Short communication: Effects of dietary addition of *N*-carbamoylglutamate on milk composition in mid-lactating dairy cows

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ABSTRACT

The present study was conducted to investigate the effect of *N*-carbamoylglutamate (NCG) on milk production and composition in mid-lactating Holstein dairy cows. Sixty multiparous cows with a mean body weight of 669 kg (standard deviation = 71) and 176 days in milk (standard deviation = 55) were blocked based on parity and milk production and randomly assigned into 4 treatments, a basal diet supplemented with 0, 10, 20, or 40 g of NCG/d per cow. Milk yield and composition were recorded weekly, whereas dry matter intake and plasma variables were determined every 2 wk. The results showed that the addition of NCG had no effect on the dry matter intake and milk yield of the cows. Milk fat content and yield increased linearly with NCG addition. The contents of milk protein and total solid also increased linearly in the cows fed NCG, whereas the yield of protein was not affected by the treatments. Conversely, dietary addition of NCG increased the plasma nitric oxide content in a quadratic manner. Moreover, addition of NCG linearly increased the plasma Arg content. Overall, the results indicate that dietary NCG addition increased the milk protein and fat contents, which improved the milk quality of lactating dairy cows.

Key words: *N*-carbamoylglutamate, arginine, milk quality, dairy cows

Short Communication

Milk yield and composition are important economical traits in dairy production and have been the focus of many studies (Wang et al., 2014; Batistel et al., 2017). Fat and protein contents are important traits in raw milk for farm economic profitability and taste after processing. A wide gap exists between China and other developed countries in terms of milk production and qual-

ity, leaving great potential for improvement in China. Many genetic (animal breed) and nongenetic factors, such as welfare, environment, and feeding (Oltenacu and Broom, 2010; Mullen et al., 2011), can affect milk fat and protein contents. Specifically, large variation in milk composition among dairy cows at different stages has been found. For example, protein decreases after calving, but slightly increases in late lactation. The fat and protein contents in milk change dramatically when cows transfer from early to late lactating stages (Gross and Bruckmaier, 2015).

Amino acids are important regulators in milk protein and fat contents. Supplementation of Lys and Met improved milk yield and N utilization efficiency in lactating dairy cows (Wang et al., 2010) and increased their milk protein content (Batistel et al., 2017). In addition to the protein precursors, some AA may have specific biological functionality, such as Arg. Despite the fact that ruminants can synthesize Arg, it is normally considered an EAA for dairy cows and is involved in body functions (Wu et al., 2009). Lassala et al. (2011) showed that parenteral administration of Arg in ewes increased lamb birth weight and improved the survival rate of fetal lambs to term in prolific ewes. As a precursor of polyamines and nitric oxide (NO), Arg can enhance angiogenesis and lactogenesis (Morris, 2009). Infusion of Arg improved N metabolism efficiency of heifers (Davenport et al., 1990), and Arg increased casein protein synthesis in bovine mammary epithelial cells (Chen et al., 2013). However, due to the fast degradation of Arg in the rumen, unprotected Arg cannot be used as an efficient feed additive in dairy cows (Chacher et al., 2012). Moreover, over-feeding Arg supplementation may cause AA imbalances, especially with lysine (Hüsier and Blum, 2002).

Alternatively, *N*-carbamoylglutamate (NCG) is a structural analog of *N*-acetylglutamate (Gessler et al., 2010), which is an allosteric activator of the essential auxiliary factor ammonia phosphate synthetase in the endogenous synthesis of Arg (Gessler et al., 2010). Compared with Arg, supplementation of NCG offered unique and important advantages, such as no negative

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effect on intestinal absorption of dietary Trp, His, or Lys and a substantially reduced cost (Wu et al., 2004). As NCG is stable in the rumen (Chacher et al., 2012), it can be regarded as a potential Arg enhancer for ruminants. Our previous study suggested that dietary addition of NCG improved the milk performance and N utilization efficiency of high-yielding Chinese Holstein dairy cows (Chacher et al., 2014). However, due to the physiological and metabolic variations between the cows in different lactating periods, whether NCG addition can improve milk production and quality in mid-lactating dairy cows was unexplored. Therefore, the aim of the present study was to investigate the effect of NCG addition on DMI and milk performance in mid-lactating dairy cows.

This experiment was approved by the Animal Care Committee of Zhejiang University and supported by Hangjiang Dairy Farm (Hangzhou, China). Sixty multiparous Holstein cows in mid lactation with a mean of 176 DIM (SD $\pm$ 55) and BW of 669 kg (SD $\pm$ 71) were selected and assigned to 15 blocks based on parity and milk yield and then randomly allocated into 4 groups with basal diets supplemented with different doses of NCG (0, 10, 20, or 40 g/d per cow). At the beginning of the experiment, the average milk yield of each group was 26.4 (SD = 2.22), 26.4 (SD = 2.25), 26.4 (SD = 2.32), and 26.4 kg (SD = 2.42), respectively, with no difference among 4 groups. The NCG (with 97% purity) was provided by Beijing Animore Sci. & Tech. Co., Ltd. (Beijing, China). Cows were housed in a barn with individual tiestalls and had free access to water. The NCG was added 3 times per day at 0600, 1400, and 2000 h by scattering it on the TMR for individual cows. Feeds were offered ad libitum to yield 5% orts. The experiment lasted for 14 wk, with the first 2 wk allowing for adaptation, during which no NCG was added.

The DMI was recorded for 2 consecutive days, and the samples of TMR and orts were collected on the same day biweekly throughout the experimental period. All the samples were analyzed for DM (105°C for 5 h), CP (method 988.05; AOAC, 1990), ADF (method 973.18; AOAC, 1990), and NDF (Van Soest et al., 1991) without sodium sulfite and amylase added. The nutrient compositions of the experimental diet are listed in Table 1.

The milk yield was recorded on d 6 and 7 weekly, and 2 subsamples of milk were also collected from individual cow. One subsample was mixed with bronopol (milk preservative; D & F Control Systems Inc., San Ramon, CA) and used for analysis of milk composition (fat, protein, and lactose) through an infrared analysis system (Laporte and Paquin, 1999) using a 4-channel spectrophotometer (MilkoScan; Foss Electric A/S, Hillerød, Denmark).

Blood samples were collected from the coccygeal vein of each cow and deposited into 5-mL tubes containing an anticoagulant (heparin lithium) at 3 h after morning feeding on d 7 of wk 0 (the beginning of the adaptation period), 6, and 12. The samples were then centrifuged at $3,000 \times g$ for 15 min at 4°C to collect the plasma, which was then frozen at -20°C for subsequent analysis. Plasma samples were analyzed using an Auto Analyzer 7020 instrument (Hitachi High-Technologies Corporation, Tokyo, Japan) with colorimetric commercial kits (Ningbo Medical System Biotechnology Co. Ltd., Ningbo, China) to determine urea N (Wang et al., 2007), total protein, albumin, nonesterified fatty acid, triglyceride, glucose, total bilirubin, and cholesterol. The plasma concentration of NO, total NO synthase, and malondialdehyde were determined by Jiancheng Bioengineering Research Institute (Nanjing, China). The plasma AA was determined using the method described by Wang et al. (2016) in an Automatic AA Analyzer (Hitachi High-Technologies Corporation).

All data were analyzed using SAS software version 2000 (SAS Institute Inc., Cary, NC) and using the covariance type AR (1) for repeated measures. A randomized complete block design with repeated measures was used for the analyses, with week, treatment, treatment $\times$ week interaction, and block as the main effects and with cow within diet as a random effect. The linear and quadratic effects of the treatment on all variables were tested through orthogonal polynomial contrast. The effect of the week was included as a repeated measure.

Table 1. Ingredients and chemical composition of the experimental diet

Items	Amount
Ingredient, g/kg of DM	
Corn grain	174
Soybean meal	103
Cottonseed meal	3
Wheat bran	35
Steam-flaked corn	119
Corn silage	176
Alfalfa hay	191
Oat hay	42
Sugar beet pulp	77
Beer grains	35
Premix ¹	45
Composition, g/kg of DM	
CP	139
NDF	352
ADF	195
Crude ash	75
NE _L , ² MJ/kg	6.48

¹Formulated to provide (per kilogram of DM): 150–180 g of salt, 250,000 IU of vitamin A, 50,000 IU of vitamin D₃, 1,100 IU of vitamin E, 3,000 mg of Zn, 17 mg of Se, 36 mg of I, 600 mg of Fe, 8 mg of Co, 630 mg of Mn, and 650 mg of Cu; water $\leq$ 100 g.

²Calculated based on the Ministry of Agriculture (2004).

The means were separated using the PDIFF option in the LSMEANS treatment. The experiment results are reported as least squares means. Significance was declared when $P < 0.05$ and trends were defined when $P < 0.10$.

The results of DMI and lactation performance of lactating dairy cows are shown in Table 2. Both DMI and milk yield were not affected by addition of NCG ($P > 0.05$). The cows administered with NCG had an increased milk fat yield ($P = 0.05$, linear) and content ($P < 0.01$, linear). Milk protein content increased linearly ($P = 0.04$) with NCG addition, but this was not the case for protein yield; addition of NCG increased TS content ($P < 0.01$, linear). The lactose content and yield as well as MUN content ($P > 0.05$) were not affected by dietary addition of NCG. Feed efficiency ($P > 0.05$) was not different between any of the treatments. The FCM ($P > 0.05$) and ECM ($P > 0.05$) were not affected by addition of NCG (Table 2).

The effects of NCG addition on plasma parameters of lactating dairy cows are shown in Table 3. The addition of NCG increased the concentration of NO ($P < 0.01$, quadratic) and total NO synthase ($P = 0.01$, linear) and tended to increase the concentration of glucose ($P = 0.08$, quadratic) and total bilirubin ($P = 0.07$, linear). An interaction between week and treatment was observed on plasma NO of lactating dairy cows ($P = 0.04$). In both wk 6 ($P < 0.01$) and 12 ($P < 0.01$), plasma NO of cows fed 40 g/d of NCG was significantly higher than in control cows (data not shown). No dif-

ference was observed on other plasma parameters of dairy cows among treatments ($P > 0.05$).

The effects of NCG addition on plasma AA of dairy cows are shown in Table 4. The concentrations of total AA ($P = 0.06$, linear) and total EAA ($P < 0.01$, quadratic) were significantly increased by NCG addition. Addition of NCG increased the concentration of Arg ($P < 0.01$, linear and quadratic), Glu ($P < 0.01$, linear and quadratic), Pro ($P = 0.05$, linear), and Cys ($P = 0.08$, quadratic) and decreased Gly ($P = 0.08$, linear); the other NEAA did not differ among treatments ($P > 0.05$). We found interactions between week and dietary NCG on the plasma concentrations of Arg ($P = 0.04$), Glu ($P = 0.01$), and Asp ($P < 0.01$) in lactating dairy cows, respectively. In wk 6, cows fed 20 g/d of NCG tended to have higher glucose concentrations than that of the control ($P = 0.09$). In wk 12, cows with NCG (10, 20, and 40 g/d) had higher Arg ($P < 0.01$) and Glu ($P < 0.01$) concentrations compared with animals without NCG. Moreover, cows fed 40 g/d of NCG tended to have greater plasma Asp than the control cows ($P = 0.06$, data not shown).

The increased the content of Arg in the plasma in the current study was consistent with our previous study (Chacher et al., 2014). The possible reason for this phenomenon could be that the NCG is used by rumen epithelial and duodenal cells for urea synthesis (Oba et al., 2005), ultimately resulting in a rise in the endogenous synthesis of Arg and its AA family, which are supported by our data (Table 4). The interactive

Table 2. Effect of dietary addition of *N*-carbamoylglutamate on the milk yield and milk composition of Holstein dairy cows

Item ¹	<i>N</i> -carbamoylglutamate, g/d					<i>P</i> -value ²			
						Treatment			
	0	10	20	40	SEM	L	Q	W	T × W
DMI, kg/d	22.3	21.5	22.0	22.4	0.56	0.71	0.29	<0.01	0.92
Yield, kg/d									
Milk	23.5	24.0	23.7	23.1	0.44	0.48	0.19	<0.01	0.11
Fat	0.91	0.92	0.95	0.97	0.03	0.05	0.58	<0.01	0.57
Protein	0.85	0.87	0.86	0.86	0.02	0.73	0.54	<0.01	0.21
Lactose	1.15	1.18	1.16	1.15	0.02	0.82	0.37	<0.01	0.36
FCM	25.0	25.1	25.7	25.7	0.53	0.23	0.93	<0.01	0.39
ECM	26.3	26.5	27.0	27.0	0.54	0.26	0.87	<0.01	0.36
Milk composition, %									
Fat	3.93	3.83	4.05	4.24	0.08	<0.01	0.06	<0.01	0.31
Protein	3.62	3.63	3.64	3.74	0.04	0.04	0.23	<0.01	0.96
Lactose	4.89	4.91	4.89	4.96	0.02	0.24	0.18	<0.01	0.43
TS	13.0	12.8	13.1	13.3	0.12	0.01	0.21	<0.01	0.59
MUN, mg/dL	15.0	15.8	14.4	15.1	0.50	0.56	0.94	<0.01	0.95
Feed efficiency	1.20	1.26	1.25	1.21	0.04	0.92	0.18	<0.01	0.70
LP, %	89.2	98.5	99.4	95.4	2.10	0.03	<0.01	<0.01	0.48

¹FCM = milk yield × 0.432 + milk fat yield × 16.216; ECM = 0.3246 × milk yield + 13.86 × milk fat yield + 7.04 × milk protein yield (Orth, 1992); Feed efficiency = kg of ECM/kg of DMI; LP = lactation persistency.

²W = week effect; W × T = interaction between week and treatment; L = linear; Q = quadratic.

Table 3. Effects of dietary *N*-carbamoylglutamate on the blood parameters of Chinese Holstein dairy cows

Item ¹	<i>N</i> -carbamoylglutamate, g/d					<i>P</i> -value ²			
						Treatment			
	0	10	20	40	SEM	L	Q	W	T × W
Total protein, g/L	79.0	78.9	80.8	78.0	1.19	0.87	0.25	0.11	0.51
BUN, μmol/L	4.17	4.52	4.11	4.14	0.20	0.61	0.43	<0.01	0.40
Glucose, mmol/L	3.27	3.35	3.51	3.34	0.07	0.25	0.08	<0.01	0.21
CHOL, mmol/mL	4.14	4.21	4.2	4.23	0.25	0.86	0.94	<0.01	0.86
NEFA, μmol/L	122	122	118	90	22.2	0.36	0.57	0.15	0.08
TG, mmol/mL	0.04	0.04	0.03	0.04	0.003	0.55	0.70	0.51	0.70
TBIL, μmol/L	0.79	0.77	0.63	0.70	0.05	0.07	0.44	<0.01	0.19
SOD, U/L	102	106	100	103	3.36	0.74	0.93	<0.01	0.08
MDA, nmol/mL	4.70	6.13	5.32	3.72	0.27	<0.01	<0.01	0.87	0.44
NO, μmol/L	21.9	24.3	31.1	29.3	0.07	0.69	<0.01	<0.01	<0.01
T-NOS, U/mL	12.6	14.3	13.9	15.4	0.70	0.01	0.88	<0.01	0.74

¹CHOL = cholesterol; NEFA = nonesterified fatty acid; TG = triglyceride; TBIL = total bilirubin; SOD = superoxide dismutase; MDA = malondialdehyde; NO = nitric oxide; T-NOS = total nitric oxide synthase.

²W = week effect; W × T = interaction between week and treatment; L = linear; Q = quadratic.

effect between week and NCG addition on Arg and its downstream metabolites (NO, Glu and Asp) indicated a delayed effect of NCG on Arg metabolism of mid-lactating cows. This occurred because more Arg, NO, Glu, and Asp were synthesized when NCG was fed long-term (12 wk) compared with short-term (6 wk). The linearly increased Pro concentration in plasma following the increased NCG addition could be attributed to the Pro synthesis from Arg in the small intestine

(Bertolo et al., 2003). As a kind of functional AA, Arg has many functions, including improvement of the production of animals and modulation of the related metabolism (Wu et al., 2004; Cao et al., 2016; Liu et al., 2016). Our study suggested that linearly increased NCG addition improved milk protein content, which is consistent with our previous work (Chacher et al., 2014). In the study of Chacher et al. (2014), protein content increased mainly because N utilization was

Table 4. Effects of dietary *N*-carbamoylglutamate on the plasma AA concentration

Item ¹	<i>N</i> -carbamoylglutamate, g/d					<i>P</i> -value ¹			
						Treatment			
	0	10	20	40	SEM	L	Q	W	T × W
EAA, ug/mL									
Arg	3.31	3.74	3.82	3.74	0.06	<0.01	<0.01	<0.01	0.04
His	2.31	2.40	2.32	2.35	0.03	0.85	0.34	0.20	0.06
Ile	2.85	2.88	2.87	2.88	0.04	0.68	0.84	0.26	0.19
Leu	5.91	5.88	5.96	5.87	0.06	0.92	0.65	0.24	0.15
Lys	5.48	5.49	5.54	5.46	0.05	0.77	0.37	0.01	0.16
Met	1.05	1.05	1.05	1.03	0.02	0.43	0.60	<0.01	0.06
Phe	4.34	4.38	4.33	4.40	0.05	0.50	0.72	0.78	0.66
Thr	5.19	5.47	5.06	5.22	0.18	0.70	0.75	0.48	0.15
Val	30.4	30.1	30.2	29.5	0.31	0.59	0.53	0.71	0.31
Subtotal	60.8	61.7	60.2	61.1	0.26	0.10	<0.01	0.28	0.82
NEAA, ug/mL									
Ser	3.31	3.27	3.29	3.30	0.05	0.96	0.62	0.18	0.47
Glu	9.94	10.3	10.5	10.4	0.07	<0.01	<0.01	<0.01	0.01
Tyr	3.33	3.28	3.29	3.12	0.10	0.26	0.56	0.80	0.81
Cys	0.63	0.67	0.64	0.65	0.65	0.25	0.08	0.02	0.51
Gly	6.21	6.12	6.13	6.02	0.06	0.08	0.94	0.05	0.27
Ala	4.21	4.21	4.13	4.09	0.06	0.13	0.78	0.66	0.09
Asp	8.50	8.34	8.63	8.48	0.08	0.59	0.96	0.22	<0.01
Pro	2.14	2.15	2.15	2.27	0.04	0.05	0.21	<0.01	0.74
Subtotal	38.2	38.3	38.7	38.4	0.17	0.16	0.28	0.66	0.73
Total AA	99.0	99.2	99.9	99.3	0.38	0.06	0.67	0.27	0.53

¹W = week effect; W × T = interaction between week and treatment; L = linear; Q = quadratic.

increased and AA were balanced when NCG was fed to high-yielding dairy cows. However, we did not find the same change of N utilization and AA profiles in our study, which may be attributed to the different lactation stages of dairy cows. In Chacher et al. (2014), the cows at early-stage lactation produced 40 kg/d of milk, which may be associated with severe oxidative stress due to the high metabolism rate at this stage (Lohrke et al., 2004). In nonruminant animals, Arg is thought to be insufficient under oxidative stress, and it can alleviate oxidative stress responses by improving antioxidative status (Cao et al., 2016). It is possible that Arg is insufficient for the dairy cows under the experimental conditions of Chacher et al. (2014). In the present study, the cows at mid lactation producing only 20 kg of milk may have a sufficient supply of Arg from their diets and a stable physiological status.

The linearly increased milk protein content in cows fed NCG in our study may be attributed to the enhanced synthesis of Arg and total AA. Chen et al. (2013) and Ding et al. (2018) found that jugular infusion of Arg increased the expression of α_S - and β -CN and was beneficial for milk protein production and milk quality. Similarly, Doepel and Lapierre (2011) found that the EAA mixture infusion tended to increase the milk protein content. Some studies found that the addition of Arg improved endocrine hormones secretion, such as insulin (Liang et al., 2017), prolactin, growth hormone (Chew et al., 1984), and leptin (Hyun-Seok et al., 2017), which are important factors in regulating milk protein synthesis. In addition, Arg is the precursor of ornithine that acts as a carbon precursor or of Pro and as an N precursor of several NEAA, notably Glu, Ala, and Ser (Mephram, 1982). These results indicated that Arg plays an important additional role in regulating milk protein synthesis. In the current study, dietary addition of NCG increased the available Arg of mid-lactating dairy cows, which is consistent with previous study (Chacher et al., 2014). Therefore, the increased milk protein content following NCG addition could be attributed to the increased available Arg in blood circulation and improved availability of the precursors for protein synthesis.

In our study, the addition of NCG improved the content of milk fat and the milk fat yield, which was inconsistent with previous study conducted on NCG (Chacher et al., 2014), and Arg (Doepel and Lapierre, 2011). However, Alumot et al. (1983) found that Pro supplementation improved milk fat contents in lactating dairy goats. In our study, the linearly increased Pro content in sera following addition of NCG might have been due to the greater Pro synthesis from Arg with NCG addition (Bertolo et al., 2003). Another study (Palmquist and Conrad, 1978) suggested that the in-

creased milk fat is accompanied by improved available energy in the body. Liu et al. (2016) found that NCG could alter energy and lipid metabolism. In our study, glucose was increased with NCG addition, possibly attributed to increased glucogenic AA, such as Glu, Arg, and Pro. With the greater glucose synthesis by NCG addition, cows fed with NCG had greater coenzyme A-SH and NADPH, which are the precursor and energy for fatty acid synthesis in the mammary gland (Zhao, 2014). These results indicate that NCG is involved in energy and lipid metabolism, but the mechanism remained to be investigated.

In conclusion, in mid-lactating dairy cows, NCG supplementation did not affect the milk yield but improved milk fat and protein compositions. The linearly increased fat and protein contents with increased NCG addition may be related to the enhanced endogenous synthesis of Arg, resulting in the improved total EAA and glucogenic AA (such as Glu, Arg, and Pro). In the current experimental conditions, addition of NCG in mid-lactating cows seemed to be more related to improved milk quality than milk yield.

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