Effects of Weaning Time on Rumen Morphology and Epidermal Growth-Related Gene Expression in Hu Sheep Lambs of Different Ages: Postprint

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Abstract

This experiment aimed to investigate the effects of weaning time on rumen morphology and epidermal growth-related gene expression in Hu sheep lambs at different ages. A two-factor experimental design was employed, with weaning time and lamb age as the two factors. Fifty-four Hu sheep lambs with similar birth weight [(3.51±0.57) kg] were selected. At 28 days of age, six lambs were randomly slaughtered, and the remaining 48 lambs were divided into a 28-day weaning group [(8.21±0.97) kg] and a 56-day weaning group [(8.06±0.53) kg] according to the principle of homogeneity. At 42, 56, 70, and 84 days of age, six lambs from each group were randomly selected for slaughter. Rumen ventral sac tissue samples were collected to determine rumen papilla length, width, and muscle layer thickness, and total RNA was extracted from rumen tissue to measure the expression of epidermal growth-related genes. The results showed that lambs in the 28-day weaning group had significantly greater rumen papilla length and width compared to those in the 56-day weaning group ($P<0.05$), while rumen muscle layer thickness was significantly lower ($P<0.05$). The interaction between weaning time and lamb age had a significant effect on rumen papilla length and muscle layer thickness ($P<0.05$). The expression levels of insulin-like growth factor binding protein (IGFBP) 3, IGFBP5, and IGFBP6 in the rumen epithelium of lambs in the 28-day weaning group were significantly higher than those in the 56-day weaning group ($P<0.05$). Rumen papilla length was significantly negatively correlated with the expression of transforming growth factor (TGF) 1 and IGFBP6 ($R=-0.318$, $P=0.001$; $R=-0.520$, $P<0.001$); rumen papilla width was significantly negatively correlated with TGF 1 expression ($R=-0.275$, $P=0.004$) and significantly positively correlated with IGFBP3 and IGFBP5 expression ($R=0.344$, $P<0.001$; $R=0.256$, $P=0.001$). In conclusion, weaning at 28 days of age promotes rumen papilla development in Hu sheep lambs, and rumen epidermal growth-related genes may be involved in the regulation of early

rumen development in lambs.

Full Text

Effects of Weaning Time on Rumen Morphology and Epidermal Growth-Related Gene Expression in Hu Lambs at Different Ages

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Abstract: This study investigated the effects of weaning time on rumen morphology and epidermal growth-related gene expression in Hu lambs at different ages using a two-factor experimental design with weaning time and age as factors. Fifty-four Hu lambs with similar birth weights [(3.51±0.57) kg] were selected. Six lambs were randomly slaughtered at 28 days of age, and the remaining 48 lambs were divided into a 28-day weaning group [(8.21±0.97) kg] and a 56-day weaning group [(8.06±0.53) kg] based on homogeneity principles. Six lambs from each group were randomly selected and slaughtered at 42, 56, 70, and 84 days of age. Rumen ventral sac tissue samples were collected to measure papilla length, width, and muscular thickness. Total RNA was extracted from rumen tissue to quantify epidermal growth-related gene expression. The results showed that lambs weaned at 28 days had significantly greater rumen papilla length and width but significantly lower muscular thickness compared to those weaned at 56 days ($P<0.05$). Significant interactions between weaning time and age were observed for papilla length and muscular thickness ($P<0.05$). Expression levels of insulin-like growth factor binding protein (IGFBP) 3, IGFBP5, and IGFBP6 were significantly higher in the 28-day weaning group ($P<0.05$). Papilla length was significantly negatively correlated with transforming growth factor (TGF) 1 and IGFBP6 expression ($R=-0.318$, $P=0.001$; $R=-0.520$, $P<0.001$), while papilla width was significantly negatively correlated with TGF 1 expression ($R=-0.275$, $P=0.004$) and positively correlated with IGFBP3 and IGFBP5 expression ($R=0.344$, $P<0.001$; $R=0.256$, $P=0.001$). In conclusion, weaning at 28 days promotes rumen papillae development in Hu lambs, and genes related to rumen epidermal growth may participate in regulating early rumen development.

Keywords: Hu lamb; early weaning; rumen morphology; development; gene expression

Rumen development is a critical aspect of lamb growth. Post-weaning changes in rumen fermentation convert indigestible fiber into volatile fatty acids (VFAs), and the absorption and transport of these VFAs by rumen epithelium largely depend on stratum corneum thickness. Research indicates that high-nutrient diets promote rumen epithelial development by increasing basal cell numbers and accelerating cell differentiation and migration, leading to increased spinous and granular cell layers [1]. Rumen epithelial cells begin differentiating during the fetal period, but this process remains incomplete at birth. At birth, rumen papillae are visible under microscopy, and their area, length, and width gradually increase with age and dietary influence [2]. Young ruminant rumen development is directly related to feed type. Calves fed only milk show no papillae development, while combined milk and forage feeding fails to effectively stimulate papillae growth [3]. Additionally, Žitňan et al. [4] demonstrated that early-weaned calves had greater papillae surface area than conventionally raised calves, though Klein et al. [5] found no similar results using two different weaning systems. This study examined rumen morphological development in Hu lambs weaned at different times while receiving starter feed, and elucidated the relationship between papillae development-related genes and rumen development to provide theoretical basis for early weaning and supplementation strategies in lambs.

Materials and Methods

1.1 Experimental Animals and Design

A two-factor experimental design was employed with weaning time and lamb age as factors. Fifty-four Hu lambs with similar birth weights [(3.51±0.57) kg] were purchased from Jinchang Zhongtian Sheep Industry Co. Ltd. and raised as twin lambs until 28 days of age. Six lambs (8.22±0.87 kg) were randomly slaughtered at 28 days, and the remaining 48 lambs were divided into a 28-day weaning group [(8.21±0.97) kg] and a 56-day weaning group [(8.06±0.53) kg] based on homogeneity principles. The 28-day weaning group was weaned on the day of grouping, while the 56-day weaning group was weaned at 56 days of age.

1.2 Experimental Diets and Management

All lambs were supplemented with starter feed 1 from 7 days of age, which was gradually replaced by starter feed 2 from 60 days of age over a 10-day transition period. Both starter feeds had a compression ratio of 1:6 and pellet diameter of 2.5 mm. Dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), calcium (Ca), and phosphorus (P) contents were determined according to the AOAC Analytical Methods Manual [6] and Feed Analysis and Feed Quality Detection Technology [7]. Digestible energy and starch content were calculated based on reference data [8-9] by multiplying the digestible energy and starch values of each ingredient for sheep by their proportions in the starter feed and summing the results. The composition and nutrient levels of the starter feeds

are shown in . The concentrate-to-forage ratios were 78:22 and 57:43 for starter feed 1 and starter feed 2, respectively.

Lambs were housed with their dams in the same pen equipped with creep feeders. Lambs were separated from their dams daily from 08:00-10:00, 12:00-14:00, and 18:00-20:00 to allow dams to consume feed. The dam diet consisted of conventional total mixed ration (TMR) composed of 40% corn silage, 12% oat hay, 10% alfalfa hay, 8% barley straw, 5% rapeseed straw, 13% soybean residue, 9% corn, and 3% soybean meal, with nutrient levels of 7.38 MJ/kg digestible energy, 7.60% CP, 0.32% Ca, and 0.25% P. After the dams finished feeding, feeders and pens were thoroughly cleaned to prevent lambs from consuming the dam feed. Lambs otherwise remained with their dams to suckle freely. Pens were completely disinfected every 15 days. The average daily dry matter intake of lambs in the 28-day and 56-day weaning groups at different ages is shown in . Since lambs were group-fed with their dams, these data were not statistically analyzed.

1.3 Sample Collection

Experimental lambs were slaughtered at 28, 42, 56, 70, and 84 days of age, with six lambs randomly selected from each group at each time point. After slaughter, five pieces of rumen ventral sac epithelial tissue (approximately 2 cm × 2 cm × 2 cm each) were rapidly collected, rinsed with ice-cold saline, immediately placed in 2.0 mL Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -80°C. Rumen ventral sac epithelial tissue was also collected, rinsed repeatedly with ice-cold phosphate-buffered saline (PBS, pH=7.4, 1×), and immediately fixed in 4% formaldehyde solution. Rumen contents were filtered through four layers of gauze, and the filtrate was placed in 5 mL Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

1.4 Major Instruments and Reagents

Microscope (IX71, Olympus, Japan), real-time PCR system (CFX96, Bio-Rad, USA), micro-volume UV-Vis spectrophotometer (NANODrop2000, Thermo, Germany), electrophoresis apparatus (PowerPac™, Bio-Rad, USA), gel imaging system (GelDoc-It310, UVP, USA), and gas chromatograph (Focus GC AI 3000, Thermo, Germany) were used in this study. Trizol, reverse transcription kit, and SYBR Green PCR Master Mix were purchased from Beijing TransGen Biotech Co., Ltd.

1.5 Experimental Procedures

1.5.1 Total RNA Extraction Total RNA was extracted from rumen ventral sac epithelial tissue using Trizol according to the manufacturer' s instructions. RNA concentration and purity were determined using a micro-volume UV-Vis spectrophotometer, and RNA quality was assessed by 1.0% agarose gel electrophoresis.

1.5.2 Real-Time Quantitative PCR Reverse transcription (RT) was performed according to the TransScript One-Step gDNA Removal and cDNA Synthesis Super Mix kit protocol. Primers were designed using Primer5 software with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. The 20 L amplification system contained 10 L SYBR Premix Ex Taq II, 0.4 L forward primer (10 mol/L) and 0.4 L reverse primer (10 mol/L) (Table 3), 1 L cDNA, and 8.2 L ddH O. Amplification conditions were: 95°C pre-denaturation for 2 min; 40 cycles of 95°C denaturation for 10 s, annealing for 20 s, and 72°C extension for 10 s with plate reading twice; final extension at 72°C for 5 min; melting curve analysis from 60-95°C with plate reading every 0.05 s. Negative controls used 1 L ddH O instead of template. Each sample was analyzed in four technical replicates.

1.5.3 Light Microscopy Observation Paraffin section preparation followed the method recommended by Shi [10]. Rumen tissue was rinsed with ice-cold PBS, and approximately 1 cm^2 of rumen ventral sac bottom was collected. Tissues were immediately fixed in 4% formaldehyde solution for sectioning and analysis. After paraffin embedding, sectioning, and hematoxylin-eosin staining, five sections were selected with five typical fields (intact tissue) per section to measure rumen papilla length and width and muscular thickness using Image-Pro Express 6.0 image analysis software.

1.5.4 Determination of Rumen Volatile Fatty Acids Rumen VFA content was determined using a gas chromatograph (AI 3000, Thermo, Germany) with an HP 19091N-213 capillary column (Agilent). Chromatographic conditions: injector temperature 220°C, nitrogen flow 2.0 mL/min, split ratio 40:1, temperature program (120°C for 3 min, then 10°C/min to 180°C, hold 1 min), flame ionization detector (FID) 250°C, with air, hydrogen, and nitrogen flows of 450, 40, and 45 mL/min, respectively.

1.6 Statistical Analysis

Real-time PCR data were processed using the 2^(-ΔΔCT) method [11]. Data were analyzed by two-way ANOVA using SPSS 19.0 software, with Tukey' s test for multiple comparisons when significant differences were detected ($P<0.05$). Pearson correlation analysis (n=54) was performed using SPSS 19.0, with $P<0.05$ considered significant.

Results

2.1 Rumen Papilla Morphology

As shown in , significant interactions between weaning time and age were observed for papilla length and muscular thickness ($P<0.05$). Lambs weaned at 28 days had significantly greater papilla length and width but significantly lower muscular thickness compared to those weaned at 56 days ($P<0.05$).

Age significantly affected papilla length and muscular thickness ($P<0.05$). Papilla length at 56, 70, and 84 days was significantly greater than at 28 and 42 days ($P<0.05$), with 42-day values significantly greater than at 28 days ($P<0.05$). Muscular thickness at 56 and 70 days was significantly greater than at 28, 42, and 84 days ($P<0.05$). Age had no significant effect on papilla width ($P>0.05$).

2.2 Effects of Weaning Time on Gene Expression Related to Rumen Epidermal Growth

As shown in , significant interactions between weaning time and age were observed for expression of transforming growth factor (TGF) 1, insulin-like growth factor binding protein (IGFBP) 3, IGFBP5, and IGFBP6 ($P<0.05$).

Weaning time significantly affected IGFBP3, IGFBP5, and IGFBP6 expression. Expression of IGFBP3, IGFBP5, and IGFBP6 was significantly higher in the 28-day weaning group compared to the 56-day weaning group ($P<0.05$). Other differences were not significant ($P>0.05$).

Age significantly affected TGF 1, IGFBP3, and IGFBP5 expression ($P<0.05$). TGF 1 expression at 42, 70, and 84 days was significantly higher than at 28 and 56 days ($P<0.05$). IGFBP3 expression at 84 days was significantly higher than at 28, 42, and 56 days ($P<0.05$), with 56-day values significantly higher than at 28 days ($P<0.05$). IGFBP5 expression at 56 days was significantly higher than at 28, 70, and 84 days ($P<0.05$), while 70- and 84-day values were significantly higher than at 28 days ($P<0.05$). Age had no significant effect on IGFBP6 expression ($P>0.05$).

2.3 Correlation Analysis

Correlation analysis was performed among rumen morphological parameters, short-chain fatty acids (SCFAs), and rumen epithelial growth-related genes in 54 lambs. As shown in , rumen SCFA content was significantly positively correlated with papilla length, muscular thickness, and expression of TGF 1, IGFBP3, and IGFBP5 ($R=0.562$, $P<0.001$; $R=0.349$, $P<0.001$; $R=0.205$, $P=0.002$; $R=0.219$, $P=0.032$; $R=0.265$, $P=0.026$), but significantly negatively correlated with IGFBP6 expression ($R=-0.616$, $P<0.001$). Propionate content was significantly positively correlated with papilla length ($R=0.226$, $P=0.007$). Butyrate content was significantly positively correlated with papilla width and IGFBP5 expression ($R=0.216$, $P=0.009$; $R=0.316$, $P<0.001$) and significantly negatively correlated with IGFBP6 expression ($R=-0.274$, $P=0.005$).

Papilla length was significantly negatively correlated with TGF 1 and IGFBP6 expression ($R=-0.318$, $P=0.001$; $R=-0.520$, $P<0.001$). Papilla width was significantly negatively correlated with TGF 1 expression ($R=-0.275$, $P=0.004$) and significantly positively correlated with IGFBP3 and IGFBP5 expression ($R=0.344$, $P<0.001$; $R=0.256$, $P=0.001$).

Discussion

TGF , a member of the growth factor superfamily, controls various processes including cell proliferation, recognition, differentiation, and apoptosis [12-13]. In this study, TGF 1 expression in rumen epithelium was significantly negatively correlated with papilla length and width. Previous studies have demonstrated that TGF 1 downregulation promotes rumen development [14], suggesting that TGF 1 may inhibit rumen epithelial cell proliferation and papillary growth, consistent with findings by Naeem et al. [15].

Research has shown that the proliferative effects of insulin-like growth factor (IGF) on rumen epithelial cells are primarily regulated by the IGFBP family [16]. Butyrate content in the rumen affects IGFBP regulation of IGF1 activity in tissue cells [17]. In this study, rumen butyrate content was significantly positively correlated with IGFBP5 expression, suggesting that butyrate may influence the IGF axis by promoting secretion of hormones that stimulate cell differentiation while reducing secretion of pro-apoptotic hormones [18]. IGFBP5 expression was significantly higher in the 28-day weaning group, and Steele et al. [19] confirmed that upregulated IGFBP5 expression promotes rumen epithelial cell proliferation, which may explain why papilla length and width were significantly greater in the 28-day weaning group. This is consistent with our finding that IGFBP5 expression was significantly positively correlated with papilla width. IGFBP6 preferentially binds IGF2 over IGF1 and inhibits IGF2 function. The significant negative correlation between IGFBP6 expression and papilla length and width observed in this study aligns with previous findings of an inhibitory role of IGFBP6 in rumen epithelium [20]. IGFBP3 inhibits IGF1 activity in rumen tissue cells, and increased rumen butyrate content downregulates IGFBP3 expression [19,21]. However, the significant positive correlation between papilla length and IGFBP3 expression observed here requires further investigation.

In rumen morphological studies, papilla length is the most important indicator, followed by papilla width and rumen wall thickness [22]. Before weaning, small intestine absorption is the primary energy source for lambs. When lambs consume solid feed, rumen papillae begin to elongate and rumen mucosa thickens [23]. In this study, average daily dry matter intake from 28-84 days was higher in the 28-day weaning group (582.81 g/d) than in the 56-day weaning group (552.97 g/d). The 28-day weaning group showed significantly greater papilla length and width, and rumen SCFA content was positively correlated with papilla length. This is because increased feed intake after weaning elevates total VFA content in the rumen, promoting rumen development [24]. Anderson et al. [25] reported that rumen propionate and butyrate contents were significantly higher in 28-day weaned calves than in unweaned calves. In this study, propionate content was positively correlated with papilla width and muscular thickness. Studies have shown that propionate and butyrate are important factors affecting papillae development [26-27]. Additionally, increased solid feed intake enhances physical stimulation of the rumen, further promoting papillae development [28]. Žitňan et al. [4] and Stobo et al. [29] also demonstrated that early weaning significantly

increased papilla length in calves. The rumen wall is thin and the reticulorumen volume is small in newborn ruminants. This study found that muscular thickness was significantly greater in the 56-day weaning group, though Stobo et al. [29] confirmed that muscular thickness did not change significantly with increased concentrate feeding. However, the interaction between age and weaning time significantly affected muscular thickness in this study. Žitňan et al. [4] found that papilla number decreased with age after weaning. This study did not measure papilla number, and how early weaning affects papilla number in Hu lambs requires further investigation.

Under the management conditions of this study, weaning at 28 days increased starter feed intake and promoted rumen papillae development. Rumen SCFAs enhanced expression of IGFBP3, IGFBP5, and IGFBP6 while inhibiting TGF 1 expression in rumen epithelium. Upregulated IGFBP5 expression was significantly positively correlated with rumen papillae development.

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