Effects of Rumen-Protected 5-Hydroxytryptophan on 5-Hydroxytryptophan and Melatonin Levels in Gastrointestinal Contents and Plasma of Sheep (Postprint)

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Abstract

This experiment aimed to investigate the distribution characteristics of melatonin in the gastrointestinal contents of sheep, explore the effects of rumen-protected 5-hydroxytryptophan on the contents of 5-hydroxytryptophan and melatonin in the gastrointestinal tract and plasma of sheep, and discuss the possibility of regulating melatonin synthesis in the sheep intestine through 5-hydroxytryptophan. The experiment selected 15 healthy 3-year-old Kazakh ewes with an average body weight of (47.79±3.70) kg, which were divided into 3 groups based on body weight, with 5 sheep per group, namely a control group and experimental groups I and II. The daily powdered concentrate feeding amount per sheep was 1.2% of body weight, corn silage feeding amount was 0.9 kg, and mixed hay was provided ad libitum. On this basis, sheep in experimental groups I and II were fed 111 and 222 mg/kg BW of rumen-protected 5-hydroxytryptophan, respectively, for a 15-day feeding trial. The results showed that the distribution characteristics of 5-hydroxytryptophan content in the gastrointestinal contents of sheep were: cecum > jejunum, colon > rumen fluid, duodenum, ileum; the distribution characteristics of melatonin content were: duodenum > rumen fluid, jejunum > ileum, cecum > colon. Except for the cecum and colon, the 5-hydroxytryptophan content in the gastrointestinal contents of sheep in experimental groups I and II was extremely significantly higher than that in the control group ($P<0.01$). The contents of 5-hydroxytryptamine and N-acetylserotonin in the duodenal contents of experimental group II were extremely significantly higher than those in the control group ($P<0.01$), and the contents of 5-hydroxytryptamine and N-acetylserotonin in the colonic contents were significantly higher than those in the control group ($P<0.05$); the contents of 5-hydroxytryptamine and N-acetylserotonin in the jejunal and ileal contents

of experimental group I were extremely significantly lower than those in the control group and experimental group II (P<0.01). The melatonin content in the colonic contents of experimental groups I and II was extremely significantly higher than that in the control group (P<0.01). The 5-hydroxyindoleacetic acid content in the duodenal and jejunal contents of experimental groups I and II was extremely significantly lower than that in the control group (P<0.01), but the 5-hydroxyindoleacetic acid content in the cecal and colonic contents of experimental group II was extremely significantly higher than that in the control group (P<0.01). The contents of 5-hydroxytryptophan, 5-hydroxytryptamine, and melatonin in the plasma of experimental group II were significantly or extremely significantly higher than those in the control group (P<0.05 or P<0.01). Therefore, the distribution characteristics of 5-hydroxytryptophan content in the gastrointestinal contents of sheep were: cecum > jejunum, colon > rumen fluid, duodenum, ileum; the distribution characteristics of melatonin content were: duodenum > rumen fluid, jejunum > ileum, cecum > colon. When the supplementation level of rumen-protected 5-hydroxytryptophan was 222 mg/kg BW, it could increase the 5-hydroxytryptophan content in the gastrointestinal contents, but its effects on the contents of 5-hydroxytryptamine and melatonin showed different patterns in various gastrointestinal segments. When the supplementation levels of rumen-protected 5-hydroxytryptophan were 111 and 222 mg/kg BW, both could increase the contents of 5-hydroxytryptophan, 5-hydroxytryptamine, and melatonin in the plasma of sheep.

Full Text

Effects of Rumen-Protected 5-Hydroxytryptophan on Contents of 5-Hydroxytryptophan and Melatonin in Gastrointestinal Tract Digesta and Plasma of Sheep

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Abstract: This study investigated the distribution characteristics of melatonin in the gastrointestinal tract digesta of sheep and examined the effects of rumen-protected 5-hydroxytryptophan (5-HTP) supplementation on 5-HTP and melatonin concentrations in both gastrointestinal digesta and plasma. The research also explored the potential for modulating intestinal melatonin synthesis in sheep through 5-HTP administration.

Fifteen healthy 3-year-old Kazak ewes with an average body weight of (47.79±3.70) kg were randomly allocated into three groups (n=5 per group): a control group and two treatment groups (Trial I and Trial II). All sheep received a basal diet consisting of powdered concentrate at 1.2% of body weight,

0.9 kg corn silage per head daily, and ad libitum access to mixed hay. The treatment groups received additional rumen-protected 5-HTP at dosages of 111 mg/kg BW (Trial I) and 222 mg/kg BW (Trial II) for a 15-day feeding period.

The distribution patterns in control sheep revealed distinct regional differences: 5-HTP content was highest in the caecum, followed by jejunum and colon, with lowest levels in rumen fluid, duodenum, and ileum. Melatonin content followed a different pattern, being highest in duodenum, followed by rumen fluid and jejunum, then ileum and caecum, with lowest levels in colon.

Supplementation with rumen-protected 5-HTP significantly altered these profiles. Except in caecum and colon, both treatment groups showed extremely significantly higher 5-HTP concentrations in gastrointestinal digesta compared to control ($P<0.01$). In duodenum, Trial II exhibited extremely significantly elevated 5-hydroxytryptamine (5-HT) and N-acetylserotonin (NAS) levels ($P<0.01$), while colon content showed significantly increased 5-HT and NAS ($P<0.05$). Conversely, Trial I demonstrated extremely significantly lower 5-HT and NAS in jejunum and ileum compared to both control and Trial II ($P<0.01$). Melatonin concentration in colon digesta was extremely significantly higher in both treatment groups ($P<0.01$). Regarding 5-hydroxyindoleacetic acid (5-HIAA), treatment groups showed extremely significantly lower levels in duodenum and jejunum ($P<0.01$), but Trial II exhibited extremely significantly higher 5-HIAA in caecum and colon ($P<0.01$). Plasma analysis revealed that Trial II had significantly or extremely significantly elevated 5-HTP, 5-HT, and melatonin concentrations compared to control ($P<0.05$ or $P<0.01$).

These findings demonstrate that rumen-protected 5-HTP at 222 mg/kg BW effectively increases 5-HTP content throughout the gastrointestinal tract, though its effects on 5-HT and melatonin vary by segment. Both dosage levels (111 and 222 mg/kg BW) successfully elevated plasma concentrations of 5-HTP, 5-HT, and melatonin in sheep.

Keywords: sheep; gastrointestinal tract; digesta; rumen-protected 5-hydroxytryptophan; melatonin

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Introduction

Melatonin (MT) is synthesized not only in the pineal gland but also in substantial quantities within intestinal tissues, where concentrations are reportedly 400–500 times higher than in the pineal gland. Intestinal melatonin originates from enterochromaffin cells in the intestinal mucosa, which secrete melatonin into the gut lumen. The distribution of melatonin throughout the gastrointestinal tract exhibits marked species-specific variations. In pigs, melatonin content decreases in the order: colon > caecum > ileum > jejunum > stomach, whereas in cattle the pattern is: ileum, colon, caecum > rumen, reticulum, omasum, abomasum > jejunum. In rats, rectal content shows relatively high melatonin

levels, while stomach, duodenum, jejunum, ileum, and colon contain lower concentrations. Notably, in pigs fasted for 30 hours and then refed, melatonin levels in intestinal tissues, mucosa, and contents increased significantly across all segments, peaking at 5 hours post-feeding. These observations indicate that fasting/refeeding cycles and intake of melatonin or its precursors can substantially influence melatonin concentrations in intestinal tissues and contents.

Melatonin synthesized from tryptophan in intestinal mucosa and secreted into the lumen plays a crucial role in regulating gut motility and maintaining tissue health. 5-Hydroxytryptophan (5-HTP) represents a key intermediate in the metabolic conversion of tryptophan to melatonin. Through sequential enzymatic reactions catalyzed by aromatic amino acid decarboxylase, serotonin N-acetyltransferase, and hydroxyindole O-methyltransferase, 5-HTP is ultimately converted to melatonin. However, the distribution patterns of melatonin and its precursors, including 5-HTP and 5-hydroxytryptamine (5-HT), in sheep intestinal digesta remain uncharacterized. Therefore, this study was designed to: (1) characterize the baseline distribution of melatonin and its precursors in sheep gastrointestinal digesta, and (2) evaluate how supplementation with rumen-protected 5-HTP, a melatonin precursor, affects the concentrations of these compounds in the gut and plasma, thereby providing a scientific foundation for understanding melatonin' s role in regulating gastrointestinal function and maintaining tissue health in ruminants like sheep.

Materials and Methods

1.1 Experimental Period and Location The feeding trial was conducted from August 1 to August 16, 2017, at the Xinjiang Huikang Animal Biotechnology Co., Ltd. sheep farm. On the day of blood sampling, sunrise occurred at 06:08 and sunset at 22:04.

1.2 Experimental Materials Pure 5-HTP (98% purity) was purchased from Wuhan Yuancheng Gongchuang Technology Co., Ltd. Rumen-protected 5-HTP was manufactured by Beijing Yahe Nutrition High-tech Co., Ltd., using 5-HTP as the active ingredient, with an effective 5-HTP content of 45.00% and a rumen bypass rate of 88.60%. The rumen bypass rate was determined by incubating 10 g of the protected product in a conical flask with 100 mL distilled water at 37°C and 50 r/min for 4 hours, followed by filtration and HPLC quantification of 5-HTP in the filtrate. The bypass rate was calculated as: [(5-HTP content in protected product - 5-HTP content in filtrate) / 5-HTP content in protected product] × 100%.

1.3 Experimental Design Fifteen healthy 3-year-old Kazak ewes weighing (47.79±3.70) kg were stratified by body weight into three groups (n=5): control, Trial I, and Trial II. All sheep received a basal diet of powdered concentrate (1.2% BW), corn silage (0.9 kg/head/day), and ad libitum mixed hay (al-

falfa:wheat straw 1:1). Treatment groups received additional rumen-protected 5-HTP at 111 mg/kg BW (Trial I) or 222 mg/kg BW (Trial II), with dosages based on Namboodiri et al. The composition and nutrient levels of the concentrate are presented in Table 1 , while those of corn silage, alfalfa, and wheat straw are shown in Table 2 .

1.4 Feeding Management During the 15-day trial, daily rations of concentrate, silage, and rumen-protected 5-HTP were divided equally into two meals provided at 07:45 and 19:45. The rumen-protected 5-HTP was mixed with 50 g concentrate for each feeding to ensure complete consumption before offering the remaining concentrate and silage. Sheep were individually penned during feeding and allowed free movement in the exercise area afterward.

1.5 Sample Collection and Processing On day 16, blood samples were collected via jugular venipuncture 6 hours after the morning feeding, and plasma was prepared. Sheep were then immediately slaughtered. After 2 minutes of exsanguination, the abdominal cavity was opened along the ventral midline. The gastrointestinal tract was ligated at the junctions of rumen, duodenum, jejunum, ileum, colon, and caecum. Digesta were immediately expelled into clean plastic beakers, mixed thoroughly, aliquoted into cryovials (rumen fluid was filtered through 60-mesh nylon), snap-frozen in liquid nitrogen, and stored until analysis.

1.6 Sample Analysis Plasma concentrations of 5-HTP, 5-HT, and melatonin were determined at Beijing Huaying Biotechnology Institute using radioimmunoassay (XH-6020 automatic gamma counter, Xi'an Nuclear Instrument Factory) for 5-HTP and melatonin, and enzyme immunoassay (Huaweidelang DR-200BS microplate reader, Wuxi Huaweidelang Instrument Co., Ltd.) for 5-HT.

Gastrointestinal digesta concentrations of 5-HTP, 5-HT, N-acetylserotonin (NAS), melatonin, and 5-hydroxyindoleacetic acid (5-HIAA) were quantified using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) with an Agilent 1290 Infinity series UHPLC System and Thermo Fisher Scientific Q Exactive Focus mass spectrometer. Chromatographic separation was achieved on a Waters ACQUITY UPLC BEH C18 column (100.0 mm × 2.1 mm, 1.7 m) using parallel reaction monitoring (PRM) mode.

Sample preparation involved homogenizing 100 mg digesta (or 1 mL rumen fluid) with 1 mL extraction solvent (acetonitrile:methanol:water = 2:2:1) in an Eppendorf tube, vortexing for 30 s, bead-beating at 45 Hz for 10 min, sonicating on ice for 10 min, and centrifuging at 12,000 r/min for 15 min at 4°C. The supernatant (100 L) was analyzed by UHPLC-MS/MS. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B was acetonitrile. Column temperature was maintained at 40°C, sample tray at 4°C, and injection volume was 1 L. Mass

spectrometry parameters included spray voltage (+3,500/-3,100 V), sheath gas flow (N) = 40, auxiliary gas flow (N) = 5, auxiliary gas temperature = 350°C, and ion transfer tube temperature = 320°C. The chromatographic gradient is detailed in Table 3 .

Prior to analysis, standard solutions of target compounds were infused to optimize PRM parameters for each analyte. The most abundant product ion was selected for quantification, with additional product ions used for confirmation. Optimized PRM parameters are listed in Table 4 .

1.7 Statistical Analysis Data were initially processed using Excel 2003 and expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA in SPSS 18.0 software, with Duncan' s multiple range test used for post-hoc comparisons between groups.

Results

2.1 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Rumen Fluid As shown in Table 5 , both Trial I and Trial II exhibited extremely significantly higher 5-HTP content in rumen fluid compared to control ($P<0.01$). Trial II also showed extremely significantly elevated 5-HT concentration ($P<0.01$). Interestingly, control group had significantly higher melatonin content than Trial II ($P<0.05$), while Trial I did not differ significantly from control ($P>0.05$). No significant differences were observed among groups for NAS or 5-HIAA concentrations ($P>0.05$).

2.2 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Duodenum Digesta Table 6 reveals that in duodenum digesta, Trial II had extremely significantly higher 5-HTP, 5-HT, and NAS contents compared to control ($P<0.01$). However, both treatment groups showed extremely significantly lower melatonin and 5-HIAA concentrations than control ($P<0.01$).

2.3 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Jejunum Digesta According to Table 7 , jejunum digesta from both treatment groups contained extremely significantly higher 5-HTP levels than control ($P<0.01$). Trial II exhibited extremely significantly elevated 5-HT, NAS, melatonin, and 5-HIAA contents compared to control and Trial I ($P<0.01$), while control group had extremely significantly higher 5-HIAA than both treatment groups ($P<0.01$).

2.4 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Ileum Digesta Table 8 demonstrates that in ileum digesta, 5-HTP

content followed the pattern Trial II > Trial I > control, with extremely significant differences among all groups (P<0.01). Both treatment groups showed extremely significantly lower 5-HT content than control (P<0.01). NAS content was extremely significantly higher in control and Trial II compared to Trial I (P<0.01). No significant differences were observed for melatonin or 5-HIAA among groups (P>0.05).

2.5 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Caecum Digesta As presented in Table 9 , control group had extremely significantly higher 5-HTP, 5-HT, and NAS contents than both treatment groups (P<0.01), with Trial II being extremely significantly higher than Trial I for 5-HTP and NAS (P<0.01). No significant differences were detected for melatonin content among groups (P>0.05). Trial II showed extremely significantly higher 5-HIAA content compared to control (P<0.01).

2.6 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Colon Digesta Table 10 indicates that colon digesta from both treatment groups had higher 5-HTP, 5-HT, NAS, melatonin, and 5-HIAA contents than control. Specifically, Trial II exhibited extremely significantly higher 5-HTP (P<0.01) and significantly higher 5-HT and NAS (P<0.05). Both treatment groups showed extremely significantly elevated melatonin content (P<0.01) and significantly or extremely significantly higher 5-HIAA (P<0.05 or P<0.01).

2.7 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Sheep Plasma Table 11 shows that 6 hours after morning feeding, plasma 5-HTP concentration increased with supplementation, being extremely significantly higher in Trial II than control (P<0.01). Plasma 5-HT was also significantly elevated in Trial II (P<0.01), and melatonin content was significantly higher in Trial II compared to control (P<0.05).

Discussion

3.1 Distribution Characteristics of 5-HTP, 5-HT, and Melatonin in Sheep Gastrointestinal Digesta Limited research has reported the distribution of 5-HTP, 5-HT, melatonin, NAS, and 5-HIAA in sheep intestinal digesta. The present study revealed distinct regional patterns for 5-HTP content: caecum > jejunum, colon > rumen fluid, duodenum, ileum. The 5-HTP detected in intestinal digesta likely originates from microbial hydroxylation of dietary tryptophan. As a crucial precursor of 5-HT, the physiological significance of these distribution differences and any additional functions of 5-HTP remain to be elucidated.

Serotonin is predominantly localized in enterochromaffin cells of the intestinal mucosa. In rats, 5-HT concentrations in intestinal tissues follow the pattern:

jejunum < stomach < ileum < colon. In the current study, control sheep exhibited highest 5-HT content in colon, with the overall pattern: rumen fluid < duodenum < jejunum < ileum < caecum < colon, which aligns with rat tissue distribution. However, in Meishan pigs, 5-HT is distributed throughout duodenum, jejunum, ileum, and colon, with highest levels in duodenum. These discrepancies may reflect species-specific differences in digestive physiology and dietary tryptophan levels.

NAS and 5-HIAA are both metabolites of 5-HT, with NAS serving as a critical intermediate in melatonin synthesis. Rat studies show 5-HIAA distribution as: stomach, ileum < jejunum < colon. In the present study, control sheep had relatively high NAS and 5-HIAA in rumen fluid and colon digesta. The gastrointestinal tract contains abundant melatonin, synthesized primarily in enterochromaffin cells of the intestinal mucosa from L-tryptophan via hydroxylation, decarboxylation, and acetylation. The distribution correlates with enterochromaffin cell density. Potential sources of intestinal melatonin include intestinal mucosa, diet, and gut microbiota. Additionally, mammalian bile contains high melatonin concentrations, suggesting enterohepatic circulation as another potential source.

The observed melatonin distribution (duodenum > rumen fluid, jejunum > ileum, caecum > colon) differs from previous reports in cattle (ileum, colon, caecum > rumen, reticulum, omasum, abomasum > jejunum) and Spanish fighting bulls (esophagus: 73, stomach: 78, anterior intestine: 20, posterior intestine: 152 pg/g). These variations may stem from differences in digestive absorption, feed intake regulation, digesta transit rates, and particularly sampling time—bovine samples were collected immediately post-feeding, whereas ours were taken 6 hours after feeding. Thus, intestinal melatonin levels appear influenced by multiple factors including feeding schedule, dietary composition, and species-specific digestive characteristics.

3.2 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Gastrointestinal Digesta Namboodiri et al. administered 5-HTP to sheep via intraperitoneal injection at 20 and 200 mg/kg BW, which directly enters systemic circulation. In contrast, dietary supplementation undergoes gastrointestinal metabolism, with uncertain ruminal degradation. Therefore, we selected 111 and 222 mg/kg BW dosages for rumen-protected 5-HTP. Post-supplementation increases in rumen fluid 5-HTP and 5-HT suggest that mechanical rumen motility and dietary pressure may rupture some protective coatings, releasing 5-HTP for conversion to 5-HT. The increased 5-HTP, 5-HT, and NAS alongside decreased melatonin in rumen fluid implies that rumen-protected 5-HTP may not regulate hydroxyindole O-methyltransferase, the rate-limiting enzyme in melatonin synthesis. The origin of ruminal melatonin remains unclear, though ruminant saliva contains melatonin that could enter the rumen during rumination and feeding.

Increased duodenal 5-HTP content following supplementation confirms release

of rumen-protected 5-HTP through rumen motility and intestinal enzymatic action. While duodenal 5-HT and NAS increased with dosage, melatonin decreased, further supporting hydroxyindole O-methyltransferase as the rate-limiting enzyme. Low duodenal pH, abundant food antigens, commensal microbes, bile salts, and pancreatic secretions may modulate enzyme activity or expression, limiting the impact of 5-HTP supplementation on duodenal melatonin synthesis. In dogs, oral 5-HTP (0.5 mg/kg BW) increased duodenal 5-HIAA within 0.5 hours, contrasting with our observed decrease, possibly reflecting species differences and dietary variations.

Supplementation increased jejunal and ileal 5-HTP content, yet Trial I showed reduced 5-HT, NAS, melatonin, and 5-HIAA in these segments. Although key enzyme activities were not measured, these results suggest potential modulation of serotonin N-acetyltransferase and hydroxyindole O-methyltransferase. In sheep pineal gland, these enzymes show diurnal variation (day: 1.1 and 0.14 nmol/(min · mg prot); night: 3.1 and 0.29 nmol/(min · mg prot)). The 222 mg/kg BW dosage significantly increased all measured compounds in jejunum, suggesting this level may provide sufficient substrate to modulate melatonin synthesis enzyme activity, whereas 111 mg/kg BW may be insufficient.

Interestingly, caecal 5-HTP, 5-HT, and NAS decreased while 5-HIAA increased with supplementation. Three factors may explain this: (1) the caecum is the shortest intestinal segment, and by 6 hours post-feeding, exogenous 5-HTP and metabolites may have transited through; (2) 5-HT promotes intestinal motility, potentially reducing caecal residence time; and (3) increased 5-HIAA suggests enhanced monoamine oxidase activity, shunting 5-HT toward 5-HIAA rather than NAS and melatonin, though this requires further investigation.

In colon, all measured compounds increased with supplementation dosage, indicating rumen-protected 5-HTP may regulate each enzymatic step in colonic melatonin synthesis. Gut microbiota can synthesize melatonin, and sheep harbor particularly diverse microbial communities in the colon. Our supplementation likely provided additional substrate for colonic microbes, increasing melatonin content. Additionally, by 6 hours post-feeding, digesta from upstream segments may have reached the colon, contributing to the observed increases. While our results demonstrate that rumen-protected 5-HTP can increase gastrointestinal 5-HT and melatonin—both critical for gut physiological function—further research is needed to determine impacts on digestive metabolism.

3.3 Effects of Rumen-Protected 5-HTP on 5-HTP and Melatonin Contents in Sheep Plasma Six hours post-administration, plasma 5-HTP and 5-HT concentrations increased, consistent with findings in humans, dogs, and sheep following intraperitoneal injection or oral 5-HTP administration. The elevated plasma 5-HT and melatonin likely originate from the gastrointestinal tract. Studies in pigs demonstrated that intestinal melatonin absorption results in substantial venous melatonin levels even after hepatic metabolism, confirming that intestinal melatonin can influence circulating levels. Similarly, tryptophan

administration to pinealectomized chickens increased plasma melatonin, suggesting extra-pineal synthesis in the gut. Our findings support the hypothesis that plasma melatonin can be derived from gastrointestinal sources and that precursor supplementation can effectively increase circulating melatonin through intestinal absorption.

Conclusion

1. The distribution of 5-HTP in sheep gastrointestinal digesta follows the pattern: caecum > jejunum, colon > rumen fluid, duodenum, ileum. Melatonin distribution follows: duodenum > rumen fluid, jejunum > ileum, caecum > colon.
2. Rumen-protected 5-HTP supplementation at 222 mg/kg BW effectively increases 5-HTP content throughout the gastrointestinal tract, though its effects on 5-HT and melatonin vary by segment.
3. Both 111 and 222 mg/kg BW dosages of rumen-protected 5-HTP significantly increase plasma concentrations of 5-HTP, 5-HT, and melatonin in sheep.

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