Changes in Rumen Epithelial Morphology and Permeability in Dairy Goats with Subacute Ruminal Acidosis (Postprint)

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Abstract

This experiment aimed to investigate the effects of subacute ruminal acidosis (SARA) on rumen epithelial morphology and permeability. Nine lactating Saanen dairy goats with good body condition and similar body weight were randomly divided into three groups (control group, SARA group, and recovery group, n=3). The control group was fed a basal diet [non-fibrous carbohydrate to neutral detergent fiber ratio (NFC/NDF) = 1.40], while the SARA and recovery groups were sequentially fed four experimental diets with NFC/NDF ratios of 1.40, 1.79, 2.31, and 3.23 to induce SARA, each for 15 days. Goats in the recovery group were allowed free access to hay for 30 days after successful SARA induction. One goat from the control group was slaughtered at each of 30, 60 (simultaneously with the three goats in the SARA group), and 90 days (simultaneously with the three goats in the recovery group). Rumen epithelial tissue from the ventral sac was collected for paraffin sectioning, transmission electron microscopy observation, and Ussing chamber studies. The results showed: 1) Histological section results showed that the thickness of the stratum corneum of the rumen epithelium in the SARA group was significantly higher than that in the control and recovery groups ($P < 0.05$), while the recovery group was significantly lower than the control group ($P < 0.05$); the thickness of the stratum granulosum in the control group was significantly higher than that in the SARA and recovery groups ($P < 0.05$), but there was no significant difference between the SARA and recovery groups ($P > 0.05$); the thickness of the stratum spinosum showed no significant difference among the three groups ($P > 0.05$); the total epithelial thickness in the recovery group was significantly lower than that in the control and SARA groups ($P < 0.05$), but there was no significant difference between the control and SARA groups ($P > 0.05$). Transmission electron microscopy results revealed that tight junctions in the rumen epithelium

of the SARA group were disrupted, intercellular spaces were enlarged, and mitochondria in the stratum spinosum cells showed degradation and vacuolation. 2) Compared with the control group, the short-circuit current (Isc), tissue conductance (Gt), and horseradish peroxidase (HRP) flux in the rumen epithelium of the SARA and recovery groups were significantly increased ($P < 0.05$), while the transmural potential difference (PD) was significantly decreased ($P < 0.05$). In conclusion, SARA disrupted the morphological and structural integrity of the rumen epithelium in dairy goats, increased rumen epithelial permeability, and caused long-term impairment of rumen epithelial barrier function.

Full Text

Effects of Subacute Ruminal Acidosis on Ruminal Epithelial Morphology and Permeability in Dairy Goats

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Abstract: This experiment aimed to investigate the effects of subacute ruminal acidosis (SARA) on ruminal epithelial morphology and permeability in dairy goats. Nine healthy lactating Saanen dairy goats with similar body weight were randomly divided into three groups (control group, SARA group, and recovery group, n=3). Goats in the control group were fed a basal diet [non-fibrous carbohydrates/neutral detergent fiber ratio (NFC/NDF) = 1.40], while those in the SARA and recovery groups were fed experimental diets with progressively increasing NFC/NDF ratios (1.40, 1.79, 2.31, and 3.23 in sequence, each for 15 days) to induce SARA. After successful SARA induction, goats in the recovery group were allowed ad libitum access to green hay for 30 days. Goats in the control group were slaughtered at 30, 60 (simultaneously with the 3 goats in the SARA group), and 90 days (simultaneously with the 3 goats in the recovery group) of feeding. Rumen epithelial tissue from the ventral sac was collected for paraffin sectioning, transmission electron microscopy observation, and Ussing chamber analysis. The results showed: 1) Histological examination revealed that the thickness of the ruminal epithelial stratum corneum in the SARA group was significantly higher than that in the control and recovery groups ($P < 0.05$), while the recovery group was significantly lower than the control group ($P < 0.05$). The thickness of the stratum granulosum in the control group was significantly higher than that in the SARA and recovery groups ($P < 0.05$), but no significant difference was observed between the SARA and recovery groups

($P > 0.05$). The thickness of the stratum spinosum did not differ significantly among the three groups ($P > 0.05$). The total epithelial thickness in the recovery group was significantly lower than that in the control and SARA groups ($P < 0.05$), but no significant difference was found between the control and SARA groups ($P > 0.05$). Transmission electron microscopy revealed that the tight junction structure of ruminal epithelium in the SARA group was disrupted, with increased intercellular spaces, mitochondrial degradation, and vacuole formation in stratum spinosum cells. 2) Compared with the control group, the short-circuit current (Isc), tissue conductance (Gt), and horseradish peroxidase (HRP) flux rate of ruminal epithelium in the SARA and recovery groups were significantly increased ($P < 0.05$), while the potential difference (PD) was significantly decreased ($P < 0.05$). In conclusion, SARA impairs the integrity of ruminal epithelial morphology in dairy goats, increases ruminal epithelial permeability, and causes long-term damage to ruminal epithelial barrier function.

Keywords: subacute ruminal acidosis; rumen epithelium; morphology; ultrastructure; permeability

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1. Materials and Methods

1.1 Experimental Animals Nine healthy lactating Saanen dairy goats with good body condition, weighing 35–40 kg and aged 2–3 years, all fitted with rumen fistulas, were selected as experimental animals. The goats were housed individually and fed equal amounts twice daily at 08:00 and 18:00, with free access to water.

1.2 Experimental Diets The diets were formulated according to the NRC (1981) nutrient requirements for goats and Jin's recommended feeding standards for dairy goats. Using corn, soybean meal, wheat bran, green hay, and alfalfa as main ingredients, four experimental diets with NFC/NDF ratios of 1.40, 1.79, 2.31, and 3.23 were designed, with concentrate-to-forage ratios of 51:49, 60:40, 68:32, and 79:21, respectively. The composition and nutrient levels of the experimental diets are shown in Table 1 .

1.3 Experimental Design A single-factor experimental design was adopted. The experimental animals were randomly divided into three groups: control group (n=3), SARA group (n=3), and recovery group (n=3). The control group was fed the basal diet (NFC/NDF = 1.40) throughout the experiment until slaughter. The SARA and recovery groups were fed diets with progressively increasing concentrate levels (NFC/NDF ratios of 1.40, 1.79, 2.31, and 3.23) to induce SARA, with each NFC/NDF diet fed for 15 days (60 days total). After successful SARA induction, goats in the SARA group were slaughtered, while those in the recovery group were allowed ad libitum access to green hay for 30 days to facilitate recovery. Goats in the control group were slaughtered at 30, 60 (simultaneously with the 3 goats in the SARA group), and 90 days

(simultaneously with the 3 goats in the recovery group) of feeding. A dynamic pH continuous monitoring system was used to monitor rumen fluid pH for 24 hours. Rumen fluid pH served as the primary parameter for determining SARA occurrence. Based on the research results of Ramanzin et al. [13], Penner et al. [6], and Penner et al. [14], SARA was considered successfully induced when rumen fluid pH remained between 5.5 and 5.2 for more than 3 hours within a continuous 24-hour period.

1.4 Sample Collection and Analysis

1.4.1 Collection and Processing of Rumen Epithelial Tissue Before slaughter, goats in the control, SARA, and recovery groups were fasted for 12 hours. Immediately after slaughter, tissue from the rumen ventral sac was collected, rinsed clean with sterile physiological saline, and surface moisture was absorbed with filter paper. Tissue blocks measuring 2 cm × 2 cm were fixed in 4% paraformaldehyde for optical microscopy observation. Rumen papillae from the ventral sac were cut into 1 mm × 1 mm × 1 mm blocks and fixed in 2.5% glutaraldehyde for transmission electron microscopy observation. Additionally, 1 cm × 1 cm rumen epithelial tissue from the ventral sac was rinsed with buffer solution and immediately placed in a thermos flask filled with mixed gas (95% O_2, 5% CO_2) for Ussing chamber analysis.

1.4.2 Morphological Structure Detection of Rumen Epithelium Tissue blocks (2 cm × 2 cm) from the rumen ventral sac were fixed in 4% paraformaldehyde for at least 48 hours, then processed into paraffin sections using conventional methods for observation of ruminal epithelial morphology under an optical microscope. Tissue blocks (1 mm × 1 mm × 1 mm) were fixed in 2.5% glutaraldehyde for 24 hours, then processed for transmission electron microscopy to observe the ultrastructure of ruminal epithelium.

1.4.3 Determination of Rumen Epithelial Permeability Fresh rumen epithelial tissue from the ventral sac was cut into 1 cm × 1 cm pieces. The muscle layer was quickly stripped on an ice bath plate, placed on a fixing ring, and inserted into the center of the two half-chambers of the Ussing chamber, connecting voltage and current electrodes. The Ussing chamber system consisted of four independent Ussing chambers. Five milliliters of pre-warmed buffer solution were added to each half-chamber, mixed gas was supplied, and after connecting to the computer and equilibrating for approximately 20 minutes until the curve stabilized, short-circuit current (Isc), tissue conductance (Gt), and potential difference (PD) were measured. After the curve stabilized, horseradish peroxidase (HRP) was added to the mucosal half-chamber to a final concentration of 2 mol/L. Every 20 minutes, 200 L of buffer solution was collected from the serosal half-chamber into prepared centrifuge tubes and stored at -20°C for HRP concentration determination. The Ussing chamber buffer solution was prepared according to reference [15].

Preparation of solutions for HRP concentration determination: 1) KH_2PO_4-$NaHPO_4$ buffer solution: 27.2 g KH_2PO_4 and 28.39 g $NaHPO_4$ were dissolved in 1,000 mL double-distilled water to a final concentration of 0.2 mol/L. 2) Tetramethylbenzidine (TMB) solution: 0.0962 g TMB was dissolved in 50 mL absolute ethanol, then diluted to 100 mL with water to a final concentration of 4 mmol/L. 3) H_2O_2 solution: 10 L of 30% H_2O_2 was thoroughly mixed with 7,990 L water to a final concentration of 0.01 mol/L.

HRP concentration determination: Based on a combination of methods by Klevenhusen et al. [7] and Wang et al. [16], 2 mL of prepared KH_2PO_4-$NaHPO_4$ buffer solution, 0.25 mL TMB solution, and 0.2 mL H_2O_2 solution were added to a 15 mL centrifuge tube, followed by 100 L of HRP-containing sample, then diluted to 5 mL with water and mixed thoroughly. The mixture was reacted in a 30°C water bath for 1 hour, then diluted to 10 mL with distilled water to terminate the reaction. Two hundred microliters of the prepared solution were added to a 96-well plate, with reagent blank as reference, and absorbance (A) was measured at 380 nm using a microplate reader. HRP concentration (C_{HRP}) was calculated using the following regression equation: A = 0.0097 C_{HRP} (g/L) - 0.0015 (R = 0.9972). HRP flux rate from the serosal to mucosal side of ruminal epithelium was calculated based on HRP concentration.

1.5 Statistical Analysis All data were organized using Excel and analyzed using SPSS 13.0 statistical software for one-way ANOVA. Duncan' s multiple comparison test was used to examine significant differences. Results are expressed as mean ± standard deviation.

2. Results

2.1 Changes in Rumen Fluid pH As shown in Table 2 , during SARA induction in dairy goats, the mean rumen fluid pH decreased from 6.20 to 5.76, the minimum pH decreased from 5.87 to 5.50, and the maximum pH decreased from 6.59 to 5.98 ($P < 0.05$), showing an overall downward trend. The duration of rumen fluid pH below 5.5 and 5.8 increased from 0 and 0.5 h/d to 3.83 and 11.33 h/d ($P < 0.05$), respectively. The area under the curve for rumen fluid pH below 5.5 and 5.8 increased from 0 and 0.09 to 1.73 and 3.33, respectively. These data indicate that the SARA model was successfully established.

2.2 Effects of SARA on Rumen Epithelial Morphology

2.2.1 Effects on Rumen Epithelial Morphology As shown in Table 3 and Figure 1 [Figure 1: see original paper], the total thickness of ruminal epithelium in the control group was higher than that in the SARA group ($P > 0.05$), while both control and SARA groups were significantly higher than the recovery group ($P < 0.05$). The thickness of the stratum spinosum did not differ significantly among the three groups ($P > 0.05$), being highest in the control group and lowest in the recovery group. The thickness of the stratum granulosum in the

control group was significantly higher than that in the SARA and recovery groups ($P < 0.05$), while the recovery group was higher than the SARA group, but the difference was not significant ($P > 0.05$). The thickness of the stratum corneum in the SARA group was significantly higher than that in the control and recovery groups ($P < 0.05$), and the control group was significantly higher than the recovery group ($P < 0.05$).

2.2.2 Effects on Rumen Epithelial Ultrastructure In the control group, ruminal epithelial cell organelles appeared normal (Figures 2-A and 2-B), with numerous intact tight junction structures (Figure 2-C). In the SARA group, the boundaries of ruminal epithelial cells were indistinct, mitochondria in stratum spinosum cells showed degradation (Figure 2-D) and vacuole formation (Figure 2-E), tight junction structures were disrupted, appearing loose and degraded (Figure 2-F). In the recovery group, the number of tight junction structures in some areas was greater than in both the SARA and control groups (Figures 2-G and 2-I), but severely damaged areas remained unrecovered (Figure 2-H), and intercellular cavities were present.

2.3 Effects of SARA on Rumen Epithelial Permeability As shown in Table 4 , Isc and Gt of ruminal epithelium in the SARA group were significantly higher than those in the control group ($P < 0.05$) and slightly higher than in the recovery group, but the differences were not significant ($P > 0.05$). PD in the SARA group was significantly lower than that in the control group ($P < 0.05$) and also lower than in the recovery group, but the difference was not significant ($P > 0.05$). Compared with the control group, Isc in the SARA and recovery groups increased by 157% and 128%, respectively; Gt increased by 24% and 20%, respectively; and PD decreased by 53% and 38%, respectively. Compared with the recovery group, Isc and Gt in the SARA group increased by 13% and 3%, respectively, while PD decreased by 25%. These results indicate that SARA significantly affected the electrophysiological parameters of ruminal epithelium, increasing its permeability, and the recovery group maintained relatively high permeability.

As shown in Figure 3 [Figure 3: see original paper], the HRP flux rate in the control group was significantly lower than that in the SARA and recovery groups ($P < 0.05$). The recovery group was lower than the SARA group, but the difference was not significant ($P > 0.05$). Compared with the control group, HRP flux rate in the SARA and recovery groups increased by 73% and 68%, respectively.

3. Discussion

3.1 Effects of SARA on Rumen Epithelial Morphological Structure The ruminal epithelium consists of four cellular layers from the mucosa to the serosa: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. Therefore, compared with intestinal monolayer epithelium, rumen endo-

toxins and bacteria are less likely to pass through the ruminal epithelium into the serosal layer and peripheral blood. Moreover, intact ruminal epithelial morphology is the structural basis for maintaining normal ruminal epithelial barrier function. The barrier formed by the stratum corneum and stratum granulosum cells can isolate harmful substances in the rumen and prevent damage to the body. The integrity of ruminal epithelium is influenced by multiple factors, with dietary concentrate-to-forage ratio being a primary factor. Excessively high concentrate-to-forage ratios can lead to incomplete keratinization and abnormal morphology of papillae. The present study found that SARA caused significant morphological changes in ruminal epithelium, characterized by reduced total epithelial thickness and stratum spinosum thickness, significantly decreased stratum granulosum thickness, but significantly increased stratum corneum thickness. These results are consistent with the findings of Steele et al. [5] that high-grain diets significantly reduced total ruminal epithelial thickness, stratum granulosum thickness, and stratum spinosum thickness. However, the results differ from those of Wu et al. [9], who reported that stratum corneum thickness in the recovery group was significantly higher than in the SARA group but significantly lower than in the control group. These discrepancies may be attributed to differences in sampling sites and SARA severity. In Wu et al.' s study [9], rumen fluid pH remained between 5.5 and 5.2 for more than 7.58 hours daily, whereas in the present study, pH fluctuated between 5.5 and 5.2 for only 3.83 hours. The different durations of pH between 5.5 and 5.2 indicate different SARA severities. Nevertheless, both Wu et al. [9] and the present study found that stratum corneum thickness in the recovery group was significantly lower than in the control group, suggesting that SARA exerts long-term effects on ruminal epithelial morphology. Additionally, the ultrastructural results in this study showed that SARA disrupted tight junction structures, increased intercellular spaces, caused mitochondrial degradation, induced vacuole formation in the stratum spinosum, and blurred epithelial cell boundaries. Moreover, severely damaged areas in the recovery group were not completely restored, and intercellular cavities were present. These results suggest that SARA damages ruminal epithelial morphological structure, weakens ruminal epithelial barrier function, and has long-lasting effects on ruminal epithelial morphology.

3.2 Effects of SARA on Rumen Epithelial Permeability Increased ruminal epithelial permeability is an important marker reflecting early damage to the ruminal epithelial barrier. Electrophysiological parameters in the Ussing chamber system can reflect ruminal epithelial permeability. Isc primarily reflects the transport capacity of ions across the epithelium, Gt reflects epithelial cell permeability to ions, and PD mainly indicates the activity of ruminal epithelial tissue. These electrophysiological parameters represent the barrier permeability of epithelial tissue. In Ussing chamber technology, using isotopic or macromolecular markers to measure the proportion passing through the gastrointestinal epithelium has become a common and important method for detecting epithelial permeability. Commonly used markers include HRP, fluorescein

isothiocyanate (FITC), and ^{3}H-mannitol [6-7]. With continuous application and improvement, this technology has become the gold standard for studying gastrointestinal barrier function.

Gastrointestinal epithelial permeability generally refers to the characteristic that mucosal epithelium is relatively easily penetrated by some molecular substances through simple diffusion. Changes in this characteristic can reflect the degree of epithelial damage and serve as an important indicator for detecting gastrointestinal barrier impairment. Increased mucosal permeability indicates compromised mucosal integrity and impaired barrier function. The present study found that compared with the control group, Isc in the SARA and recovery groups significantly increased by 157% and 128%, respectively; Gt significantly increased by 24% and 20%, respectively; PD significantly decreased by 53% and 38%, respectively; and HRP flux rate in the SARA and recovery groups was significantly higher than in the control group, increasing by 73% and 68%, respectively. Penner et al. [6] used the Ussing chamber system to study the short-term effects of mild SARA on ruminal epithelial barrier function and reported increased Gt. Klevenhusen et al. [7] used the Ussing chamber system to investigate the effects of high-grain diets on goat ruminal epithelial permeability and found that Isc, Gt, and HRP concentration in the high-grain diet group were all significantly higher than in the control group. Aschenbach et al. [21] used the Ussing chamber system under in vitro conditions and found that at pH 5.1, ruminal epithelial Gt increased while Isc decreased. Penner et al. [6] induced SARA in sheep by direct glucose infusion and reported increased Gt and ^{3}H-mannitol flux rate from the serosal to mucosal side of ruminal epithelium, decreased Isc, and increased ruminal epithelial permeability with increasing acidity. The present results partially agree with these findings, demonstrating that SARA can disrupt ruminal epithelial integrity, increase ruminal epithelial permeability, and impair ruminal epithelial barrier function.

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