Effects of Rumen Fluid Preparation Supplementation on Immunoglobulin Levels in Intestinal Mucosa and Plasma of Lambs Postprint

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Abstract

This experiment aimed to investigate the effects of supplementary feeding of rumen fluid preparations on immunoglobulin content in the intestinal mucosa and plasma of lambs, and to explore the influence of rumen fluid preparations on intestinal mucosal immunity and humoral immunity in newborn lambs.

Fifty newborn lambs with similar birth weights were selected as model animals and randomly divided into 5 groups with 10 lambs per group. Lambs in the experimental groups began receiving supplementary feeding of rumen fluid preparations from healthy adult sheep at 1 day of age [rumen fluid ( ), sterilized rumen fluid ( ), ultrasonicated rumen fluid ( ), and sterilized ultrasonicated rumen fluid ( )], while lambs in the control group received an equal volume of physiological saline, once daily for 5 consecutive days. At 24 days of age, 3 lambs from each group were selected for slaughter to collect intestinal mucosa; at 14 and 28 days of age, jugular vein blood was collected and plasma was separated. The immunoglobulin content in intestinal mucosa and plasma was determined.

The results showed: 1) In small intestinal mucosal proteins, the total amounts of immunoglobulin A (IgA), secretory immunoglobulin A (SIgA), and immunoglobulin G (IgG) in all experimental groups were higher than those in the control group. Among them, the total IgA in experimental group was extremely significantly higher than that in the control group ($P<0.01$) and significantly higher than that in other experimental groups ($P<0.05$); there was no significant difference in the total amounts of SIgA and IgG between the experimental groups and the control group ($P>0.05$). Overall, the trend of immunoglobulin content change was ileum > duodenum > jejunum, and the total immunoglobulin content in experimental group increased more compared with the control group.

2) In plasma, the immunoglobulin content of lambs at 28 days of age was higher than that at 14 days of age. At 14 days of age, there was no sig-

nificant difference in plasma IgA content among the experimental groups (P>0.05), but at 28 days of age, experimental group was significantly higher than the control group (P<0.05) and extremely significantly higher than other experimental groups (P<0.01); there was no significant difference in IgG content among the experimental groups at both 14 and 28 days of age (P>0.05).

The results suggest that supplementary feeding of differently treated rumen fluid preparations to newborn lambs can improve the intestinal mucosal immunity of lambs, with supplementary feeding of ultrasonicated rumen fluid showing the best effect.

Full Text

Effects of Oral Administration of Ruminal Fluid Preparations on Immunoglobulin Contents in Intestinal Mucosa and Plasma of Lambs

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Abstract: This experiment aimed to investigate the effects of oral administration of ruminal fluid preparations on immunoglobulin contents in the intestinal mucosa and plasma of lambs, and to explore the influence of these preparations on intestinal mucosal and humoral immunity in newborn lambs. Fifty newborn lambs with similar birth weights were selected as model animals and randomly divided into five groups of ten lambs each. Lambs in the treatment groups received oral supplementation of ruminal fluid preparations from healthy adult sheep [ruminal fluid (Group I), autoclaved ruminal fluid (Group II), ultrasonicated ruminal fluid (Group III), and autoclaved ultrasonicated ruminal fluid (Group IV)] starting at one day of age, while control group lambs received an equivalent volume of physiological saline. Supplementation occurred once daily for five consecutive days. At 24 days of age, three lambs from each group were slaughtered to collect intestinal mucosa samples. Blood was collected via jugular venipuncture at 14 and 28 days of age to obtain plasma samples. Immunoglobulin contents in both intestinal mucosa and plasma were measured. The results showed: (1) In small intestinal mucosal protein, the total contents of immunoglobulin A (IgA), secretory immunoglobulin A (SIgA), and immunoglobulin G (IgG) in all treatment groups were higher than those in the control group. Specifically, the total IgA content in Group III was extremely

significantly higher than that in the control group (P<0.01) and significantly higher than that in the other treatment groups (P<0.05). No significant differences were observed in total SIgA and IgG contents between treatment and control groups (P>0.05). Overall, immunoglobulin contents followed the trend of ileum > duodenum > jejunum, with Group III showing the greatest increase in total immunoglobulin content compared to the control. (2) In plasma, immunoglobulin contents at 28 days of age were higher than those at 14 days of age. At 14 days, no significant differences in plasma IgA content were detected among groups (P>0.05). However, at 28 days, Group I showed significantly higher IgA content than the control group (P<0.05) and extremely significantly higher content than the other treatment groups (P<0.01). No significant differences in plasma IgG content were observed among groups at either 14 or 28 days of age (P>0.05). These results indicate that oral administration of variously processed ruminal fluid preparations can enhance intestinal mucosal immune function in newborn lambs, with ultrasonicated ruminal fluid demonstrating the most effective results.

Keywords: ruminal fluid preparation; newborn lamb; intestinal mucosa; plasma; immunoglobulin

Introduction

The intestinal mucosal immune system represents the largest and most complex component of the body' s immune system. For newborn animals, the development of this system depends heavily on microbial exposure, as bacterial microorganisms stimulate the production of numerous lymphocytes and lymphoid tissues, promoting the normal development and gradual maturation of the mucosal immune system. Early oral administration of beneficial bacteria (such as microbial preparations derived from the gastrointestinal tract of healthy animals) or their components can regulate intestinal mucosal immunity, and early bacterial stimulation after neonatal inoculation can promote the development and maturation of the small intestinal mucosal immune system. Previous studies have demonstrated that ruminal microorganisms or ruminal fluid inoculants have positive effects on calves and lambs, including increased body weight, reduced diarrhea incidence, and enhanced rumen activity. However, researchers have not fully examined the potential immunological effects of ruminal fluid or rumen bacteria and their components on newborn lambs and calves. This study administered four different ruminal fluid preparations to newborn lambs and measured immunoglobulin contents in both intestinal mucosa and plasma to investigate the effects of ruminal fluid and its preparations on small intestinal mucosal immunity in newborn lambs, providing insights into whether rumen microbes from adult ruminants can serve as appropriate microbial antigens to promote the development of the lamb intestinal mucosal immune system.

1.1 Collection and Processing of Ruminal Fluid Samples

Ruminal fluid was collected from 1.5-year-old Chinese Merino (Xinjiang type) rams fitted with permanent rumen fistulas. The donor animals were fed a diet with a concentrate-to-forage ratio of 40:60 (dry matter basis), with the concentrate supplement composed of 64.5% yellow corn, 32% cottonseed meal, 1% salt, 0.5% calcium hydrogen phosphate, and 2% premix; the primary roughage was corn straw. Ruminal fluid was collected six hours after feeding and filtered through a 60-mesh nylon bag. The filtrate was centrifuged at 1,100×g for 5 minutes at 4 °C to remove rumen protozoa, and the supernatant containing abundant rumen bacteria was collected as the experimental ruminal fluid. A portion of this fluid was used to prepare other ruminal fluid preparations as follows: Autoclaved ruminal fluid was prepared according to the method reported by Muscato et al. by autoclaving a portion of the ruminal fluid at 121 °C for 30 minutes, cooling it, stirring with a magnetic stirrer, and aliquoting. Ultrasonicated ruminal fluid was prepared according to the method reported by Zhai Weishuang by subjecting a portion of ruminal fluid to ultrasonication (4 seconds on, 2 seconds off, 90 cycles, 400 W frequency, repeated three times), followed by magnetic stirring and aliquoting. Autoclaved ultrasonicated ruminal fluid was prepared by autoclaving a portion of the ultrasonicated ruminal fluid at 121 °C for 30 minutes, cooling, stirring with a magnetic stirrer, and aliquoting.

1.2 Experimental Animals and Design

The experiment was conducted at the China-Australia Sheep Breeding Center affiliated with the Xinjiang Academy of Animal Science. Texel lambs with similar birth weights were selected and randomly divided into five groups of ten lambs each: a control group, Group I, Group II, Group III, and Group IV, which received physiological saline, ruminal fluid, autoclaved ruminal fluid, ultrasonicated ruminal fluid, and autoclaved ultrasonicated ruminal fluid, respectively. Each lamb received supplementation within 24 hours after birth (after colostrum feeding) according to its group assignment. Lambs were supplemented once daily with 5 mL of ruminal fluid preparation mixed with 5 mL of boiled commercial milk, warmed to approximately 37 °C before administration. During supplementation, the operator knelt down, held the lamb with the left arm to keep its head naturally elevated, and used the right hand to tilt a feeding bottle so the nipple could be naturally taken into the lamb' s mouth, allowing the lamb to suckle voluntarily for five consecutive days. Except for ruminal fluid supplementation, all other management practices followed the standard protocols of the sheep farm.

1.3 Sample Collection and Processing

1.3.1 Blood Collection and Processing Blood samples (5 mL) were collected via jugular venipuncture from lambs at 14 and 28 days of age using heparin sodium as an anticoagulant. Samples were left to stand at 4 °C for 2

hours before centrifugation at 3,500×g for 15 minutes. The supernatant was collected, aliquoted into 1.5 mL Eppendorf tubes, and stored at -20 °C.

1.3.2 Lamb Slaughter and Intestinal Sample Collection At 24 days of age, three male lambs from each group were slaughtered by exsanguination. The carcasses were positioned on the operating table with the abdomen facing upward, and the abdominal cavity was opened along the midline with a scalpel. The gastrointestinal tract was immediately separated, and each segment was rinsed with chilled physiological saline to remove residues. A 5 cm segment from the distal end of each intestinal section was collected, snap-frozen in liquid nitrogen, and stored at -70 °C for subsequent determination of protein and immunoglobulin contents in the mucosa.

1.4 Determination of Immunoglobulin Contents

1.4.1 Intestinal Mucosal Protein, IgA, SIgA, and IgG Frozen samples were transferred from -70 °C to -20 °C for 2 hours, then thawed slowly at 4 °C. Once completely thawed, intestinal segments were placed in white porcelain trays and opened longitudinally. The mucosa was scraped off using a glass slide and transferred to 1.5 mL Eppendorf tubes, weighed, and mixed with sterile phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.2) at a 1:4 ratio (w/v). The mixture was homogenized and centrifuged at 13,800×g for 10 minutes at 4 °C, and the supernatant was collected. Protein content was determined using Bradford' s method with bovine serum albumin as the standard. IgA, SIgA, and IgG contents in intestinal mucosa were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer' s instructions using kits purchased from Shanghai Huayi Biological Technology Co., Ltd.

1.4.2 Plasma IgA and IgG Plasma IgA and IgG contents were determined by ELISA according to the manufacturer's instructions using kits from Shanghai Huayi Biological Technology Co., Ltd.

1.5 Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA in SPSS 16.0, with Duncan' s multiple range test used for post-hoc comparisons. Differences were considered significant at $P<0.05$ and extremely significant at $P<0.01$.

Results

2.1 Effects of Ruminal Fluid Preparations on Intestinal Mucosal Immunoglobulin Contents in Newborn Lambs

2.1.1 IgA Content in Small Intestinal Mucosal Protein at 24 Days of Age The effects of ruminal fluid preparations on IgA content in small intestinal mucosal protein of 24-day-old lambs are presented in Table 1 . In the

duodenum, Group II showed the highest IgA content, which was significantly different from Groups I, IV, and the control group ($P<0.05$) but not significantly different from Group III ($P>0.05$). Group III was significantly higher than Group I ($P<0.05$). In the jejunum, Group III exhibited the highest IgA content, which was extremely significantly different from Groups I, IV, and the control group ($P<0.01$), while no significant differences were observed among the other groups ($P>0.05$). In the ileum, IgA content was higher than in the duodenum and jejunum, with Group III showing the highest value and the control group the lowest, though differences among groups were not statistically significant ($P>0.05$). Overall, IgA content followed the distribution trend of ileum > duodenum > jejunum. The total IgA content in Group III was extremely significantly higher than that in the control group ($P<0.01$) and significantly higher than that in the other treatment groups ($P<0.05$). Groups I, II, and IV showed higher total IgA contents than the control group, but these differences were not significant ($P>0.05$).

2.1.2 SIgA Content in Small Intestinal Mucosal Protein at 24 Days of Age The effects of ruminal fluid preparations on SIgA content in small intestinal mucosal protein of 24-day-old lambs are shown in Table 2 . In the duodenum, Group III had the highest SIgA content while Group IV had the lowest, with a significant difference between these two groups ($P<0.05$); no significant differences were found among the other groups ($P>0.05$). In the jejunum, SIgA contents varied among groups, with Groups II and III showing relatively higher values, but no significant differences were detected ($P>0.05$). In the ileum, all treatment groups had higher SIgA contents than the control group, though differences were not significant ($P>0.05$). Overall, SIgA content followed the distribution pattern of ileum > duodenum > jejunum, with Groups I and IV showing relatively greater increases in the ileum. Total SIgA contents in all treatment groups were higher than that in the control group, but differences were not significant ($P>0.05$). Specifically, total SIgA contents in Groups I, II, III, and IV were 7.14%, 23.70%, 27.60%, and 14.29% higher than the control group, respectively.

2.1.3 IgG Content in Small Intestinal Mucosal Protein at 24 Days of Age The effects of ruminal fluid preparations on IgG content in small intestinal mucosal protein of 24-day-old lambs are presented in Table 3 . In the duodenum, Groups II and III showed relatively higher IgG contents, while Group I had the lowest value. Groups II and III were significantly different from Group I ($P<0.05$), with no significant differences among other groups ($P>0.05$). In the jejunum, IgG contents varied among groups but differences were not significant ($P>0.05$), with Group I remaining the lowest and Group III the highest. In the ileum, Group IV had the highest IgG content, which was significantly different from Group II and the control group ($P<0.05$), but not significantly different from the other groups ($P>0.05$). Overall, IgG content followed the distribution trend of ileum > duodenum > jejunum, with Groups

I, III, and IV showing relatively greater increases in the ileum. Groups III and IV had higher total IgG contents, but no significant differences were observed among groups (P>0.05). Compared to the control group, total IgG contents in Groups I, II, III, and IV increased by 4.74%, 23.81%, 37.19%, and 38.66%, respectively.

2.2 Effects of Ruminal Fluid Preparations on Plasma Immunoglobulin Contents in Newborn Lambs

The effects of ruminal fluid preparations on plasma IgA and IgG contents in 14- and 28-day-old lambs are shown in Table 4 . Plasma IgA and IgG contents were higher at 28 days than at 14 days of age. At 14 days, no significant differences in plasma IgA content were observed among groups (P>0.05), with the control group showing the highest value and Group II the lowest. At 28 days, Group I had the highest IgA content, which was significantly higher than that in the control group (P<0.05) and extremely significantly higher than that in Groups II, III, and IV (P<0.01); no significant differences were found among the other groups (P>0.05). No significant differences in plasma IgG content were detected among groups at either 14 or 28 days of age (P>0.05), although IgG contents in all treatment groups increased from 14 to 28 days.

Discussion

3.1 Effects of Ruminal Fluid Preparations on Intestinal Mucosal Immunity in Newborn Lambs

Husband and Peterson et al. have confirmed that antibodies produced by plasma cells in the intestinal mucosal immune system are primarily IgA, which combines with secretory components from epithelial cells to form SIgA after synthesis in plasma cells. SIgA serves as a crucial effector molecule in mucosal immune responses, mainly synthesized by B cells in the lamina propria, while IgG is closely associated with disease resistance and susceptibility. The development of the mucosal immune system largely depends on microbial exposure, and the innate immune response induced by commensal bacteria plays an important role in the maturation and development of both mucosal and peripheral immune systems. Microbial exposure is essential for mucosal immunity and intestinal epithelial cell development, stimulating the secretion of more IgA+ plasma cells in gut-associated lymphoid tissue. The intestines of newborn infants and animals are sterile at birth. In germ-free mice, SIgA secretion is significantly reduced due to the lack of stimulation from intestinal microbiota, while serum IgA content only decreases to half of normal levels. However, SIgA secretion increases after colonization with a certain amount of commensal bacteria. In specific pathogen-free (SPF) mice, despite the absence of pathogenic bacteria in the intestinal flora, substantial SIgA secretion still occurs in the intestinal lumen, demonstrating the stimulatory effect of commensal bacteria on SIgA production. Perdigón et al. reported that probiotics can increase mucosal IgA content, and Dogi et al. found that both Gram-positive bacteria such as *Lactobacillus acidophilus*

CRL1462 and A9, and Gram-negative bacteria such as *Escherichia coli* 129 and 13-7, can significantly increase the number of IgA+ B cells in BALB/c mice. Che Chuanyan observed that oral inoculation of human-derived flora into newborn SPF piglets increased the number of IgA and IgG-secreting cells in the piglet intestine.

Traditionally, research has focused primarily on the nutritional value of rumen microorganisms, with little attention paid to the potential immunological effects of ruminal fluid or rumen bacteria and their components on newborn lambs and calves. Rumen fluid from ruminants contains a diverse array of bacteria and microorganisms, including hundreds of bacterial polysaccharide molecules. In this study, newborn lambs were supplemented with variously processed adult sheep ruminal fluid preparations within 24 hours after birth, a period when small intestinal permeability is particularly high and intestinal epithelial development is immature, creating an "open" state. The bacteria present in the ruminal fluid preparations, acting as antigenic substances, entered the offspring' s intestine and were absorbed, potentially altering the intestinal microbial environment and inducing corresponding changes in the mucosal immune system of gut-associated lymphoid tissue.

In this experiment, IgA and SIgA contents in intestinal mucosa were higher in all treatment groups than in the control group, indicating that rumen bacteria possess "probiotic" effects that can stimulate the development and maturation of intestinal mucosal immunity in newborn lambs, resulting in greater IgA release. The autoclaved ruminal fluid group released more immune effector molecules than the non-autoclaved group, consistent with the report by Muscato et al. that autoclaved ruminal fluid has more positive effects on calves. This may be attributed to two factors: first, autoclaving reduces harmful substances in ruminal fluid, and second, the sterilization process partially disrupts bacterial structures, releasing more heat-resistant immune effector factors such as peptidoglycan and lipopolysaccharide from bacterial cell walls. In particular, the treatment in Group III (ultrasonicated ruminal fluid) ruptures bacterial cell walls, releasing peptidoglycan, lipopolysaccharide, and teichoic acid. Peptidoglycan acts as an immune system activator, and small amounts are crucial for maintaining and promoting important physiological functions in the host.

In addition to IgA, IgG is also an important effector molecule in ruminant intestinal mucosal immunity. In this study, IgG was found to be the most abundant immunoglobulin in the intestinal mucosa of 24-day-old lambs, consistent with findings by Cripps et al. and Butler. Bouvet' s research indicates that IgG in the intestinal mucosa of early offspring partially originates from maternal IgG that enters the offspring' s bloodstream through milk and then reaches the intestinal lumen via the liver and bile, while another portion is produced locally by intestinal mucosal B cells, providing immune protection before autonomous IgA synthesis. Passive mucosal protection in newborn mammals before weaning depends on the continuous supply of maternal dimeric IgA and IgG1, which play anti-infective roles in the body. Although SIgA from breast milk maintains its

local immune and anti-infective capabilities after entering the digestive tract, incomplete development of the intestinal mucosal immune system may result in relatively low SIgA content in the intestine.

The distribution pattern of immunoglobulins in the small intestinal mucosa of 24-day-old lambs in this study was ileum > duodenum > jejunum. This differential distribution across intestinal segments may be related to the distribution of plasma cells in the intestinal lamina propria and could also result from different microbial antigens encountered in various segments stimulating increased plasma cell secretion. The duodenum, located at the proximal small intestine, first contacts antigens and interacts with foreign antigens earliest and most directly, generating intense immune responses and thus higher immunoglobulin content. The ileal mucosa and submucosa contain abundant aggregated lymphoid nodules with numerous B cells, providing substantial potential for plasma cell production, which may also contribute to higher immunoglobulin levels in the ileum.

Supplementation with variously processed ruminal fluid preparations resulted in higher immunoglobulin contents in small intestinal mucosal protein compared to the control group. This may be due to the presence of immunologically active substances in the ruminal fluid preparations that stimulated the intestinal mucosa to produce more immune effector molecules. Alternatively, some bacteria present in the ruminal fluid may have colonized the intestine, promoting the development of gut-associated lymphoid tissue and enhancing local humoral immunity and overall immune function. However, the specific immunologically active substances in ruminal fluid were not further analyzed in this study and warrant future investigation. Additionally, data on immunoglobulin content and distribution characteristics in low-age lambs are currently limited, and further research in this area is needed.

3.2 Effects of Ruminal Fluid Preparations on Plasma Immunoglobulin Contents in Newborn Lambs

IgG is an important antibody in serum that plays a key role in humoral immunity by neutralizing toxins and viruses, agglutinating particulate antigens (such as bacteria and viruses) to facilitate phagocytosis, and activating complement. Serum IgG antibodies can prevent corresponding antigens from penetrating the mucosa into tissues. IgA exists in two forms: serum-type (mostly monomers, some dimers) and secretory-type (all dimers containing secretory pieces), accounting for only 10-15% of total serum immunoglobulins. Newborn lambs lack disease resistance and must obtain passive immunity by acquiring sufficient immunoglobulins from colostrum within 24 hours after birth. This passive immunity gradually weakens with age, and autogenous immune capacity begins to establish at approximately four weeks of age. In this study, plasma immunoglobulin contents were higher at 28 days than at 14 days, indicating that lambs' immune function gradually strengthened with increasing age.

Supplementation with variously processed ruminal fluid preparations had no significant effect on plasma IgG content, likely because IgG in early offspring plasma primarily originates from the dam, resulting in minimal differences among treatment groups. In contrast, plasma IgA contents differed substantially among treatment groups at 28 days of age, with the ruminal fluid group (Group I) showing the highest value, while the autoclaved and ultrasonicated ruminal fluid groups had lower values. This pattern was inconsistent with IgA distribution in intestinal mucosa. Previous studies have reported that in healthy organisms, serum monomeric IgA antibodies respond to mucosal or vascular space immune stimulation, but these antibodies do not originate from intestinal mucosa. The present results also suggest that plasma IgA content in lambs is minimally affected by intestinal mucosal IgA, although ruminal fluid supplementation can enhance humoral immunity to some extent, and the underlying mechanisms require further investigation.

Conclusion

Oral administration of variously processed ruminal fluid preparations can increase immunoglobulin contents in the small intestinal mucosa of newborn lambs, thereby enhancing intestinal mucosal immune function, with ultrasonicated ruminal fluid demonstrating the most effective results.

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