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Effects of Oral Supplementation with N-Carbamylglutamate on Serum Biochemical Indices and Intestinal Morphology with its Proliferation in Weanling Piglets

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Abstract: The experiment was conducted to evaluate the effects of N-Carbamylglutamate (NCG) on intestinal mucosa with its proliferation in weanling piglets. About 12 Duroc x Landrace x Yorkshire piglets (average BW 5.67±0.14 kg; weaned at 21 days) were grouped into 2 treatments and fed one of the following diets for 7 days: a Standard Diet (SD), SD+NCG (0.05%). All the piglets were sacrificed for blood and tissue collection. The results showed that compared with the control group, NCG decreased serum urea nitrogen and ammonia concentration ($p<0.05$). Adding NCG to the diet resulted in a higher villus height in jejunum ($p<0.05$). The RT-PCR results showed that NCG significantly increased PCNA mRNA abundance in both jejunum ($p<0.05$) and ileum ($p<0.01$). These results indicated that oral supply of NCG improved intestinal mucosa morphology and had favorable effects on proliferation of intestinal mucosa.

Key words: N-carbamylglutamate, arginine, mucosal morphology, piglets, proliferation

INTRODUCTION

Weaning stress of piglets is often associated with reduced food consumption as well as temporary reductions in weight gain which can result in postweaning lag, a time of depressed feed intake and of increased diarrhea and disease intestinal dysfunction and atrophy and mortality in piglets (Boudry *et al*., 2004; Castillo *et al*., 2007; Gu *et al*., 2002).

Arginine is a nutritionally essential amino acid for piglets, particularly under stressfull conditions (Tong and Barbul, 2004; Wu *et al*., 2004a). An arginine deficiency can result in hyperammonemia and intestinal and immunological dysfunction (Tong and Barbul, 2004; Wu *et al*., 2004a). N-Carbamylglutamate (NCG) is a metabolically stable analogue of N-Acetylglutamate (NAG) which activates Carbamylphosphate Synthase-1 (CPS-1), a key enzyme in arginine synthesis in enterocytes (Caldovic *et al*., 2010). Previous studies indicate that NCG may help prevent ammonia buildup, promotes removal and disposal of ammonia and therefore helps enhance GH activity (Tuchman *et al*., 2008). The mechanism of NCG may lie in increasing the endogenous synthesis of Arg (Wu *et al*., 2004b; Wu *et al*., 2010; Frank *et al*., 2007).

In the earlier studies, researchers found that dietary supplementation with Arg and NCG partially increased intestine weight induced by weaning stress in piglets (Peng and Cai, 2011). The present experiment was conducted to evaluate the effects of NCG on serum biochemical indices and intestinal mucosa with its proliferation in weanling piglets.

MATERIALS AND METHODS

Animal and treatment: About 12 piglets (average weight 5.67±0.14 kg) were randomly grouped into 2 treatments and fed diets with a Standard Diet (SD), SD+NCG (6 g kg^{-1}), respectively. All diets were made iso-nitrogenous with addition of appropriate amounts of alanine. All the nutrients were adequate for piglets met the NRC (1998) recommended requirement within the weight range used in the present study (Table 1). The diets were administered three times daily at 08:00 and 12:00 and 18:00, respectively, close to *ad libitum* intake (5.0% of BW/day). All animals had free access to drinking water.

NCG used in the current research was provided by Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China. The experiment was carried out in accordance with the Chinese guidelines for animal welfare.

Samples collection: The Average Daily Gain (ADG) and Average Daily Feed Intake (ADFI) were collected for all pigs throughout the study. On age of 28 days, 10 mL blood samples were collected into heparinized tubes, followed immediately by centrifugation at 3,000×g for

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Table 1: Composition and nutrient content of based diet (%)

Ingredients	Content (%)
Corn (CP 8% H_2O<13%)	50.20
Soybean expanded (CP 43%)	26.00
Fish meal (CP 65%)	6.00
Whey (100%)	9.00
Cream (EE 50%)	6.00
Limestone	0.50
Monocalcium phosphate	1.00
Moldproofant	0.10
NaCl	0.20
Premix*	1.00
Total	100.00
Nutritional level	
DE (MJ kg^{-1})	14.21
CP (%)	20.00
Ca (%)	0.71
P (%)	0.48
Arg (%)	1.09
Lys (%)	1.35
Met (%)	0.42
Thr (%)	0.90

*Supplied per kg diet: flavor, 70 g; antioxidant, 20 g; choline chloride (50%), 80 g; trace mineral premix, 300 g; vitamin premix, 40 g; L-Lysine·HCl, 310 g; Met, 60 g; Thr, 120 g

10 min at 4°C. The supernatant fluid (serum) were collected and immediately frozen at -20°C for biochemical analyses. Immediately after the last blood sample was collected, all the pigs were anaesthetised with an i.v. injection of sodium pentobarbital (50 mg kg^{-1} BW) and bled by exsanguination. The entire intestine was then rapidly removed and dissected free of mesenteric attachments, weighted and then placed on a smooth, cold surface tray. The entire intestine was then rapidly removed and dissected free of mesenteric attachments, weighed and then placed on a smooth, cold surface tray.

The jejunum and ileum were separated as described by Wu *et al.* (2010). Their contents were manually rinsed. Samples were taken at 5 cm from the pylorus (duodenum) and at 50% of the small intestine's length (mid-jejunum) for intestinal immuno-histochemistry assessment. Other samples were taken for the assessment of intestinal morphology (Wu *et al*., 1996).

Serum biochemical indices: An Automated Biochemistry Analyzer (Synchron CX Pro, Beckman Coulter, Fullerton, CA, USA) was used to determine the concentrations of serum urea nitrogen, ammonia and glucose according to the commercial kits and manufacturer's instructions. All the kits were purchased from Beijing Chemlin Biotech Co., Ltd. (Beijing, China).

Intestinal morphology: Formalin-fixed jejunum and ileum samples were embedded in paraffin; cross-sections of the segments were cut approximately 5 μm thick with a microtome and stained with haematoxylin and eosin. In each section, the villus height and the associated crypt depth were measured using a light microscope with a computer-assisted morphometric system. Villus height is defined as the distance from the villus tip to crypt mouth and crypt depth from crypt mouth to base.

Real-time PCR for PCNA in jejunum and ileal mucosa: Total RNA in small intestine was isolated using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's recommendation. Total RNA was reversed into cDNA using a SuperScript First-Strand Synthesis System kit (Invitrogen Life Technologies). PCR amplification was performed in a total volume of 50 μL including Taq DNA polymerase and specific primers.

To amplify Proliferating Cell Nuclear Antigen (PCNA) (target gene) and GAPDH cDNA fragments, the following sequences of PCR primer pairs were used: forward 5'-CAACTCCGCCACCATGTTTGA-3', reverse 5'-AAGCTGCACTAAGGAGACGTGAGAC-3' for PCNA (163 bp); forward 5'-GGCAAGTTCAACGGCACAG-3', reverse 5'-CGCCAGTAGACTCCACGACAT-3' for GAPDH (142 bp). The RT-PCR conditions were: About 30 sec denaturation at 94°C, 30 sec annealing at 60°C and 30 sec extension at 72°C for 30 cycles. The relative quantification of gene amplification by RT-PCR was performed using Cycle threshold (Ct) values. The comparative Ct value method was employed to quantitate expression levels for PCNA relative to those for GAPDH. For the relative comparison of mRNA expression levels, the data from real-time PCR were analyzed with a $\Delta\Delta$Ct method and normalized to the amount of GAPDH cDNA as an endogenous control.

Statistical analysis: Data are presented as the mean±SEM obtained from triplicate experiment. Differences between mean values of multiple groups were analyzed by one-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test performed using SPSS 13.0. Statistical significance was considered at $p<0.05$.

RESULTS AND DISCUSSION

Effects of NCG on growth performance: The reuslts of the effects of NCG on growth performance were shown in Table 2. NCG significantly increased ADG and ADFI ($p<0.05$).

Effect of NCG on plasma biochemical indices: Comparing to the control piglets, NCG significantly increased ($p<0.05$) serum ALP and decreased ($p<0.05$) urea nitrogen.

Table 2: Effects of oral supplementation with NCG on the growth performance of piglets

Variables	Control	NCG diet
Initial body weight (kg)	5.68±0.12	5.66±0.15
Final body weight (kg)	6.16±0.18[a]	6.45±0.19[b]
Average daily gain (g)	68.60±5.20[a]	112.90±6.50[b]
Daily feed intake (g day^{-1})	168.20±12.1	182.30±15.5

Values are expressed as mean±SEM, n = 6 piglets for body weights and n = 6 for intestinal weights per treatment. [a, b]Means within a row with different letters differ ($p<0.05$)

Table 3: Effect of NCG on plasma biochemical indices

Items	Control	NCG
Glucose	7.82±1.21	8.06±1.60
Urea nitrogen (mmol L^{-1})	6.42±1.12[a]	5.56±1.12[b]
Ammonia (μmol L^{-1})	89.72±5.68	78.68±6.61

Table 4: Effects of oral supplementation with NCG on intestinal morphology in weanling pigs (n = 6)

Items	Control	NCG diet
Villus height (μm)		
Jejunum	302.18±31.23[a]	362.62±30.22[b]
Ileum	318.12±28.25	353.20±32.65
Crypt depth (μm)		
Jejunum	108.21±18.32	129.32±12.62
Ileum	116.22±15.21	121.62±13.85
Villus height:crypt depth		
Jejunum	2.79±0.180	2.80±0.240
Ileum	2.73±0.160	2.90±0.210

Values are expressed as mean±SEM, n = 6. [a, b]Means within a row with different letters differ ($p<0.05$)

Compared with the piglets in the control group, serum urea N level of piglets fed a NCG was lower ($p<0.05$) (Table 3). NCG significantly decreased serum nitrogen and ammonia concentration ($p<0.05$).

Intestinal morphology: Intestinal morphology results were showed in Table 4. Compared to the control group, NCG significantly enhanced villus height in jejunal and crypt depth ($p<0.05$). Compared to the control group, although there was a trend toward an increase in jejunal and ileal crypt depth in the NCG group, there was no difference between villus height:crypt depth. Figure 1 is representative staining of jejunal mucosal morphology.

mRNA expression for PCNA in the jejunum and ileal mucosa: The data for mRNA expression of PCNA are shown in Fig. 2. Compared with control group, NCG significantly increased PCNA mRNA abundance in jejunum ($p<0.05$) and ileum ($p<0.01$).

Arg is essential when Arg turnover increases as in growth inflammation or tissue repair in rodents, dietary supply can become rate-limiting for the Arg metabolizing pathways (Wu *et al*., 2004b). Indeed, low Arg levels have been documented in the weaning age of piglet. It is reported that intestinal synthesis of citrulline and Arg from glutamine and glutamate decreases by 70-73% in 7 days old suckling pigs in comparison with newborn pigs and declines further in 14-21 days old pigs (Wu *et al*., 2004b).

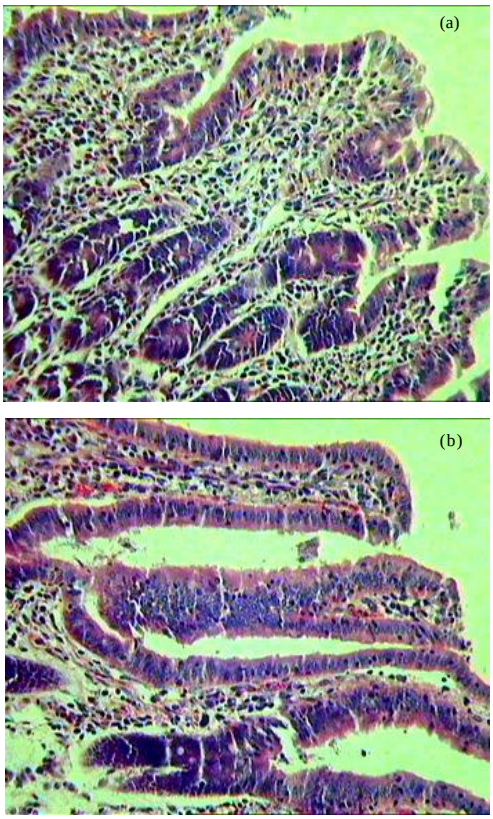

Fig. 1: Representative staining of jejunal mucosal morphology. The results showed that oral supplementation with NCG a significant decrease in villus height and crypt depth in jejunum and ileum; a) control piglets treated with saline; b) control piglets treated with NCG

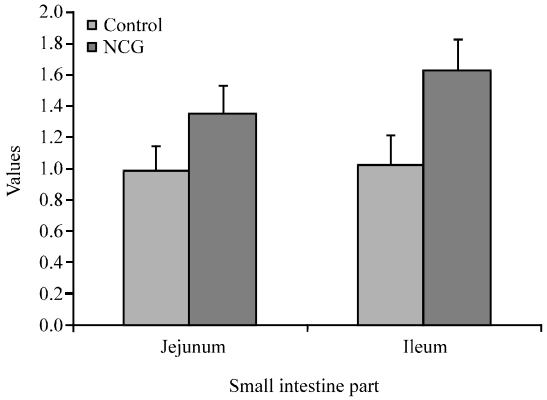

Fig. 2: Real-time PCR for PCNA mRNA in jejunum and ileum mucosa in weaned piglets. Values are expressed as mean±SEM, n = 6. *Means differ significantly ($p<0.05$) and **differ very significantly ($p<0.01$)

NCG is a metabolically stable analog of NAG. NCG has been used as for the treatment of hyperammonemia in rare inherited disorders: Carbamyl Phosphate Synthetase I (CPSI) deficiency, NAGS deficiency, Ornithine Transcarbamylase (OTC) deficiency, propionic acidemia and methylmalonic acidemia (Tuchman *et al*., 2008). NCG improves the acute therapy of decompensated propionic aciduria by increasing ammonia detoxification and avoiding hyperammonaemia (Gebhardt *et al*., 2005). Consistent with this NCG also decreased plasma ammonia in weanling piglets. In the present study, NCG.

NCG can activate intestinal or endogenous synthesis of Arg and citrulline. Most recently, the suckling piglets treated with NCG (oral administration of 50 mg of NCG kg^{-1} BW every 12 h for 7 days) had greater plasma concentrations of Arg than the control piglets (Frank *et al*., 2007). The mechinism of the NCG improved the growth performance may lied in its activate intestinal endogenous synthesis of Arg and citrulline.

PCNA expression could be a marker of dysplasia in oral mucosa indicating a special proliferative cellular state (Martinez-Lara *et al*., 1996). But it is interestingly, compared to the control group, supplementation of NCG as performed in this study increase PCNA mRNA which indicated that NCG contributed in inducing the cell growth and proliferation in intestinal mucosa.

CONCLUSION

The results of this study shows that NCG supplementation may facilitate a more spatially precise in improving intestinal mucosa morphology and had favorable effects on proliferation of intestinal mucosa. Judged on the the above results, the finding from this study that oral supplementation with NCG appears to be a new effective support for intestinal mucosa of weaning piglets.

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