

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Summary

Laminitis is regarded as an important underlying cause of lameness disorders, yet its specific pathogenesis remains unclear. Consistent histological changes in cows with laminitis consist in the degradation of the basement membrane (BM). The breakdown of BM is accomplished by numerous proteases, especially MMP-9 and MMP-2. This study recruited 45 cows according to veterinary diagnostic criteria and divided them into three groups: subclinical laminitis cows (SCL, $n = 15$), chronic laminitis cows (CL, $n = 15$), and healthy cows (CON, $n = 15$). After blood samples were collected from the jugular vein, the serum was separated and frozen at -80°C . The serum samples were analyzed by gelatin zymography and reverse zymography to evaluate the activities of MMP-9, MMP-2, TIMP-1, and TIMP-2. In the SCL group, the activity of MMP-9 significantly increased ($P < 0.01$) and the activity of TIMP-1 significantly decreased ($P < 0.05$), compared with those in the CON group, while the activities of MMP-2 and TIMP-2 were not significantly different. In the CL group, the activities of MMP-9 ($P < 0.001$) and MMP-2 ($P < 0.05$) significantly increased, and the activities of TIMP-1 ($P < 0.01$) and TIMP-2 ($P < 0.05$) significantly decreased compared with those in the CON group. This is the first study to report changes in the content of MMP-2 and MMP-9 and their inhibitors, TIMP-2 and TIMP-1, in the serum of dairy cows with laminitis. These results indicate that inadequate regulation of the activities of MMPs and TIMPs in serum may play a role in the development of laminitis in dairy cows.

Keywords: cow, lameness, laminitis, basement membrane, MMP-2, MMP-9

Lameness is one of the most serious health problems in dairy cattle around the world (7), which significantly affects animal welfare and causes enormous economic losses in the dairy industry. Laminitis in dairy cows is the most common hoof disease, which can be defined as a diffuse, sterile inflammation, located in the hoof wall of the dermal nipple and vascular layer (2). Laminitis in cows is normally divided into clinical laminitis and subclinical laminitis (SCL), whereas clinical laminitis can be divided into acute laminitis, subacute laminitis, and chronic laminitis (CL). SCL and CL have a severe adverse effect on milk production in dairy cows.

SCL can cause bovine hoof bottom bleeding, yellow dyeing, and other symptoms (9). If not diagnosed and promptly treated, it can easily turn into CL. Cows with CL develop hoof deformations and eventually have to be culled. Therefore, early detection and treatment are essential in cows with laminitis.

Matrix metalloproteinases (MMPs) are a zinc-dependent family of proteolytic enzymes that degrade the extracellular matrix (ECM) and have at least 25 members (10). Gelatinase (MMP-2, MMP-9) is one of them (3). The function of the inhibitors of metalloproteinases (TIMPs) is to inhibit the activity of MMPs. TIMP-1 inhibits the activity of MMP-9, and TIMP-2 inhibits the activity of MMP-2 (1, 3). TIMPs inhibit MMP activity by binding to the active site of MMPs with a 1 : 1 stoichiometry (16).

The activity of MMPs in biological samples can be assessed by gelatin zymography. Reverse zymography, developed as a modification of gelatin zymography, makes it possible to detect protease inhibitors. Characterized by simplicity and sensitivity, zymography is widely used to identify proteolytic activity under non-reducing conditions. Although enzyme-linked immunosorbent assays (ELISAs) can quantitatively evaluate the content of MMPs, they do not distinguish between intact molecules, complexes, and degradation products (8).

* This project was supported with funds by the National Key R&D Program of China (Project No. 2017YFD0502200) and Heilongjiang Provincial Funding for National Subjects (GX18B023).

The hoof lamellar basement membrane (BM) belongs to extracellular matrices (ECMs) and its main function is to maintain the integrity of tissue structure, regulate the migration of extracellular molecules, and store a variety of cytokines and growth factors. The principal histological change in horses and bovines with laminitis is the degradations of BM in hoof tissue (15). BM is composed mainly of collagens IV and laminin (22). It has been reported that collagens IV, laminin, and collagens III are destroyed by laminitis, and the degradation of BM is related to gelatinase (14). MMP-2 (Gelatinase A) degrades gelatin, collagen (IV-VI, X), elastin, and fibronectin. MMP-9 (Gelatinase B) degrades gelatin, collagen (IV, V, VII, X, XIV), elastin, fibrillin, and osteonectin (26).

Increased levels of MMP-9 in horses with laminitis can be observed in various biological samples, such as serum, plasma, and hoof tissue (27). However, changes in MMP-2 vary in different studies. Previous research has showed that the levels of MMP-2 in the hoof tissue of horses with laminitis are significantly increased (13), but it has also been reported that there was no significant increase in the levels of MMP-2 in the hoof tissue of horses with laminitis (25). Changes in MMP-9, MMP-2 and TIMP-2 levels have also been found in cattle with hoof tissue ulcers (25). But changes in MMP-2, MMP-9 and their inhibitors in the serum of cows with laminitis remain unclear.

Therefore, the purpose of this study was to explore changes in the activities of MMP-9, MMP-2, and their inhibitors, TIMP-1 and TIMP-2, in cows with subclinical laminitis and chronic laminitis.

Material and methods

Animals. A total of 45 cows were recruited from a large-scale dairy farm in Harbin, Heilongjiang Province. The cows were examined by dairy veterinarians according to SCL and CL standards (7). Diagnostic criteria for SCL were hemorrhage in the hoof, white line, and non-traumatic ulcer (7). Diagnostic criteria for CL were hoof deformation, hoof ball weight, hoof bottom weight is not accurate, and extended hoof (7). The cows were divided into three groups of 15 cows each: healthy cow group (CON), SCL cow group, and CL cow group. All of the three groups of cows were fed with the same feed formula and were provided with free access to fresh water, as described in previous studies (12).

Sample processing. Blood was collected from the jugular vein at the same time every day (from 8 a.m. to 12 a.m.) over a period of seven days. After blood collection, the samples were left to rest for 30 minutes and then centrifuged for 10 min at 4000 rpm at 4°C. Serum was separated from blood and immediately stored at -80°C.

Zymography and reverse zymography. The activities of MMP-9 and MMP-2 were measured by gelatin zymography according to previous reports, (11, 23), and the activities of TIMP-1 and TIMP-2 were measured by reverse zymography (18, 24). Serum was diluted with deionized water at a ratio of 1 : 6. The protein concentration was regulated by the BCA protein assay, then serum was mixed

with a sample buffer of equal volume (50 mM Tris/HCl, 20% glycerol, 4% SDS, 0.005% bromophenol blue). Gelatin zymogram: an equal amount of protein sample was loaded on an 8% polyacrylamide gel (containing 1% bovine gelatin), and electrophoresis was carried out at constant voltage (150 V) at 4°C for 90 minutes. After electrophoresis was completed, the gels were washed 4 times on a shaker with renaturing buffer (2.5% Triton X-100) for 15 minutes every time and then washed twice in the development buffer (50 mM Tris/HCl, 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij35, 1 μM ZnCl₂) for 20 minutes each time. Finally, the gels were soaked in the development buffer and placed in a thermostat at 37°C for 16 h. Reverse zymography was basically the same as the gelatin zymogram, but a 12% polyacrylamide gel (containing 1% bovine gelatin) was used, and recombinant human MMP-2 (C377, Novoprotein technology, China) was added at a concentration of 0.1 μg/mL. After incubation was completed, the gels were dyed with Coomassie blue R-250 (0.25%). This was followed by fading the gels in a decolorization liquid (methanol : acetic acid : distilled water = 5 : 1 : 4), and then the gels were imaged using a Gel 1000 imager system (Sage Creation Science, China). The ImageJ software was used to analyze the grayscale values of the stripe. The activities of MMP-9 and MMP-2 were determined by transparent bands against a dark blue background, while the activities of TIMP-1 and TIMP-2 were determined by dark stripes against a light background.

Statistical analysis. All statistical analyses were performed with SPSS 22.0. All data were analyzed with the Independent-Samples T-Test. The results were presented as mean ± SD, and differences were assumed statistically significant at $P < 0.05$.

Results and discussion

The activity of serum MMP-9 in the SCL group was significantly increased ($P < 0.01$) compared with that in the CON group, while the level of serum MMP-2 in the SCL group ($P = 0.058$) showed no change. In addition, the CL group showed a significant increase in serum MMP-9 ($P < 0.001$), as well as in serum MMP-2 ($P < 0.05$) (Fig. 1).

In reverse zymography analysis, compared with CON group, the level of serum TIMP-1 was significantly reduced ($P < 0.05$), while the level of serum TIMP-2 had no change. In addition, the results of inhibitors in CL group showed significant decrease in the activity of serum TIMP-1 ($P < 0.01$) and a significant decreased in the level of serum TIMP-2 ($P < 0.05$) (Fig. 2).

There is no clear pathogenesis of cow laminitis. Many predisposing factors are being associated with the occurrence of laminitis, including farm management, housing, genetics, breeding, and nutrition. Nutrition is considered to be an important factor. It has been suggested that rumen acidosis is one of the most important factors in the etiology of cow laminitis (5). Although the specific relationship between rumen acidosis and laminitis is hitherto unknown, one possible mechanism may be that as a large amount of lactic acid begins to accumulate excessively in

the rumen, toxic compounds, such as endotoxins or histamine, are released into the circulation and then may directly or indirectly trigger blood reflow obstruction and microcirculation disorders in the corium of the hoof, followed by tissue hypoxia and metabolic

disorders, which results in the occurrence of laminitis (2, 20).

The elasticity of BM is due mainly to collagen IV. It has been reported that collagen IV and laminin of BM can be destroyed to varying degrees during laminitis (11). MMP-9 and MMP-2 can degrade the basement membrane. After the integrity of BM is compromised, the distal phalanx of the hoof may be predisposed to rotation and displacement (19).

Several growth factors and pro-inflammatory cytokines can stimulate neutrophils to release gelatinase which is activated by protein hydrolyzing (4, 21). Although the changes of MMP-9 and MMP-2 activities have been studied extensively in horse laminitis, very little research focus on the role of MMP-9 and MMP-2 in cow laminitis. According to reports, MMP-9 is stored in neutrophil granulocytes and is released once stimulated. The increase in the level of MMP-9 in the serum may be caused mainly by neutrophil granulocytes (17). Once the balance between matrix metalloproteinases and their inhibitors is broken, it can lead to a variety of pathological changes, such as the degradation of BM, which gives rise to laminitis (13).

The content of MMPs is strictly regulated by inhibitors of metalloproteinases (TIMPs) (6, 19). The results of this study also reveal the same trend: in the SCL and CL cow groups, the activity of MMP-9 in serum increased significantly, compared with that in the CON cows, and the inhibitor TIMP-1 decreased significantly, corresponding to the changes in MMP-9. This seems to indicate that MMP-9 plays an important role in the occurrence of laminitis.

However, in contrast with the CON cows, although the content of MMP-2 was significantly increased in the serum of the CL cows, it was not significantly elevated in the SCL cow group. In addition, in comparison with the CON cows, the inhibitor TIMP-2 activity in the SCL cows was not significantly reduced, while the level of the inhibitor TIMP-2 in the CL cows was significantly lower. Previous studies have revealed no

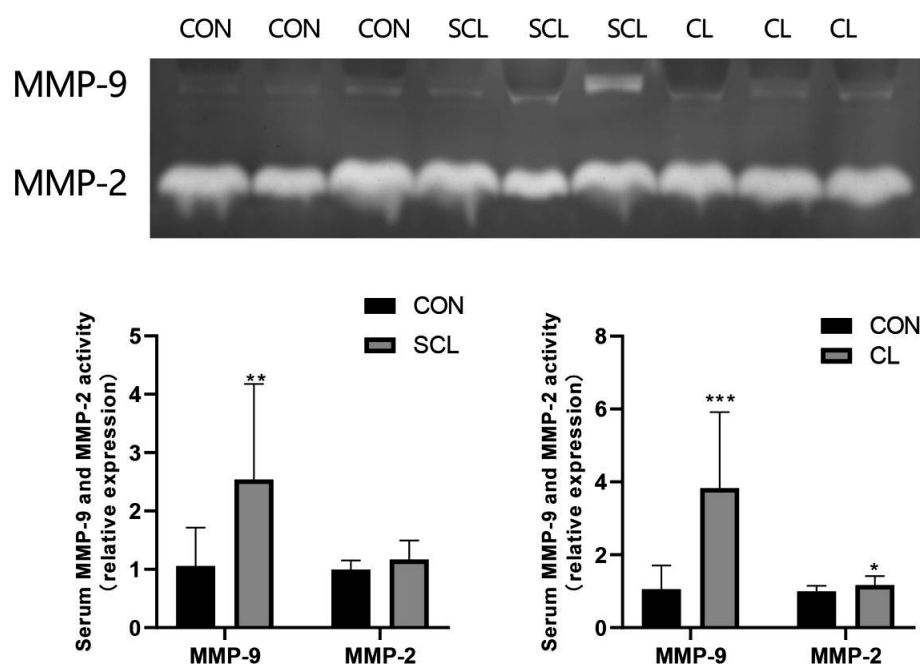


Fig. 1. Representative gelatin zymography and densitometry analysis of MMP-2 and MMP-9 in serum from healthy dairy cows (CON), subclinical laminitis dairy cows (SCL), and chronic laminitis dairy cows (CL)

Explanations: *significantly different ($P < 0.05$); **significantly different ($P < 0.01$); ***significantly different ($P < 0.001$) from CON. (A) Representative gelatin zymography and densitometry analysis of MMP-2 and MMP-9 in serum from healthy dairy cows, subclinical laminitis dairy cows, and chronic laminitis dairy cows. (B) Representative reverse zymography and densitometry analysis of TIMP-1 and TIMP-2 in serum from healthy dairy cows, subclinical laminitis dairy cows, and chronic laminitis dairy cows

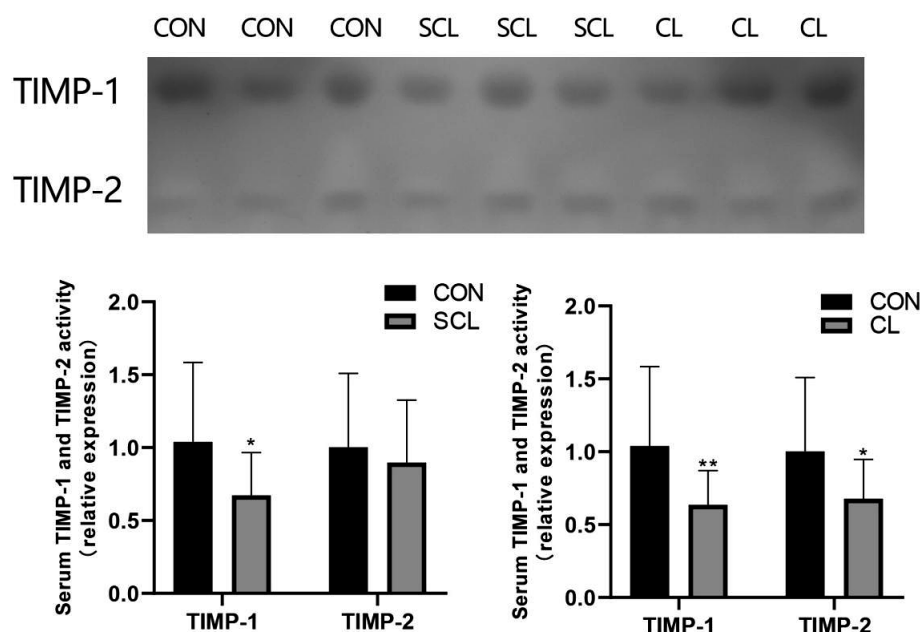


Fig. 2. Representative reverse zymography and densitometry analysis of TIMP-1 and TIMP-2 in serum from healthy dairy cows (CON), subclinical laminitis dairy cows (SCL), and chronic laminitis dairy cows (CL)

Explanations: *Significantly different ($P < 0.05$); **significantly different ($P < 0.01$) from CON

significant increase in MMP-2 levels in the serum, plasma, and hoof tissue of horses with laminitis, possibly due to the regulatory effect of TIMP-2 (27). Therefore, the absence of a significant increase in the content of MMP-2 in the SCL cow group may also be due to the regulatory effect of TIMP-2 in this study.

Furthermore, there were no significant changes in the activities of MMP-2 and TIMP-2 in the SCL cow group in this study. It seems that MMP-2 does not play a major role in SCL cows. This may be caused by the different pathogenesis of subclinical laminitis and chronic laminitis in dairy cows, so it is necessary to further explore that difference.

In summary, the current study found that the level of MMP-9 in the serum of cows with SCL and CL increased significantly and the level of TIMP-1 decreased significantly. In addition, the level of serum MMP-2 in cows with CL was elevated and the level of TIMP-2 was reduced. However, there was no significant change in the levels of serum MMP-2 and TIMP-2 in the SCL cows.

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