Effects of Oligofructose-Induced Acute Ruminal Acidosis on Rumen Fermentation, Hoof Tissue Structure, and Expression of Inflammatory Cytokines and Metalloproteinases in Goats: Post-print

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Abstract

This experiment aimed to investigate the effects of oligofructose-induced rumen acidosis on rumen fermentation, hoof tissue structure, hoof inflammatory factors, and metalloproteinase expression in goats. A randomized block design was adopted, and 8 healthy Boer crossbred goats (Boer goat × Yangtze River Delta white goat) fitted with permanent rumen fistulas were randomly divided into a control group and an acidosis-induced experimental group, with 4 goats in each group. The oligofructose infusion dose for the experimental group goats was 21 g/kg BW. Rumen fluid was collected at pre-infusion (0 h) and at 4, 8, 12, 24, and 48 h post-infusion, while blood was collected via the jugular vein at 0, 4, 8, 24, and 48 h. Goats from both groups were slaughtered at 48 h post-infusion, and hoof tissue was collected. The results showed that: compared with the control group, the experimental group exhibited significantly lower average rumen fluid pH and volatile fatty acid concentrations ($P<0.05$), and significantly higher average concentrations of lactic acid and lipopolysaccharide in blood and rumen fluid ($P<0.05$); histomorphological results revealed that, compared with the control group, the lengths of secondary epidermal hoof lamellae and secondary dermal hoof lamellae in the experimental group goats were shortened, with irregular hoof lamella shape; real-time quantitative PCR detection results demonstrated that, compared with the control group, the relative mRNA expression of tissue inhibitor of metalloproteinases-1 in goat hoof tissue was significantly decreased ($P<0.05$), while the relative mRNA expression levels of interleukin-6, membrane-type matrix metalloproteinase-1, and matrix metalloproteinase-2 were significantly increased ($P<0.05$). These results suggest that oligofructose-induced acute rumen acidosis in goats can cause rumen fermentation disorder, increase lipopolysaccharide and lactic acid concentrations

in rumen fluid and blood, alter the expression of related inflammatory factors and metalloproteinases in goat hoof tissue, and ultimately induce acute laminitis in goats.

Full Text

Effects of Fructo-Oligosaccharide-Induced Ruminal Acute Acidosis on Ruminal Fermentation, Hoof Tissue Structure, and Expression of Inflammatory Cytokines and Matrix Metalloproteinases in Goats

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Abstract

This experiment aimed to investigate the effects of fructo-oligosaccharide-induced ruminal acidosis on ruminal fermentation, hoof tissue structure, and expression of inflammatory cytokines and matrix metalloproteinases in goats. Using a randomized block design, eight healthy crossbred goats (Boer goat × Yangtze River Delta white goat) fitted with permanent ruminal fistulas were randomly divided into a control group and an acidosis-induced treatment group (n=4 per group). The treatment group received an intraruminal infusion of fructo-oligosaccharide at 21 g/kg body weight. Ruminal fluid was collected at 0 (pre-infusion), 4, 8, 12, 24, and 48 h post-infusion, while blood samples were collected via jugular venipuncture at 0, 4, 8, 24, and 48 h. All goats were slaughtered at 48 h post-infusion for hoof tissue collection. The results showed that compared with the control group, the treatment group exhibited significantly lower mean ruminal pH and volatile fatty acid concentrations ($P<0.05$), along with significantly higher mean concentrations of lactic acid and lipopolysaccharide (LPS) in both blood and ruminal fluid ($P<0.05$). Histological examination revealed that compared with the control group, the lengths of secondary epidermal and dermal lamellae were shortened and appeared irregular in shape in the treatment group. Real-time quantitative PCR analysis demonstrated that the treatment group had significantly decreased mRNA expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) ($P<0.05$), while showing significantly increased mRNA expression of interleukin-6 (IL-6), membrane type-1 matrix metalloproteinase (MT1-MMP), and matrix metalloproteinase-2 (MMP-2) in hoof tissues ($P<0.05$). These findings indicate that fructo-oligosaccharide-induced acute ruminal acidosis disrupts ruminal fermentation, elevates LPS and lactic acid concentrations in ruminal fluid and peripheral blood, alters the expression of inflammatory cytokines and matrix metalloproteinases in hoof tissues, and ultimately triggers acute laminitis in

goats.

Keywords: goat; fructo-oligosaccharide; inflammatory cytokines; matrix metalloproteinase; laminitis

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The hoof is a critical support and locomotor organ in ruminants such as cattle and sheep, and its health directly affects animal performance. Laminitis is a diffuse, non-infectious dermal injury affecting the lamellar structures (including dermal and epidermal lamellae) of the hoof [1-2]. Epidemiological surveys indicate that laminitis is a common disease in intensively farmed dairy cows, sheep, and goats [3]. While numerous factors have been implicated in the pathogenesis of laminitis, many researchers consider high-concentrate feeding as one of the primary predisposing factors in herbivores.

Current evidence linking high-concentrate diets to laminitis primarily derives from equine studies. Research has shown that intragastric infusion of fructo-oligosaccharide disrupts hindgut fermentation, damages intestinal mucosal barrier function, and facilitates translocation of abnormal metabolites such as lipopolysaccharide (LPS) into systemic circulation. This process elevates matrix metalloproteinase (MMP) content in hoof tissues. Since MMPs primarily function to degrade extracellular matrix and basement membrane components [4-8], increased MMP activity promotes degradation of these structures, disrupting the lamellar connection between epidermal and dermal tissues and ultimately causing laminitis. However, as these findings originate exclusively from equine research, whether similar mechanisms operate in ruminants such as cattle and sheep remains unclear.

Based on these considerations, we hypothesized that intraruminal infusion of fructo-oligosaccharide in goats would induce acute acidosis and subsequently trigger laminitis. Therefore, this study investigated the effects of fructo-oligosaccharide infusion on ruminal fermentation, hoof tissue structure, and expression of inflammatory cytokines and matrix metalloproteinases in goats, aiming to elucidate the pathogenic mechanisms of laminitis in ruminants and provide a theoretical basis for establishing a low-cost laminitis research model. The experiment was conducted in September 2015 at the animal facility of Nanjing Agricultural University.

1.1 Experimental Animals and Diet

Eight healthy 2-year-old crossbred intact male goats (Boer goat × Yangtze River Delta white goat) weighing approximately 26 kg and fitted with permanent ruminal fistulas were selected for the study. All animals were dewormed and housed individually. The basal diet was formulated according to the "Feeding Standard of Meat-Producing Sheep" (NY/T 816-2004) [9]. Diet composition and nutrient levels are presented in Table 1 . Each goat received 450 g of diet daily, fed in two equal portions at 08:00 and 17:00, with free access to water.

1.2 Experimental Design

At the start of the experiment, the eight goats were randomly allocated to either a control group or a treatment group (n=4 per group). Fructo-oligosaccharide was dissolved in deionized water and infused intraruminally at a dose of 21 g/kg body weight. To allow ruminal microbes to adapt, treatment goats received 5% of the final dose daily for 3 days before the formal experimental period. During the formal period, ruminal fluid was collected at 0 (pre-infusion), 4, 8, 12, 24, and 48 h post-infusion, while blood samples were collected via jugular venipuncture at 0, 4, 8, 24, and 48 h. All goats were slaughtered immediately at 48 h post-infusion.

1.3 Sample Collection

At each sampling time point, 20 mL of ruminal fluid was collected from each goat using a custom-made negative pressure device. The collected fluid was filtered through four layers of cheesecloth, mixed thoroughly in a clean beaker, and immediately analyzed for pH. Subsequently, the filtrate was transferred to centrifuge tubes and stored at -20°C for subsequent analysis of volatile fatty acids, lactic acid, and LPS concentrations. Blood samples were collected from the jugular vein into vacuum tubes, allowed to clot at room temperature for 30 min, then centrifuged at 3,500×g for 10 min. The serum was harvested and stored at -20°C for lactic acid and LPS analysis. At 48 h post-infusion, all goats were slaughtered. Hoof tissues were collected using an electric saw [11] and processed as follows: one portion was immediately snap-frozen in liquid nitrogen, while another was fixed in 4% formalin solution for histological examination of lamellar structures.

1.4.1 Determination of Ruminal Fermentation Parameters

Ruminal pH was measured using a pH meter (HI-9125, HANNA Instruments, Italy). Volatile fatty acid concentrations were determined by gas chromatography (GC-14B, Shimadzu, Japan; column temperature 130°C, injector temperature 180°C, detector temperature 180°C) [13]. Lactic acid concentrations in ruminal fluid and blood were measured using a colorimetric method [14]. LPS concentrations were quantified using a chromogenic limulus amebocyte lysate assay kit (Xiamen Limulus Reagent Experimental Factory) [15].

1.4.2 Tissue Section Processing

Hoof tissues were fixed in 4% paraformaldehyde, then processed through washing, graded alcohol dehydration, paraffin infiltration, embedding, sectioning, mounting, dewaxing, rehydration, hematoxylin-eosin (HE) staining, and coverslipping. The stained sections were examined under light microscopy to observe lamellar morphology.

1.4.3 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from hoof tissues using TRIzol reagent (TaKaRa, Japan) [16]. RNA concentration was measured using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). Samples with OD260/OD280 ratios between 1.8 and 2.0 were accepted and stored at -80°C. Total RNA was reverse transcribed into cDNA using the PrimeScript® RT reagent kit (TaKaRa, Japan).

1.4.4 Real-Time Quantitative PCR

Primer sequences for inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor- (TNF-) were adopted from Liu et al. [17]. Primers for MMP-2, MMP-9, membrane type-1 matrix metalloproteinase (MT1-MMP), and tissue inhibitor of metalloproteinases-1 (TIMP-1) were designed based on conserved nucleotide sequences of cattle and sheep in GenBank using Primer 3.0 software. All primer sequences are listed in Table 2 and were synthesized by Shanghai Invitrogen Biotechnology. Real-time quantitative PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) to quantify target genes and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative mRNA expression levels were normalized to GAPDH and calculated using the 2-ΔΔCt method.

1.5 Data Processing

Data were initially processed using Excel 2010. Ruminal pH, volatile fatty acid concentrations, and lactic acid and LPS concentrations in ruminal fluid and blood were analyzed using repeated measures in the general linear model of SPSS 20.0. mRNA expression data for inflammatory cytokines and matrix metalloproteinases were analyzed using independent samples t-tests. Differences were considered significant at $P<0.05$. Gene expression data are presented as means with standard errors, while other data are expressed as means ± standard deviations.

2.1 Ruminal Fluid pH

As shown in Figure 1 [Figure 1: see original paper], ruminal pH in the treatment group decreased immediately after infusion, reached its nadir at 24 h, and then gradually increased from 24 to 48 h. The mean ruminal pH was significantly lower in the treatment group compared with the control group ($P<0.05$). Ruminal pH <5.6 is generally considered indicative of acidosis; pH between 5.0 and 5.6 represents subacute or chronic ruminal acidosis, while pH <5.0 (approaching 4.5 or lower) indicates acute acidosis. In this study, ruminal pH in the treatment group fell below 5.0 after 8 h and remained at this level until the end of the experiment, demonstrating that the treatment goats developed acute ruminal acidosis.

2.2 Ruminal Volatile Fatty Acid Concentrations

As illustrated in Figure 2 [Figure 2: see original paper], concentrations of acetate, propionate, butyrate, and valerate in the treatment group began to decline at 4 h post-infusion. Acetate reached its lowest point at 12 h before slowly recovering, whereas propionate, butyrate, and valerate reached their minima at 24 h and remained stable thereafter. Isobutyrate and isovalerate concentrations decreased immediately after infusion, reaching their lowest levels at 24 h. Compared with the control group, the treatment group showed significantly lower mean concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate ($P<0.05$).

2.3 Lactic Acid and LPS Concentrations in Ruminal Fluid and Blood

As depicted in Figure 3 [Figure 3: see original paper], ruminal lactic acid concentration in the treatment group increased continuously during the first 24 h, reaching a mean peak of 12 mmol/L at 24 h, while blood lactic acid concentration also increased progressively, peaking at 48 h. Both ruminal fluid and blood LPS concentrations in the treatment group increased during the first 12 h, reached their maxima at 12 h, and then gradually declined. The treatment group exhibited significantly higher mean concentrations of lactic acid and LPS in both blood and ruminal fluid compared with the control group ($P<0.05$).

2.4 Effects of Acute Acidosis on Hoof Tissue Morphology

As shown in Figure 5 [Figure 5: see original paper], the control group clearly displayed the typical lamellar architecture comprising primary epidermal lamellae (PEL), secondary epidermal lamellae (SEL), secondary dermal lamellae (SDL), and primary dermal lamellae (PDL). In contrast, the treatment group exhibited shortened and irregularly shaped secondary epidermal and dermal lamellae that appeared asymmetrical.

A (10×), B (40×): Control group; C (10×), D (40×): Test group.

2.5 Relative mRNA Expression of Inflammatory Cytokines in Hoof Lamellae

As presented in Table 3 , the treatment group showed significantly increased relative mRNA expression of the inflammatory cytokine IL-6 in hoof lamellae compared with the control group ($P<0.05$), whereas no significant differences were observed in IL-1 or TNF- mRNA expression ($P>0.05$).

2.6 Relative mRNA Expression of Matrix Metalloproteinases in Hoof Lamellae

As shown in Table 4 , the treatment group demonstrated significantly increased mRNA expression of MMP-2 and MT1-MMP ($P<0.05$) and significantly decreased expression of TIMP-1 ($P<0.05$) in hoof lamellae compared with the control group, while no significant difference was found in MMP-9 mRNA expression ($P>0.05$).

Behavioral observations in this study revealed that treatment goats exhibited obvious lameness at 24 h post-infusion. Combined with subsequent histological examination of hoof tissues, these results confirm that the experimental protocol successfully induced acute laminitis in goats, providing a low-cost research model for future investigations of laminitis in ruminants.

Ruminal acidosis is a digestive disorder characterized by abnormal ruminal fermentation resulting in excessive production of lactic acid and organic acids, clinically manifested as acidosis and reduced activity of certain ruminal microbial populations. Based on ruminal pH values, acidosis is classified as acute (persistent pH <5.0) or subacute (pH maintained between 5.0 and 5.6) [18-19]. In this study, ruminal pH in the fructo-oligosaccharide-infused goats rapidly decreased below 5.0 and remained at this level for approximately 40 h, confirming the induction of acute ruminal acidosis. Previous research has demonstrated that *Streptococcus bovis* proliferates rapidly under acute acidosis conditions, producing large quantities of lactic acid that cause sustained declines in ruminal pH [20]. Consistent with these reports, ruminal lactic acid concentration in our treatment group increased continuously after fructo-oligosaccharide infusion, achieving the expected experimental outcome. The subsequent decline in lactic acid concentration after 24 h may be attributed to either carbohydrate depletion or gradual recovery of lactate-utilizing bacteria.

LPS is a major component of the outer membrane of Gram-negative bacteria. Our previous studies have shown that high-grain feeding increases ruminal LPS concentration and impairs ruminal epithelial barrier function [21]. The present study demonstrated significantly elevated LPS concentrations in both ruminal fluid and peripheral blood of treatment goats, indicating that acute acidosis disrupted ruminal microflora, increased LPS production, damaged digestive tract epithelial barrier integrity, and triggered LPS translocation into systemic circulation.

Equine laminitis research has demonstrated significantly increased metalloproteinase activity in hoof tissues under laminitic conditions. Metalloproteinase activity is regulated at multiple levels, including transcriptional regulation, proenzyme activation, and enzymatic inhibition [22]. At the transcriptional level, pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and TNF- can induce and/or stimulate metalloproteinase expression [23-24]. Regarding proenzyme activation, MMPs are synthesized as inactive zymogens that require extracellular activation by other enzymes. Specifically, inactive pro-MMP-2 must

be activated by free MT1-MMP to form active MMP-2, while MMP-9 activation involves MT1-MMP-mediated activation of pro-MMP-13 to active MMP-13, which subsequently activates pro-MMP-9 [25]. Enzymatic activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to the zinc-containing active center of activated MMPs at a 1:1 ratio through cysteine residues in their functional domains, thereby blocking MMP-substrate interactions. Our results showed that compared with the control group, the treatment group exhibited significantly increased mRNA expression of IL-6, MMP-2, and MT1-MMP, along with significantly decreased TIMP-1 expression in hoof tissues. Combined with the observed increases in LPS and lactic acid, these findings suggest that under ruminal acidosis conditions, circulating inflammatory factors such as LPS and lactic acid may activate metalloproteinase expression at the transcriptional level via inflammatory cytokines like IL-6, while also modulating the expression of MT1-MMP and TIMP-1 to enhance MMP activity, ultimately leading to lamellar degradation, hoof tissue destruction, and laminitis.

Intraruminal infusion of fructo-oligosaccharide induces acute ruminal acidosis in goats, resulting in increased LPS and lactic acid concentrations in both ruminal fluid and peripheral circulation. These inflammatory factors likely enhance inflammatory cytokine expression, which in turn modulates the expression of matrix metalloproteinases in hoof tissues, ultimately disrupting normal lamellar architecture and triggering acute laminitis.

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Note: Figure translations are in progress. See original paper for figures.