Effects of Dietary Nutrient Restriction on Intestinal Tissue Morphology and Serum Insulin-like Growth Factor-1 and Glucagon-like Peptide-2 Concentrations in Lambs (Postprint)

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Abstract

This study aimed to investigate the effects of dietary nutrient restriction on intestinal tissue morphology and serum concentrations of insulin-like growth factor-1 (IGF-1) and glucagon-like peptide-2 (GLP-2) in lambs. Sixty-four 17-day-old Hu sheep lambs were randomly allocated to 4 groups and fed milk replacer and starter feed with different nutrient levels: control (CON) group, 20% protein restriction (PR) group, 20% energy restriction (ER) group, and 20% protein + 20% energy restriction (BR) group, with 4 replicates per group, 4 lambs per replicate, and equal numbers of males and females. Serum was collected at 20, 40, and 60 days of age for determination of IGF-1 and GLP-2 concentrations. At 40 and 60 days of age, 4 lambs from each group were slaughtered, and duodenum, jejunum, and ileum tissues were collected to observe small intestinal morphological structure. The results showed: 1) At 40 days of age, small intestine weight in PR, ER, and BR groups was significantly lower than in CON group ($P<0.05$); by 60 days of age, only small intestine weight in ER group remained significantly lower than in CON group ($P<0.05$), with the reduction primarily due to decreased jejunum weight. 2) Villus height in duodenum and jejunum at 40 days of age, and duodenal villus height at 60 days of age in PR, ER, and BR groups were significantly lower than in CON group ($P<0.05$); jejunal villus height at 60 days of age in ER and BR groups was significantly lower than in CON group ($P<0.05$). 3) The duodenal villus height/crypt depth ratio at 40 days of age in BR group was significantly lower than in CON group ($P<0.05$). 4) Serum GLP-2 concentration at 60 days of age in ER group was significantly lower than in the other three groups ($P<0.05$), while no significant difference in serum IGF-1 concentration was observed among the four groups ($P>0.05$). In conclusion, dietary nutrient restriction inhibited morphological

development of duodenal and jejunal tissues in lambs, while energy restriction decreased serum GLP-2 concentration.

Full Text

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Abstract: This study investigated the effects of dietary nutrition restrictions on intestinal morphology and serum concentrations of insulin-like growth factor-1 (IGF-1) and glucagon-like peptide-2 (GLP-2) in lambs. Sixty-four healthy 17-day-old Hu lambs with similar birth dates and initial body weights [(2.5$±0.2)*kg*](*bodyweight*(6.2±$0.2) kg at 17 days) were randomly allocated to four groups with four replicates per group and four lambs per replicate (half male and half female). The groups included a control group (CON) fed a basal diet, and three restriction groups: 20% metabolizable energy restriction (ER), 20% crude protein restriction (PR), and simultaneous 20% restriction of both metabolizable energy and crude protein (BR). The CON group nutritional levels were established according to the recommended requirements for Hu sheep with 10 kg body weight and 300 g average daily gain by Yang et al. The experimental diets consisted of milk replacer and starter feed. The CON group milk replacer nutritional level was determined based on our laboratory' s research findings (metabolizable energy 20 MJ/kg, crude protein 24%), which then informed the metabolizable energy and crude protein levels in the starter feed. A 3-day pre-trial period was conducted from 17 to 19 days of age, followed by the formal experimental period starting at 20 days until 60 days of age.

The results demonstrated that dietary nutrition restrictions significantly reduced small intestinal weight at 40 days of age, particularly through decreased jejunum weight, and inhibited morphological development of the duodenum and jejunum, with energy restriction showing the most severe inhibitory effect. Energy restriction significantly decreased serum GLP-2 concentration at 60 days of age, whereas serum IGF-1 concentration was not significantly affected by any restriction treatment.

Keywords: lamb; nutrition restriction; morphology; insulin-like growth factor-1; glucagon-like peptide-2

1.1 Experimental Design

This experiment employed a randomized design with dietary nutrition level as the experimental factor. Sixty-four purebred Hu lambs at (17$±1)$daysofagewithsimilarbirthdates, initialbodyweights[(2.5±0.2)kg], andgoodhealthstatus[bodyweight(6$ kg] were randomly divided into four groups, with four replicates per group and four lambs per replicate (half male and half female), with each replicate housed in one pen. The control group (CON) was fed the basal diet, while the other three groups received diets with 20% metabolizable energy restriction (ER group), 20% crude protein restriction (PR group), or simultaneous 20% restriction of both metabolizable energy and crude protein (BR group). A pre-trial period was conducted from 17 to 19 days of age, with the formal trial commencing at 20 days of age and continuing until lambs reached 60 days of age.

The nutritional levels for the CON group were established according to the recommended requirements for 10 kg Hu sheep with an average daily gain of 300 g by Yang et al., with corresponding adjustments made for the ER, PR, and BR groups. The experimental diets comprised milk replacer and starter feed. The CON group milk replacer nutritional level (metabolizable energy 20 MJ/kg, crude protein 24%) was determined based on our laboratory' s research findings, which then informed the determination of metabolizable energy and crude protein levels in the starter feed. The milk replacer was provided by Beijing Precision Animal Research Center, with its nutritional composition presented in Table 1 . Lambs in the CON group had ad libitum access to starter feed, while PR, ER, and BR groups were fed starter amounts based on the previous day' s intake of the CON group to maintain similar feed intake across all groups. The starter feed was pelleted (diameter 4 mm, length 10 mm) and formulated in-house, with its composition and nutritional levels shown in Table 2 .

1.2 Feeding Management

Before 17 days of age, experimental lambs suckled their dams. From 17 days of age, lambs were gradually transitioned from dam nursing to milk replacer feeding, with complete weaning from maternal milk by 20 days of age. Milk replacer feeding amounts were set at 2.0% and 1.5% of lamb body weight during 21-50 days and 51-60 days of age, respectively. Lambs were fed three times daily (08:00, 12:00, and 18:00) from 21 to 30 days of age, and twice daily (09:00 and 18:00) from 31 to 60 days of age. Starter feed supplementation began at 17 days of age. Lambs were ear-tagged before the experiment and vaccinated according to routine farm procedures. The sheep house was a semi-open warm shed with good ventilation, and disinfection was performed every two weeks with 0.5% Baiedusha and 0.1% Xinjiermie.

1.3.1 Milk Replacer and Starter Intake and Nutrient Levels

Milk replacer intake was accurately weighed and recorded. For starter feed, the amount offered and previous day' s residual feed were recorded daily to calculate intake, with feed and residual samples collected. Metabolizable energy was determined using a Parr-6400 oxygen bomb calorimeter, crude protein content was measured with a KDY-9830 automatic Kjeldahl nitrogen analyzer, and dry matter, ether extract, crude ash, crude fiber, calcium, and phosphorus contents were analyzed according to "Feed Analysis and Feed Quality Detection Technology."

1.3.2 Intestinal Morphology

At 40 and 60 days of age, one healthy lamb per replicate with body weight close to the group average was selected for slaughter (half male and half female). After slaughter, small intestinal segments were rapidly separated, contents removed and washed, and each segment weighed. Two-centimeter samples were taken from the middle of the duodenum, jejunum, and ileum, fixed in tissue fixative (120 mL of 40% formaldehyde, 880 mL distilled water, 4 g $NaH_2PO_4 \cdot H_2O$, and 13 g Na_2HPO_4), dehydrated with conventional alcohol, and embedded in paraffin with 6 m sections. After hematoxylin-eosin (HE) staining, small intestinal mucosal morphology was observed under light microscopy. Using an Image-Pro Express image analysis system, three non-consecutive slices were examined per sample, measuring the longest villus height (V) and crypt depth (C) in the field of view, with at least five fields selected per slice. The average values were calculated as the final measurement data.

1.3.3 Serum Insulin-Like Growth Factor-1 (IGF-1) and Glucagon-Like Peptide-2 (GLP-2) Concentrations

Blood samples were collected via jugular venipuncture before morning feeding (09:00) at 20, 40, and 60 days of age. Blood was placed in silica-containing coagulation-promoting tubes, allowed to stand for 30 minutes, then centrifuged at 1,358×g for 15 minutes. Serum was collected and stored at -20°C for analysis. Serum IGF-1 and GLP-2 concentrations were determined using commercial sheep enzyme-linked immunosorbent assay (ELISA) kits (Kamai Shu (Shanghai) Co., Ltd.) according to the manufacturer' s instructions.

1.4 Statistical Methods

Experimental data were processed using Excel and analyzed with SAS 8.1 statistical software using the ANOVA model. Duncan' s multiple range test was used for significant differences. All data were considered significantly different at $P<0.05$.

2.1 Effects of Dietary Nutrition Restrictions on Growth Performance and Feed Intake

As shown in Table 3 , average daily gain (ADG) of lambs in the PR, ER, and BR groups during 21-40 days and 41-60 days of age was lower than that in the CON group ($P<0.05$). Milk replacer intake was consistent across all four groups at each stage, while starter feed intake showed no significant differences ($P>0.05$). Metabolizable energy intake in the PR group did not differ significantly from the CON group ($P>0.05$), whereas ER and BR groups were significantly lower ($P<0.05$). Crude protein intake in PR and BR groups was significantly lower than in the CON group ($P<0.05$), while the ER group showed no significant difference ($P>0.05$). The results for ADG and feed intake met the experimental design requirements.

2.2 Effects of Dietary Nutrition Restrictions on Small Intestinal Weight

Table 4 shows that at 40 days of age, jejunum and total small intestine weights in PR, ER, and BR groups were significantly lower than in the CON group ($P<0.05$), while duodenum and ileum weights showed no significant differences among the four groups ($P>0.05$). At 60 days of age, jejunum, ileum, and total small intestine weights in the ER group were significantly lower than those in the CON, PR, and BR groups ($P<0.05$).

2.3 Effects of Dietary Nutrition Restrictions on Small Intestinal Morphology

Table 5 indicates that villus height in the duodenum and jejunum at 40 days of age, and in the duodenum at 60 days of age, was significantly lower in PR, ER, and BR groups compared to the CON group ($P<0.05$). Villus height in the jejunum at 60 days of age was significantly lower in ER and BR groups than in the CON group ($P<0.05$). Ileal villus height showed no significant differences among groups at either 40 or 60 days of age ($P>0.05$). Duodenal crypt depth showed no significant differences among the four groups at 40 days of age ($P>0.05$), but was significantly lower in ER and BR groups than in the CON group at 60 days of age ($P<0.05$), with BR group also significantly lower than PR group ($P<0.05$).

Jejunal crypt depth was significantly lower in PR, ER, and BR groups than in the CON group at both ages ($P<0.05$). Ileal crypt depth was significantly lower in ER and BR groups than in the CON group at 40 days of age ($P<0.05$), and significantly lower in PR, ER, and BR groups than in the CON group at 60 days of age ($P<0.05$). Villus height/crypt depth (V/C) ratio in the duodenum at 40 days of age was significantly lower in PR, BR, and ER groups compared to the CON group ($P<0.05$), with no other significant differences observed among groups at either age ($P>0.05$).

Jejunal villus morphology is shown in Figure 1 [Figure 1: see original paper].

Lambs in the CON group exhibited slender, finger-like villi with good development. PR group lambs showed villus adhesion and irregular shapes, while ER and BR groups displayed severe epithelial shedding and irregular villus morphology.

2.4 Effects of Dietary Nutrition Restrictions on Serum IGF-1 and GLP-2 Concentrations

Table 6 shows that serum IGF-1 concentrations did not differ significantly among the four groups ($P>0.05$). Serum GLP-2 concentrations at 20 and 40 days of age showed no significant differences among groups ($P>0.05$), but at 60 days of age, GLP-2 concentration in the ER group was significantly lower than in the other three groups ($P<0.05$). Serum concentrations of both IGF-1 and GLP-2 decreased significantly with age ($P<0.05$).

3.1 Effects of Dietary Nutrition Restrictions on Small Intestinal Weight

Each 1 g increase in small intestine weight increases animal maintenance requirements by 290–350 g/d. When nutritional requirements, particularly energy, are not met, lambs reduce visceral organ maintenance needs to support growth. In this study, the BR group reduced small intestinal weight at 40 days of age, especially jejunum weight. However, at 60 days of age, small intestinal weight was lower than in the CON group but the difference was not significant. Inconsistent results regarding the effects of nutrition restriction on small intestinal weight may be attributed to increased maintenance nutrients required as lamb body weight increases, leaving insufficient nutrients for significant small intestinal growth. Li et al. reported that 40% protein restriction and 20% energy restriction did not reduce small intestinal weight in weaned lambs. Discrepancies among studies may result from different nutritional levels in control groups, leading to varying restriction levels. For instance, this study used a reference of 300 g/d average daily gain, whereas Li et al. used 145 g/d. Reducing dietary energy levels in ruminants decreases the efficiency of converting plant protein to rumen microbial protein, while high-protein plant diets contain anti-nutritional factors that can cause intestinal damage. Consequently, the ER group consistently showed lower small intestinal weight than the CON group, significantly lower than the BR group at 60 days of age. Wang et al., using similar feeding management, reported that lambs consuming 80.58 g/d protein had significantly lower small intestinal weight than those consuming 117.82 g/d, which contradicts our PR group findings. However, Wang et al.' s study involved different intake levels among lambs, whereas our study maintained similar intake across groups, providing stronger evidence that protein restriction alone does not alter small intestinal weight. Therefore, energy restriction appears more effective than protein restriction in reducing small intestinal weight and causing intestinal damage.

3.2 Effects of Dietary Nutrition Restrictions on Small Intestinal Morphological Development

Villus height reflects cellular proliferation, and increased villus height enhances intestinal absorptive surface area. Studies have shown that restricting nutrition to 40% of normal requirements significantly reduces villus height in ewes and limits intestinal development in their offspring, while increased supplementation under grazing conditions improves small intestinal villus height and promotes intestinal development in weaned calves. Our study demonstrated that simultaneous 20% energy and protein restriction significantly reduced duodenal and jejunal villus height, consistent with Sun et al. However, Sun et al. found no significant effects of protein or energy restriction alone on villus height, which differs from our results. These inconsistencies may be due to differences in metabolic type affecting intestinal morphological development, breed differences, or rumen development stage. Sun et al. studied lambs with mature rumen development, whereas our lambs were in transition from non-ruminant to ruminant status, with some dietary components directly stimulating the intestine. Protein and energy restriction reduce villus height primarily by limiting small intestinal cell hypertrophy and proliferation. Reduced dietary energy or protein levels decrease sodium, potassium-ATPase activity in small intestinal tissue. Sodium, potassium-ATPase is a carrier protein for active transmembrane transport of sodium and potassium ions, and increased activity promotes normal cell hypertrophy and proliferation.

Undifferentiated cells in crypts are the regenerative source for intestinal glands and villus epithelial cells. The number and location depth of these undifferentiated cells are crucial for maintaining intact villus morphology and normal function. Li et al. reported that nutrition restriction reduced jejunal crypt depth in weaned lambs. In our study, crypt depth in all small intestinal segments was lower in restriction groups than in the CON group, with the lowest jejunal crypt depth in the BR group, consistent with Reed et al.' s report that restricting maternal nutrition during pregnancy significantly reduced jejunal crypt cell area and total protein synthesis in newborn lambs. Intestinal mucosal damage typically manifests as shortened villi and increased crypt depth. Our study observed consistent changes in villus height and crypt depth, suggesting that increased crypt depth resulted from increased cell proliferation rather than hyperplasia, though further investigation is needed. Montanholi et al. suggested that reduced feed conversion efficiency decreases crypt cell numbers. Our lambs had similar feed intake, with PR, ER, and BR groups showing lower feed conversion efficiency than the CON group, lowest in the BR group, which also had the lowest crypt depth. However, Sun et al. reported that 40% nutrition restriction for 48 days in 3-month-old weaned lambs did not reduce intestinal crypt depth, possibly due to differences in breed, age, dietary ingredients, and management practices. The V/C ratio comprehensively reflects small intestinal functional status; a decreased ratio indicates reduced digestive and absorptive function, often accompanied by diarrhea, while an increased ratio indicates enhanced function and reduced diarrhea incidence. The BR group showed the lowest jejunal V/C ratio, and observation confirmed that BR group lambs were more prone to diarrhea.

3.3 Effects of Dietary Nutrition Restrictions on Serum IGF-1 Concentration

IGF-1 is widely present in ruminant body fluids and promotes growth and development of the body, organs, and tissues. Both protein and energy affect serum IGF-1 concentration. Yan et al. reported a positive correlation between dietary protein levels (15%, 18%, 21%) and serum IGF-1, consistent with Pell et al. However, Sun et al. found that energy or protein restriction alone did not reduce serum IGF-1 concentration in weaned lambs, while simultaneous energy and protein restriction significantly decreased it. In our study, nutrition restriction reduced serum IGF-1 concentration at 20, 40, and 60 days of age, but differences were not significant. Under nutritional deficiency, animals adapt to environmental impacts through endocrine hormone secretion, primarily by inhibiting somatostatin secretion and increasing growth hormone secretion, which can elevate IGF-1 concentration. However, severe nutrition restriction reduces hepatic growth hormone receptor (GHR) expression, decreasing IGF-1 secretion. In our study and Sun et al.' s study, the restricted nutrition levels in energy or protein restriction groups represented deficiency insufficient to reduce hepatic GHR expression, while simultaneous energy and protein restriction may reduce hepatic GHR gene expression. Therefore, nutrition restriction did not significantly reduce serum IGF-1 concentration in lambs. Lu et al. reported that IGF-1 participates in epithelial cell proliferation. Our study showed that nutrition restriction reduced intestinal villus height and crypt depth without significantly affecting serum IGF-1 concentration, suggesting that IGF-1 is not the sole hormone promoting intestinal morphological development.

3.4 Effects of Dietary Nutrition Restrictions on Serum GLP-2 Concentration

While IGF-1 plays an important role in maintaining intestinal development, it is not specific to intestinal epithelial cells, whereas GLP-2 is a specific promoter of intestinal epithelial cells. Studies indicate that maintaining normal GLP-2 secretion requires that at least 40% of total nutrient intake be provided through the gastrointestinal tract. In our study, serum GLP-2 concentration at 60 days of age was significantly lower in the ER group than in the other three groups, though no significant difference was observed at 40 days of age. This is consistent with Wang' s research in piglets, which reported that short-term dietary changes had minor effects on serum GLP-2 concentration. Górka et al. demonstrated that long-term (29 days) maintenance of high intake levels increased serum GLP-2 concentration. Therefore, short-term energy restriction does not significantly reduce serum GLP-2 concentration in lambs. Studies in rats have shown that GLP-2 promotes repair of intestinal mucosal damage, and Jin et al. reported that villus height in weaned piglets was highly correlated with serum GLP-2 concentration. Observations of intestinal villus morphology combined with intestinal weight revealed severe villus shedding and shorter villi in the ER group, along with significantly lower small intestinal weight compared to the CON group, confirming that lamb small intestinal development is related to GLP-2 concentration. Although nutrition restriction inhibited intestinal morphological development in our study, only the ER group showed reduced serum GLP-2 concentration at 60 days of age. Further research is needed to investigate the effects of nutrition restriction on serum GLP-2 concentration or GLP-2 gene expression, particularly regarding protein restriction.

Conclusion

Dietary nutrition restrictions reduced small intestinal weight at 40 days of age and inhibited morphological development of the duodenum and jejunum, with energy restriction causing the most severe inhibition. Energy restriction decreased serum GLP-2 concentration in lambs at 60 days of age but had no significant effect on serum IGF-1 concentration.

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