Effects of Calcium Folinate Supplementation in a Rumen-Protected Tryptophan Diet on Plasma Tryptophan, Kynurenine, and Melatonin Concentrations in Sheep (Postprint)

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Abstract

This study investigated the effects of dietary supplementation with two doses of calcium folinate (CF) in a rumen-protected tryptophan (RPTrp)-based diet on plasma tryptophan (Trp), kynurenine (Kyn), and melatonin (MT) concentrations in sheep, aiming to explore methods for regulating MT synthesis in ruminants. The experiment utilized 15 healthy Suffolk sheep aged (3.0±0.5) years with an average body weight of (64.45±3.48) kg, which were allocated into three groups (n=5 per group) based on body weight: a control group and experimental groups I and II. The daily ration per sheep consisted of concentrate supplement at 12 g/kg BW, corn silage at 1.8 kg, RPTrp at 222.2 mg/kg BW, and mixed hay ad libitum. Experimental group I received an additional 50 mg CF, while experimental group II received 100 mg CF. The feeding trial lasted for 15 days. The results demonstrated: 1) During the 0–12 h period following morning feeding, no significant differences in plasma Trp or Kyn concentrations were observed among groups ($P>0.05$). However, at 6 h and 8 h, plasma Trp concentrations in the experimental groups exhibited a decreasing trend ($P=0.0872$ and $P=0.0531$, respectively). Similarly, at 4.5, 6, 8, and 10 h, plasma Kyn concentrations in the experimental groups showed a decreasing trend ($P=0.0948$, $P=0.0667$, $P=0.0909$, and $P=0.0542$, respectively). 2) At 4.5 and 8 h post-feeding, plasma 5-hydroxytryptamine (5-HT) concentrations in the experimental groups displayed an increasing trend compared with the control group ($P=0.0807$ and $P=0.0541$, respectively), and at 10 h, the increase was highly significant ($P=0.0057$). At 6 and 8 h post-feeding, MT concentrations in the experimental groups also showed an increasing trend ($P=0.0890$ and $P=0.0704$, respectively), and at 10 h, they were highly significantly higher than those in the control group ($P=0.0002$). 3) At 0 h (pre-feeding), experimental

group II exhibited significantly elevated plasma total antioxidant capacity and glutathione peroxidase activity, along with significantly reduced malondialdehyde content, compared with the control group ($P<0.05$). Experimental group I also demonstrated significantly increased plasma total antioxidant capacity ($P<0.05$). Therefore, daily supplementation with 50 or 100 mg CF per sheep in addition to a RPTrp diet (222.2 mg/kg · BW) showed a trend toward reduced plasma Kyn concentrations during the 4.5–10 h post-feeding period, exerted no significant overall effect on plasma Trp, 5-HT, and MT concentrations, but enhanced the plasma antioxidant capacity of sheep.

Full Text

Effects of Dietary Calcium Folinate Supplementation on Plasma Tryptophan, Kynurenine, and Melatonin Contents in Sheep Fed Rumen-Protected Tryptophan

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Abstract: This study investigated the effects of two doses of calcium folinate (CF) supplementation on plasma tryptophan (Trp), kynurenine (Kyn), and melatonin (MT) concentrations in sheep fed rumen-protected tryptophan (RPTrp) to explore methods for regulating MT synthesis in ruminants. Fifteen healthy Suffolk sheep aged (3.0±0.5) years with an average body weight of (64.45±3.48) kg were divided into three groups (n=5 per group): a control group, trial group I, and trial group II. All sheep received concentrate supplement at 12 g/kg BW, 1.8 kg corn silage, and RPTrp at 222.2 mg/kg BW daily, with free access to mixed hay. Trial groups I and II received additional CF supplementation at 50 mg and 100 mg per sheep per day, respectively, during a 15-day feeding trial.

The results showed: (1) During the 0–12 h period after morning feeding, no significant differences were observed in plasma Trp or Kyn contents among groups ($P>0.05$). However, plasma Trp content in the trial groups showed a decreasing trend at 6 h and 8 h ($P=0.0872$ and $P=0.0531$, respectively). Plasma Kyn content also exhibited a decreasing trend at 4.5, 6, 8, and 10 h ($P=0.0948$, $P=0.0667$, $P=0.0909$, and $P=0.0542$, respectively). (2) At 4.5 h and 8 h after morning feeding, plasma 5-hydroxytryptamine (5-HT) content in the trial groups showed an increasing trend compared with the control group ($P=0.0807$ and $P=0.0541$, respectively), and was significantly elevated at 10 h ($P=0.0057$). Plasma MT content in the trial groups also showed an increasing trend at 6 h and 8 h ($P=0.0890$ and $P=0.0704$, respectively), and was significantly higher than the control group at 10 h ($P=0.0002$). (3) At 0 h before morning feed-

ing, compared with the control group, trial group II showed significantly increased plasma total antioxidant capacity (T-AOC) and glutathione peroxidase (GSH-Px) activity ($P<0.05$), significantly decreased malondialdehyde (MDA) content ($P<0.05$), while trial group I also showed significantly improved T-AOC ($P<0.05$).

In conclusion, supplementation with 50 or 100 mg CF per sheep per day on top of RPTrp (222.2 mg/kg BW) tended to reduce plasma Kyn content during the 4.5–10 h period after feeding. While no significant overall effects were observed on plasma Trp, 5-HT, or MT content, CF supplementation improved the plasma antioxidant capacity of sheep.

Keywords: sheep; rumen-protected tryptophan; calcium folinate; kynurenine; melatonin

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Melatonin (MT) in mammals is an indole hormone primarily synthesized and secreted by the pineal gland, widely distributed in numerous organs, tissues, and cells. Research has demonstrated that MT can promote oocyte maturation, maintain sperm function, enhance embryonic development, and improve antioxidant capacity in sheep. Therefore, appropriately increasing plasma MT content may have important implications for sheep reproduction and health. Tryptophan (Trp) serves as the precursor for MT synthesis in animals, undergoing hydroxylation, decarboxylation, acetylation, and methylation to form MT. Previous studies from our laboratory have shown that supplementation with rumen-protected tryptophan (RPTrp) increases plasma total and free Trp content, while also elevating kynurenine (Kyn) content. Enhancing Trp conversion to MT requires first increasing 5-hydroxytryptamine (5-HT) synthesis, and tetrahydrobiopterin (BH4) is a critical cofactor for tryptophan hydroxylase (TPH), the key enzyme converting Trp to 5-HT. Since calcium folinate (CF) is a folate derivative that directly provides the activated form of folate after absorption and stabilizes BH4, this study used sheep as experimental animals to investigate whether CF supplementation could reduce the Trp-Kyn metabolic pathway and enhance Trp conversion to MT when intestinal Trp absorption was increased.

1.1 Experimental Period and Location

The experiment was conducted from July 30 to August 14, 2017, at the sheep farm of Xinjiang Huikang Animal Husbandry Biotechnology Co., Ltd. under natural lighting conditions. On the day of blood sampling, sunrise was at 07:12 and sunset at 21:15, with a day length of 14.03 hours.

1.2 Experimental Animals

Fifteen healthy Suffolk sheep aged (3.0±0.5) years with an average body weight of (64.45±3.48) kg were selected.

1.3 Experimental Design

The 15 Suffolk sheep were randomly divided into three groups (n=5 per group): control group, trial group I, and trial group II. All sheep received the same concentrate supplement (purchased from Xinjiang Tiankang Animal Husbandry Biotechnology Co., Ltd.) at 12 g/kg BW daily, RPTrp (purchased from Beijing Yahe Nutrition High-tech Co., Ltd., Trp content 45%, rumen bypass rate 85%) at 222.2 mg/kg BW, and 1.8 kg corn silage, with free access to mixed hay (alfalfa:wheat straw=1:1) and water. Trial groups I and II received additional CF supplementation at 50 mg and 100 mg per sheep per day, respectively (purchased from Shanghai Kewei Chemical Technology Co., Ltd.). The supplementation levels were based on reference studies. The composition and nutrient levels of the experimental diets are shown in Table 1 .

Table 1 Composition and nutrient levels of experimental diets (DM basis) %

Items	Concentrate supplement[1])	Corn silage	Alfalfa hay	Wheat straw	Total
Nutrient levels[2])					
Dry matter					
Ash					
Crude protein					
Neutral detergent fiber					
Acid detergent fiber					
Tryptophan					
Total					

[1]) One kg of concentrate supplement contained: corn 0.44 kg, oat 0.16 kg, barley 0.15 kg, soybean meal 0.20 kg, CaHPO 0.03 kg, NaCl 0.01 kg, premix 0.01 kg. The premix provided per kg of concentrate supplement: VA 480 IU, VB 816 mg, VB 333 mg, VB 49 mg, VD 70 U, VE 21,333 IU, pantothenic acid 20 mg, nicotinamide 485 mg, Cu (as copper sulfate) 11 mg, Fe (as ferrous sulfate)

35 mg, Mn (as manganese sulfate) 33 mg, Zn (as zinc sulfate) 31 mg, I (as potassium iodide) 2 mg, Se (as sodium selenite) 6 mg, Co (as cobalt chloride) 1 mg.

[2]) The Trp content was a calculated value, while other nutrient levels were measured values.

1.4 Animal Management

Sheep were housed individually. Daily RPTrp, CF, concentrate supplement, and corn silage were divided into two equal portions fed at 08:00 and 20:00. To ensure complete consumption of RPTrp and CF, they were first mixed with 50 g of concentrate supplement and fed; after consumption, the remaining concentrate and corn silage were provided, with free access to hay and water. Pens were cleaned regularly according to farm management protocols.

1.5 Sample Collection and Processing

Blood samples were collected on day 16 of the experiment at 0 h before morning feeding (07:30) and at 1.5, 3, 4.5, 6, 8, 10, and 12 h after morning feeding. Blood was collected via jugular venipuncture into heparinized tubes, centrifuged at 3,500 r/min for 15 min to prepare plasma, aliquoted into 1.5 mL Eppendorf tubes, and stored at -20 °C.

1.6 Index Determination

Plasma Trp and Kyn contents were determined by high-performance liquid chromatography. Plasma 5-HT and MT contents were measured by enzyme-linked immunosorbent assay. Plasma total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) content at 0 h before morning feeding were determined by colorimetric methods. All samples were analyzed at Beijing Huaying Biotechnology Research Institute.

1.7 Data Processing

Data were analyzed using one-way ANOVA with SAS 8.0 statistical software. Duncan's multiple comparison test was used when significant differences were detected. Significance was set at $P<0.01$ for highly significant differences, $P<0.05$ for significant differences, and $0.05<P<0.10$ for trends.

2.1 Effects of CF Supplementation on Plasma Trp Content in Sheep Fed RPTrp

As shown in Table 2 , no significant differences in plasma Trp content were observed among groups during the 0–12 h period after morning feeding ($P>0.05$), with similar changing trends. However, plasma Trp content in trial groups

showed a decreasing trend compared with the control group at 6 h and 8 h (P=0.0872 and P=0.0531, respectively).

Table 2 Effects of supplementation with CF on plasma Trp content in sheep fed RPTrp (n=5) mol/L

Sampling time	Control group	Trial group I	Trial group II	P-value
0 h before morning feeding (07:30)	45.46±4.87	40.55±4.35	44.96±8.24	
1.5 h after morning feeding (09:30)	35.29±2.15	34.88±3.14	34.54±1.43	
3 h after morning feeding (11:00)	37.46±2.05	33.42±1.95	35.44±6.00	
4.5 h after morning feeding (12:30)	43.84±2.48	36.26±6.02	37.70±8.69	
6 h after morning feeding (14:00)	47.38±4.37	39.04±4.39	42.42±4.21	
8 h after morning feeding (16:00)	47.77±3.74	41.03±4.76	44.22±3.94	
10 h after morning feeding (18:00)	46.99±3.60	40.35±3.88	43.89±4.23	
12 h after morning feeding (20:00)	43.74±3.50	41.84±5.03	40.92±2.62	

In the same row, values with no letter or the same letter superscripts indicate no significant difference (P>0.05), different lowercase letters indicate significant difference (P<0.05), and different uppercase letters indicate highly significant difference (P<0.01). The same applies below.

2.2 Effects of CF Supplementation on Plasma Kyn Content in Sheep Fed RPTrp

As shown in Table 3 , no significant differences in plasma Kyn content were observed among groups during the 0-12 h period after morning feeding (P>0.05). However, during the 4.5-10 h period, plasma Kyn content in trial groups showed a decreasing trend compared with the control group (P=0.0948, P=0.0667, P=0.0909, and P=0.0542 at 4.5, 6, 8, and 10 h, respectively).

Table 3 Effect of supplementation with CF on plasma Kyn content in sheep fed RPTrp (n=5) mol/L

Sampling time	Control group	Trial group I	Trial group II	P-value
0 h before morning feeding (07:30)	4.89±0.42	4.46±0.61	4.02±0.15	
1.5 h after morning feeding (09:30)	4.00±0.66	3.59±0.59	3.91±0.78	
3 h after morning feeding (11:00)	3.82±0.87	3.43±0.62	3.81±0.87	
4.5 h after morning feeding (12:30)	4.15±0.36	3.52±0.17	3.33±0.57	
6 h after morning feeding (14:00)	4.31±0.45	3.96±0.28	3.54±0.39	
8 h after morning feeding (16:00)	4.55±0.56	4.12±0.49	3.72±0.32	
10 h after morning feeding (18:00)	4.40±0.43	4.06±0.16	3.66±0.32	
12 h after morning feeding (20:00)	4.48±0.28	4.02±0.42	3.98±0.47	

2.3 Effects of CF Supplementation on Plasma 5-HT Content in Sheep Fed RPTrp

As shown in Table 4 , plasma 5-HT content in trial groups was higher than the control group during the 0–8 h period after morning feeding, with similar changing trends among groups, but no significant differences were observed (P>0.05). Plasma 5-HT content showed an increasing trend at 4.5 h and 8 h (P=0.0807 and P=0.0541, respectively), and was highly significantly higher than the control group at 10 h (P=0.0057), with no significant difference between trial groups (P>0.05).

Table 4 Effect of supplementation with CF on plasma 5-HT content in sheep fed RPTrp (n=5) mol/L

Sampling time	Control group	Trial group I	Trial group II	P-value
0 h before morning feeding (07:30)	320.88±46.71	328.21±53.39	331.46±70.62	
1.5 h after morning feeding (09:30)	223.96±76.23	264.24±40.32	255.33±49.02	
3 h after morning feeding (11:00)	155.89±8.34	182.53±29.25	185.26±35.92	
4.5 h after morning feeding (12:30)	230.35±23.38	279.01±33.96	312.57±36.78	
6 h after morning feeding (14:00)	273.37±16.26	285.57±55.92	296.94±56.72	
8 h after morning feeding (16:00)	179.73±11.17Bb	204.53±27.72Aa	230.40±15.00Aa	
10 h after morning feeding (18:00)	209.28±28.97Bb	301.12±17.32Aa	314.57±23.82Aa	

Sampling time	Control group	Trial group I	Trial group II	P-value
12 h after morning feeding (20:00)	360.46±70.45	319.97±64.40	282.47±43.54	

2.4 Effects of CF Supplementation on Plasma MT Content in Sheep Fed RPTrp

As shown in Table 5 , no significant differences in plasma MT content were observed among groups during the 0–8 h period after morning feeding (P>0.05), though trial groups showed an increasing trend at 6 h and 8 h (P=0.0890 and P=0.0704, respectively). During the 8–10 h period, plasma MT content in the control group decreased, reaching the minimum daytime value at 10 h, while trial groups showed an increasing trend and were highly significantly higher than the control group at 10 h (P=0.0002).

Table 5 Effect of supplementation with CF on plasma MT content in sheep fed RPTrp (n=5) pg/mL

Sampling time	Control group	Trial group I	Trial group II	P-value
0 h before morning feeding (07:30)	73.78±3.98	73.58±6.99	75.98±4.56	
1.5 h after morning feeding (09:30)	79.40±8.64	92.14±2.93	88.45±10.59	
3 h after morning feeding (11:00)	66.00±6.25	59.71±2.79	61.05±6.90	
4.5 h after morning feeding (12:30)	87.51±9.72	78.29±2.69	86.20±8.73	
6 h after morning feeding (14:00)	45.80±1.02	52.82±7.16	56.20±6.08	

Sampling time	Control group	Trial group I	Trial group II	P-value
8 h after morning feeding (16:00)	66.40±5.80	77.77±1.73	71.20±6.40	
10 h after morning feeding (18:00)	42.36±6.98Bc	81.72±10.45Ab	112.88±19.28Aa	
12 h after morning feeding (20:00)	63.03±10.51	61.63±7.17	62.68±4.46	

2.5 Effects of CF Supplementation on Plasma Biochemical Indexes in Sheep Fed RPTrp

As shown in Table 6 , at 0 h before morning feeding, compared with the control group, trial group II showed significantly increased plasma T-AOC and GSH-Px activity ($P<0.05$) and significantly decreased MDA content ($P<0.05$), while trial group I also showed significantly improved T-AOC ($P<0.05$). No significant differences were observed in plasma SOD activity among groups ($P>0.05$).

Table 6 Effects of supplementation with CF on plasma biochemical indexes in sheep fed RPTrp (n=5)

Items	Control group	Trial group I	Trial group II	P-value
Total antioxidant capacity T-AOC (U/mL)	9.86±1.44b	12.29±1.38a	15.53±2.70a	
Glutathione peroxidase GSH-Px (U/mL)	820.61±102.01b	848.78±49.60b	1,054.48±93.35a	

Items	Control group	Trial group I	Trial group II	P-value
Superoxide dismutase SOD (U/mL)	72.51±8.70	65.90±7.05	81.52±5.83	
Malondialdehyde MDA (nmol/mL)	3.79±0.57a	3.40±0.34ab	2.78±0.43b	

3.1 Effects of CF Supplementation on Plasma Trp and Kyn Contents in Sheep Fed RPTrp

During the 0–12 h period after morning feeding, no significant differences were observed in plasma Trp content among groups, though trial groups had lower values than the control group, with a decreasing trend at 6 h and 8 h. Research indicates that CF, as a folate derivative, stabilizes and promotes BH4 synthesis. These results may be attributed to CF supplementation increasing BH4 content and TPH activity in sheep, promoting Trp conversion to 5-hydroxytryptophan along the 5-HT pathway. During the 4.5–10 h period, plasma Kyn content in trial groups showed a decreasing trend compared with the control group, consistent with the trend in plasma Trp content. Studies show that approximately 95% of L-Trp is converted to Kyn by tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO). Under physiological conditions, TDO is the key enzyme catalyzing Trp conversion to Kyn in the liver, with activity primarily regulated by substrate Trp and hormone levels (glucocorticoids and estrogen), while IDO mainly functions in extrahepatic tissues during infection, inflammation, or stress. In this study, CF supplementation may not have significantly affected hepatic TDO activity, and the decreased plasma Kyn content may be related to increased TPH activity or expression and reduced plasma Trp content.

3.2 Effects of CF Supplementation on Plasma 5-HT and MT Contents in Sheep Fed RPTrp

During the 0–8 h period after morning feeding, plasma 5-HT content in trial groups was not significantly higher than the control group, but showed an increasing trend at 4.5 h and 8 h. Research shows that TPH has two genetically encoded isoforms: TPH1 and TPH2. TPH1 is mainly distributed in enterochromaffin cells and the pineal gland, while TPH2 is primarily found in enteric and central nervous system 5-HT neurons. In the central nervous system, BH4 levels do not saturate TPH2; therefore, direct intracerebroventricular injection of 10 L of 20 nmol/L BH4 or microdialysis perfusion of BH4 analog tetrahydrobiopterin dihydrochloride can significantly increase TPH2 activity and 5-HT content in mouse brain tissue. Whether increasing plasma BH4 content can enhance TPH1 activity and plasma 5-HT content remains unreported. The current

results may be due to insufficient CF supplementation or ruminal degradation, resulting in no significant effect on TPH1 activity. The CF dosage was based on oral CF administration in gastric cancer patients undergoing chemotherapy (90 mg/d), and no studies have investigated CF degradation in the rumen of ruminants. Future studies could verify these findings by increasing CF dosage or using rumen-protected CF. The results may also be related to TPH expression levels in sheep. If TPH expression is low and already saturated by BH4, increasing BH4 content through CF supplementation may not affect TPH1 activity. Studies indicate that over 95% of 5-HT in mammals is distributed in the gastrointestinal tract, primarily synthesized by enterochromaffin cells. At 10 h after morning feeding, plasma 5-HT content in trial groups was highly significantly higher than the control group, possibly due to CF promoting TPH1 expression in enterochromaffin cells and 5-hydroxytryptophan synthesis. Since intestinal mucosal tissue was not collected in this study, future experiments could verify this by measuring TPH1 content in sheep intestinal mucosa.

TPH1 is not only the rate-limiting enzyme for 5-HT synthesis but also a key enzyme for MT synthesis. During daytime, plasma MT in mammals mainly originates from enterochromaffin cells. Namboodiri et al. confirmed that intraperitoneal injection of 20 or 200 mg/kg BW of 5-hydroxytryptophan can significantly increase plasma MT content in sheep. In this study, plasma MT content in trial groups showed an increasing trend at 6 h and 8 h, and was highly significantly higher than the control group at 10 h. This may be related to CF increasing TPH1 expression in enterochromaffin cells and elevating plasma 5-hydroxytryptophan content. Additionally, 5-HT is both a conversion product of Trp and 5-hydroxytryptophan and a precursor for MT synthesis. Studies have found that monoamine oxidase widely present in enterochromaffin cells, enteric neurons, platelets, liver, and kidneys can convert 5-HT to 5-hydroxyindoleacetic acid, which is eventually excreted in urine. In this study, plasma 5-HT content showed an increasing trend at 4.5 h, while MT content did not increase, possibly due to CF increasing 5-hydroxyindoleacetic acid content. Future studies could further detect 5-hydroxyindoleacetic acid content in sheep plasma and urine to verify the effects of different CF dosages on plasma 5-HT content when supplemented with rumen-protected RPTrp.

3.3 Effects of CF Supplementation on Plasma Biochemical Indexes in Sheep Fed RPTrp

Supplementation with CF on top of RPTrp improved the antioxidant capacity of sheep plasma. Studies have shown that folate promotes the conversion of BH2 to BH4, and BH4 has antioxidant functions. Since CF directly provides the activated form of folate in vivo, it promotes BH2 conversion to BH4. These results may be related to increased plasma BH4 content. Additionally, the results may be associated with increased plasma MT content, as MT is an effective antioxidant that reduces reactive oxygen and nitrogen species and increases antioxidant enzyme expression and activity.

4 Conclusion

Supplementation with 50 or 100 mg CF per sheep per day on top of RPTrp (222.2 mg/kg BW) tended to reduce plasma Kyn content during the 4.5–10 h period after feeding. While no significant overall effects were observed on daytime plasma Trp, 5-HT, or MT content, CF supplementation improved the plasma antioxidant capacity of sheep.

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