Effects of arginase inhibition via jugular infusion of *N*^ω-hydroxy-nor-L-arginine on metabolic and immune indices in lactating dairy cows

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ABSTRACT

Transformation of Arg to nitric oxide and ornithine (Arg-Orn) constitutes the main route of Arg metabolism in mammals. The primary objective of this work was to determine the effects of inhibiting the Arg-Orn pathway via *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) on health of lactating cows. Furthermore, we also explored the effect of Arg-Orn inhibition on the efficiency of nitrogen utilization to find support for previous research that showed the inhibition of Arg-Orn inhibited milk protein synthesis. Six healthy Chinese Holstein cows of similar body weight (550.0 ± 20 kg), parity (4.0 ± 0), body condition score (3.0 ± 0), milk yield (21.0 ± 1.0 kg), and days in milk (80 ± 2 d) were selected and randomly assigned to 3 treatments in a replicated 3×3 Latin square design with 22 d for each period (7 d for infusion and 15 d for washout). The treatments were (1) saline infusion (control); (2) infusion of 125 mg/L of nor-NOHA; and (3) infusion of 125 mg/L of nor-NOHA with 9.42 g/L of Arg. Dry matter intake, apparent digestibility of nutrients, urinary N, N in milk, and blood indices of metabolism and immune function were determined. Compared with the control, the infusion of nor-NOHA had no effect on the concentrations of cholesterol, high-density lipoproteins, IgA, IL-1 β , tumor necrosis factor- α , and alanine transaminase. In addition, the dry matter intake, apparent digestibility of N, and the concentration of milk protein N in the Nor-NOHA did not differ from the control; however, the infusion of nor-NOHA and Arg resulted in greater concentrations of high-density lipoprotein, IgA, IL-1 β , and tumor necrosis factor- α , and lower concentrations of cholesterol in serum compared with the control. Moreover, the addition of Arg to cows infused

with nor-NOHA increased the concentration of nitrate (the indicator of nitric oxide) in serum and was associated with greater milk protein N production due to greater milk yield compared with those infused with nor-NOHA. Overall, the results indicated important roles of Arg in immunity and mammary N utilization, whereas a minor role of the Arg-Orn pathway in these physiologic processes was found.

Key words: arginine, immune function, *N*^ω-hydroxy-nor-L-arginine, N utilization efficiency

INTRODUCTION

Previous studies in goats and cows have reported that Arg, a semi-essential AA, plays important roles in the regulation of milk protein synthesis (Mabjeesh et al., 2002; Doepel and Lapierre, 2011). A recent study in bovine mammary epithelial cells (BMEC) also found the addition of Arg to growth medium promoted the synthesis of casein (Wang et al., 2014); similar results were also reported by Zhao et al. (2018) in lactating dairy cows. In addition to the effects on mammary protein synthesis in ruminants, Arg was also reported to have positive effects on the health in animals. For instance, high concentrations of Arg in blood increased the cytotoxicity of monocytes and natural killer cells in rats (Cynober, 2014), and induced death of tumor cells by activating macrophages (Waithe et al., 1975). In the study of Tan et al. (2014), using broiler chickens, supplementation with 1.42% Arg in the diet attenuated the overexpression of proinflammatory cytokines. Some of the functional effects of Arg seem to be related, at least in part, to its ability to reduce the concentration of IL-1 β , IL-6, and tumor necrosis factor- α (TNF- α) in BMEC induced by LPS (Wu et al., 2016). Recent work (Zhao et al., 2018) has demonstrated that, in lactating cows, a jugular infusion of Arg alters indices of immune function in those challenged with LPS. However, the relationship between Arg metabolism and the health in cattle is still unclear.

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Catabolism of Arg occurs via 2 main routes in mammals (Castillo et al., 1996; Wu and Morris, 1998): the transformation of Arg to nitric oxide (**Arg-NO**), which is regulated by nitric oxide synthase (**NOS**), and the transformation of Arg to ornithine (**Arg-Orn**) and polyamines, which is regulated by arginase and ornithine decarboxylase. The regulatory function of Arg on various physiologic processes could be related to its metabolites. For instance, putrescine and spermidine, the metabolic products of Arg-Orn have been reported to be associated with protein synthesis in porcine trophectoderm cells (Oka and Perry, 1974; Kong et al., 2014). Furthermore, proline, another metabolic product of Arg-Orn, was reported to play a key role in collagen synthesis in patients (Barbul et al., 1990), protecting lymphocytes from apoptosis and stimulating cell growth and antibody production (Li et al., 2007). Arginine supply and the synthesis of polyamines were also implicated in mucosal repair in humans (Moinard et al., 2005).

A recent study found that inhibition of the Arg-Orn pathway induced with *N*^ω-hydroxy-nor-L-arginine (**nor-NOHA**; a powerful specific inhibitor of arginase, as detailed by Custot et al., 1997) decreased the production of casein in BMEC (Wang et al., 2017). That study verified this result in vivo by infusing nor-NOHA to dairy cattle and found the Arg-Orn pathway and the daily production of casein in milk were inhibited (Ding et al., 2018). However, it is still unclear what effect inhibition of the Arg-Orn pathway has on the whole body.

Taking into consideration previous work, we hypothesized that Arg might partly regulate health status via the Arg-Orn pathway. This hypothesis is important given recent indicating a benefit of enhancing essential amino acid supply to dairy cows during critical stages of lactation such as the periparturient period (Zhou et al., 2016a,b; Batistel et al., 2018). Our specific objective was to verify the importance of the Arg-Orn pathway by exploring the effects of Arg-Orn inhibition on immune and liver functions in lactating cows. At the same time, data regarding the effects of Arg-Orn inhibition on N utilization were explored in an attempt to support the study of Ding et al. (2018).

MATERIALS AND METHODS

This study was a follow-up to Ding et al. (2018), which reported the effects of inhibition of Arg-Orn by nor-NOHA infusion on milk production and milk protein synthesis in lactating cows. The same experimental cows were used for the present study.

Animals, Diets, and Experiment Designs

The Yangzhou University Animal Care and Use Committee of Jiangsu Province approved the animal experimental protocol. The experiment was carried out with 6 Chinese lactating Holstein cows of similar BW (550.0 ± 20.0 kg; mean $\pm$ SE), BCS (3.0 ± 0 , measured using a 5-point scale method; Marija et al., 2011), parity (4.0 ± 0), milk yield (21.0 ± 1.0 kg), and DIM (80 ± 2 d) at the Experimental Farm of Yangzhou University (Yangzhou, Jiangsu, China).

The experimental cows were randomly assigned to 3 groups, with 2 cows in each group. The animals were fed a basal diet formulated according to NRC (2001). The composition and nutrient level of basal diets were the same as described in Ding et al. (2018). The 6 cows were individually separated by fences and housed in a large barn, which was designed for 30 cows; cows had ad libitum access to the TMR and fresh water. The experimental cows were fed twice daily, at 0600 and 2000 h, and milked thrice daily, at 0700, 1500, and 2300 h.

Before the infusion, indwelling catheters (L13712, Jiangxi Huali Medical Instrument Company, Ganzhou, China) were placed in the jugular vein during the experimental period. Catheters were flushed with 150 IU/mL of heparin and physiological saline twice daily and sealed with 750 IU/mL of heparin and physiological saline. The cows were then assigned, in a replicated 3×3 Latin square design with 22 d for each period (1 wk for infusion and 2 wk for washout), to 1 of 3 treatments with infusions of (1) saline (control); (2) saline + 125 mg/L nor-NOHA; or (3) saline + 125 mg/L nor-NOHA + 9.42 g/L L-Arg. The composition of perfusates used here was designed according to a previous study (Ding et al., 2018). The perfusates were prepared by Cambridge Biological Company (Nanjing, China) in sterile conditions 2 d before the infusion period and stored at 4°C. The solutions were infused continuously through a peristaltic pump (Longer, Hebei, China) with a speed of 0.5 L/h for 8 h/d (from 0600 to 1400 h). The infusions of different perfusates started together with feeding in the morning. The experimental cows were tied in separate pens when they were fed at 0600 h. Infusion began once the cows were tied and continued for 8 h/d. This work took almost an hour, and the first milking began around 0700 h by a portable milker (HL-JN02, Chuangpu Machinery Limited Company, Lianyungang, China) together with feeding and infusing. The experimental cows were fed again at 2000 h and milked at 1500 and 2300 h before being released to move around.

Sampling of Feed and Feces and Chemical Analysis

Within each period during infusions, the daily amount of TMR offered and residual feed for each cow were recorded for calculation of feed intake. Samples of fresh TMR and refusals of each cow were collected daily during the last 3 d of each infusion period. Fecal grab samples were collected 3 times daily (0600, 1300, and 2000 h) and a pool of 100 g of feces was made from the combination of each time point; samples were stored at -20°C . Diet ingredients and feces were dried in a forced-air oven (DHG9626A, Jinhong Co., Shanghai, China) at 60°C and ground to 1-mm particle size for analyses. One gram of sample was weighed and dried in a forced-air oven at 105°C for 4 h to measure DM and CP with methods described by Hach et al. (1987). Ether extract (EE) was measured with the Ankom fat analysis system (XT10, Ankom Technology, Macedon, NY); ADF and NDF were measured using amylase and sodium sulfite on an Ankom 200 fiber analyzer (Ankom Technology) according to Van Soest et al. (1991). Ash content was analyzed by incinerating a 1-g sample at 450°C in a muffle furnace (SX2-12-10, Jinqi Co., Shanghai, China) for 8 h. Organic matter was then calculated as $\text{OM} = (100 - \text{ash } \%)$ according to AOAC International standards (Parkany, 2002). Apparent digestibility coefficients of DM, CP, EE, ADF, NDF, and OM were calculated using the formula:

$$\begin{aligned} \text{Apparent digestibility coefficient } (\%) = \\ [1 - (\text{marker in diet } \% / \text{marker in feces } \%) \\ \times (\text{nutrient in feces } \% / \text{nutrient in diet } \%)] \times 100, \end{aligned}$$

using 4 mol/L of HCl acid-insoluble ash as described previously (Lee and Hristov, 2013).

Blood Sampling and Analysis

In the last day of the infusion period, blood from the coccygeal artery, instead of external pudendal artery (Cant et al., 1993), was collected into 10-mL serum tubes (Becton Dickinson Vacutainer System, Franklin Lakes, NJ) every 3 h from 0600 to 1500 h. The sampling of blood from coccygeal artery was carried out strictly by the following procedures. First, the needle for blood collection was inserted into the vessel, and the blood in artery (bright red) was differentiated from that in vein (dark red) by color. Furthermore, we observed whether the blood remained in the pipe following puncture, as high pressure in the artery would cause the blood to recede back into the artery. We then connected the needle

to the serum tube to ensure blood was drawn from the coccygeal artery.

Samples were centrifuged (10 min, $2,810 \times g$, 4°C) 2 to 3 h after collection and the serum was aliquoted and stored at -20°C before analysis of blood biochemical indices. An extra 2 mL of blood was sampled with a Vacutainer containing EDTA-K3 anticoagulant (Becton Dickinson Vacutainer System) and delivered to the Disease Control and Prevention Center (Yangzhou, China) immediately for analyses of white blood cell, red blood cell, blood platelet, and hemoglobin concentrations with an automatic hematology analyzer (HB-7201, Sinnova, Nanjing, China). The serum and blood samples were collected at 0600, 0900, 1200, and 1500 h from each cow. Pooled samples were generated by mixing equal amounts before biochemical analyses.

The concentrations of IgA, IgM, and IgG in serum were measured with commercial ELISA kits [MBS702497 (detection range: 1.56–100 $\mu\text{g/mL}$), MBS282896 (detection range: 0.16–10.00 $\mu\text{g/mL}$), and MBS285353 (detection range: 31.25–2,000 $\mu\text{g/mL}$) respectively, MyBioSource Inc., San Diego, CA]. The intra-assay coefficient of variation (CV) of the MBS702497 kit was lower than 8% and interassay CV was lower than 10%. The intra-assay CV of the MBS282896 kit was lower than 10% and interassay CV was lower than 12%. The intra-assay CV of the MBS285353 kit was lower than 8% and interassay CV was lower than 12%. The concentrations of IL-1 β , IL-6, and TNF- α were measured with bovine ELISA kits [LS - F11758 (detection range: 78–5,000 pg/mL, intra-assay CV <10%, interassay CV <12%); LS - F6140 (detection range: 7.81–500 pg/mL, intra-assay CV <10%, interassay CV <12%); LS - F5014 (detection range: 7.81–500 pg/mL, intra-assay CV <10%, interassay CV <12%)], LifeSpan BioScience Co., Seattle, WA]. The protocols used were those provided by the manufacturers (<https://www.lsbio.com/products/elisakits>). The absorbance to calculate concentrations was measured on a microplate spectrophotometer (ELx 800; BioTek Instruments, Winooski, VT). The concentrations of albumin, alanine transaminase, glutamic-oxaloacetic transaminase, total cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and glucose were assayed by commercial kits (AB7502, AL7930, AS7938, CH7532, TR7534, CH7536, CH7538, and GL7546, respectively, Zhicheng Biological Company, Shanghai, China) with an automatic biochemical analyzer (Hitachi 7060, Hitachi Group, Tokyo, Japan). The concentration of nitrate in serum was measured with the nitrate reductase method (A012-1, Jiancheng Biogineering Institute, Nanjing, China).

Table 1. Effect of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) infusion on the content of nitric oxide (NO) in serum

Item ¹	Treatment ²			SEM	P-value
	Control	Nor-NOHA	Nor-NOHA + Arg		
NO, μmol/L	67.54 ^b	72.59 ^{ab}	82.06 ^a	2.24	0.02

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Blood samples (10 mL) for serum isolation were collected every 3 h from 0600 to 1500 h on the last day of each infusion period. Pooled samples for each cow were made with equal amounts of samples at 0600, 0900, 1200, and 1500 h before analysis of NO concentration.

²Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

Data Collection and Milk Sample Analysis

Milk production was recorded at each milking and sampled on the last 2 d of each infusion period (d 6 and 7). Milk samples were collected 3 times a day and a pool was made from each sample in proportion to milk yield at each milking. A subsample of milk was used for measuring protein (Bentley FTS/FCM 400 Combi; Bentley Instrument, Chaska, MN). Another subsample was centrifuged at $2,810 \times g$ at 4°C for 10 min to remove fat before measuring urea nitrogen with a Urea Assay Kit (C013–2, Jiancheng Bioengineering Institute). Milk protein N content was calculated after determination of protein using a 6.38 factor, in which $N \text{ (g/kg)} = \text{protein (g/kg)} \times 6.38$.

Urine Sampling and Detection of Urea Nitrogen

Total urine was collected by using a simple urine cup method. Urine was weighed and 5% of the total volume was sampled on the last 2 d of each infusion period. Urine samples were acidified with 50% H₂SO₄ to achieve a pH between 2 and 4 before storage at 4°C to minimize volatilization (Freudenberger et al., 1994). Immediately after the collection of urine on the last day, samples were pooled within animals and mixed thoroughly for analysis. The concentration of urea N was measured with the Urea Assay Kit (C013–2, Jiancheng Bioengineering Institute).

Statistical Analysis

Data were analyzed using the general linear model procedures of SPSS 16.0 (SPSS Inc., Chicago, IL):

$$Y_{ijk} = \mu + T_i + P_j + C_k + e_{ijk},$$

where Y_{ijk} = response variable value of the *k*th cow subjected to the *i*th treatment in the *j*th period, μ is the grand mean, T_i = fixed effect of the *i*th treatment

(*i* = control, nor-NOHA, and nor-NOHA + Arg), P_j = random effect of the *j*th period (*j* = 1, 2 and 3), C_k = random effect of the *k*th cow, and e_{ijk} = the random error. Treatment differences were determined by the Tukey multiple comparison test and were considered significant if $P < 0.05$.

RESULTS

Effects of nor-NOHA Infusion on Concentration of Nitrate in Serum

As described in Table 1, the infusion of nor-NOHA had no effect on the content of nitrate in serum compared with the control ($P = 0.51$) or nor-NOHA + Arg ($P = 0.12$). However, the concentration of nitrate in response to nor-NOHA + Arg was significantly higher than that in the control ($P = 0.01$).

Effects of nor-NOHA Infusion on Plasma and Blood Indices

We found no difference in the concentrations of IgM, IgG, IL-6, or albumin as well as the activity of alanine transaminase and glutamic-oxaloacetic transaminase in serum among groups (Table 2). The infusion of nor-NOHA + Arg increased the concentration of IgA and TNF-α in serum compared with the control ($P = 0.03$ and $P = 0.02$, respectively) and nor-NOHA alone ($P = 0.03$ and $P = 0.04$, respectively). Furthermore, no difference in serum IgA ($P = 1.00$) and TNF-α ($P = 0.96$) was found between the control and nor-NOHA alone. The infusion of nor-NOHA had no effect on the concentrations of IL-1β in serum compared with the control ($P = 0.06$); the infusion of nor-NOHA plus Arg increased the content of IL-1β compared with that in the control ($P = 0.01$). We observed no difference in the number of white blood cells, red blood cells, and blood platelets or the concentration of hemoglobin in blood between different groups (Table 3).

Table 2. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) infusion on immunoglobulins, cytokines, alanine transaminase, glutamic-oxaloacetic transaminase, and albumin in dairy cows

Item ¹	Treatment ²			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
IgA, g/L	0.69 ^b	0.68 ^b	0.95 ^a	0.05	0.02
IgM, g/L	1.01	1.14	0.96	0.11	0.28
IgG, g/L	23.33	22.33	23.83	1.28	0.51
IL-1 β , pg/mL	214.70 ^b	245.07 ^{ab}	255.18 ^a	6.31	0.01
IL-6, pg/mL	131.39	133.76	140.57	3.17	0.50
TNF- α , pg/mL	83.63 ^b	84.70 ^b	95.83 ^a	2.07	0.02
ALT, U/L	24.67	25.33	29.33	1.97	0.07
AST, U/L	58.83	64.33	63.33	4.30	0.42
ALB, g/L	28.17	28.52	28.93	1.89	0.92

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Blood samples (10 mL) for serum isolation were collected every 3 h from 0600 to 1500 h on the last day of each infusion period. Pooled samples for each cow were made with equal amounts of samples at 0600, 0900, 1200, and 1500 h before analysis of immune parameters. TNF- α = tumor necrosis factor- α ; ALT = alanine transaminase; AST = glutamic-oxaloacetic transaminase; ALB = albumin.

²Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

Effects of nor-NOHA Infusion on Plasma Parameters

No treatment effects were found for the concentrations of glucose, triglycerides, and LDL in serum (Table 4). Furthermore, compared with the control, the infusion of nor-NOHA had no effect on the concentration of cholesterol ($P = 1.00$) and HDL ($P = 0.96$) in serum; however, the concentration of cholesterol in the serum of cows infused with nor-NOHA + Arg was lower than that in control ($P = 0.048$). The concentration of HDL in nor-NOHA + Arg was higher compared with control ($P = 0.049$).

Effects of nor-NOHA Infusion on DMI and Apparent Digestibility of Nutrients

We found no significant difference in DMI or apparent digestibility of DM, CP, NDF, ADF, and OM among

different groups (Table 5). Compared with control ($P = 0.046$) and nor-NOHA alone ($P = 0.047$), the infusion of nor-NOHA plus Arg increased the apparent digestibility of EE. Digestibility of EE between control and nor-NOHA alone was not significant ($P = 0.47$).

Effects of nor-NOHA Infusion on Digestion, Absorption, and Utilization of N

Effects on Partitioning of N. We noted no differences in dietary N intake, digestible N intake, and total N intake across treatments (Table 6); the daily quantities of feces N, urine N, MUN, and retained N were also similar across groups. Furthermore, the daily production of protein N in milk of the cows infused with nor-NOHA was similar to that in the control ($P = 0.06$), but significantly lower compared with nor-NOHA + Arg ($P = 0.02$). Furthermore, as described

Table 3. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) infusion on blood routine indices of lactating cows

Item ¹	Treatment ²			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
White blood cells, 10 ⁹ /L	8.36	9.00	8.72	1.79	0.37
Red blood cells, 10 ¹² /L	7.16	6.95	6.77	0.32	0.57
Hemoglobin, g/L	102.73	88.83	104.33	11.60	0.36
Blood platelets, 10 ⁹ /L	361.33	298.17	348.67	44.77	0.35

¹Blood samples (10 mL, EDTA-K3 anticoagulant) for serum isolation were collected every 3 h from 0600 to 1500 h on the last day of each infusion period. Pooled samples for each cow were made with equal amounts of samples at 0600, 0900, 1200, and 1500 h before analysis.

²Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

Table 4. Effects of *N*²-hydroxy-nor-L-arginine (nor-NOHA) infusion on the concentrations of glucose, cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein

Item, ¹ mmol/L	Treatment ²			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
Glucose	3.92	3.74	3.70	0.45	0.88
Cholesterol	3.30 ^a	3.31 ^{ab}	2.85 ^b	0.09	0.03
Triglyceride	0.22	0.18	0.21	0.04	0.53
High-density lipoprotein	1.87 ^b	1.90 ^{ab}	2.14 ^a	0.10	0.04
Low-density lipoprotein	0.80	0.77	0.85	0.14	0.83

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Blood samples (10 mL) for serum isolation were collected every 3 h from 0600 to 1500 h on the last day of each infusion period. Pooled samples for each cow were made with equal amounts of samples at 0600, 0900, 1200, and 1500 h before analysis of liver function indices.

²Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

in our previous study (Ding et al., 2018), the infusion of nor-NOHA + Arg had no effects on milk yield compared with control ($P = 0.11$) but led to greater milk yield compared with nor-NOHA alone ($P < 0.01$). The difference in daily production of milk protein N and milk yield between control and nor-NOHA + Arg was not significant ($P = 0.86$ and $P = 0.35$, respectively).

Effects on Utilization of N. We found no difference in the ratios of fecal N to dietary N intake, urine N to total N intake, and retained N to total N intake among the different groups (Table 7). The ratio of milk protein N to total N intake in the nor-NOHA group was significantly lower than the cows treated with nor-NOHA plus Arg ($P = 0.01$); however, we observed no difference in the milk protein N-to-total N intake ratio between the control and nor-NOHA alone ($P = 0.42$) or the control and the nor-NOHA + Arg groups ($P = 0.23$).

DISCUSSION

Effects of nor-NOHA Infusion on Arg-Orn and Arg-NO Pathway and Metabolites

As reported in a previous study with the same animals (Ding et al., 2018), the infusion of nor-NOHA or nor-NOHA + Arg through the jugular vein inhibited the activity of total arginase, but had no effects on the activity of ornithine decarboxylase and total NOS in the blood of dairy cows. Although the activity of total NOS in different groups was similar, the addition of Arg to cows infused with nor-NOHA increased the concentration of nitrate in blood compared with control, indicating an elevated concentration of NO. This result was similar to the report by Jeyabalan et al. (2008) in mice that received an intraperitoneal injection (100 mg/kg) of nor-NOHA. This effect may have been caused by

Table 5. Effect of *N*²-hydroxy-nor-L-arginine (nor-NOHA) infusion on feed intake and apparent digestibility of nutrients

Item, ¹ % (unless noted)	Treatment ²			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
DMI, kg/d	22.40	21.26	22.36	1.41	0.66
DM digestibility	78.98	78.04	79.56	1.70	0.67
CP digestibility	73.86	74.75	75.35	1.07	0.40
Ether extract digestibility	76.60 ^b	73.03 ^b	81.81 ^a	4.76	0.04
NDF digestibility	73.22	70.13	74.02	2.12	0.19
ADF digestibility	42.99	38.88	43.31	2.23	0.13
OM ³ digestibility	81.46	80.62	81.81	1.53	0.73

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Samples of fresh TMR, refusals, and feces for each cow were collected daily during the last 3 d of each infusion period.

²Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

³OM was calculated as OM = (100 – ash %) according to AOAC International (Parkany, 2002).

Table 6. Effects of *N*²-hydroxy-nor-L-arginine (nor-NOHA) infusion on the intake and output of nitrogen in dairy cows

Item, ¹ g/d	Treatment ²			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
Dietary N intake	501.79	476.16	500.86	12.33	0.66
Digestible N intake	370.06	356.26	377.13	8.97	0.66
Total N intake ³	501.79	476.16	512.99	12.61	0.51
Feces N	131.73	119.90	123.73	4.19	0.54
Urine N	193.60	203.17	198.55	5.29	0.79
Milk protein N	123.96 ^{ab}	108.34 ^b	127.48 ^a	3.77	0.01
MUN	10.09	10.89	9.75	0.20	0.05
Retained N	42.42	33.86	53.15	8.80	0.70

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Samples of fresh TMR, refusals, and feces for each cow were collected daily during the last 3 d of each infusion period. Total urine was collected daily during the last 2 d of each infusion period.

²Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

³Total N intake = dietary N intake + N in perfusate (12.13 g/d).

the increase in concentration of substrate for arginase and NOS in blood. As described in our previous study (Ding et al., 2018), the Arg-Orn pathway was inhibited by the infusion of nor-NOHA. The addition of Arg to perfusate with nor-NOHA increased the concentration of Arg, resulting in an increase of product from the other pathway (Arg-NO) for Arg metabolism.

Effects of nor-NOHA Infusion on Immune Function Indices

The supplementation of Arg can play beneficial roles in the immune system, particularly on T cell-dependent and thymus-dependent immune reactions (Barbul et al., 1980). Other positive effects of Arg on the immune system are not limited to T cells. For instance, studies in athymic mice indicated that the addition of Arg increased IL-1 and IL-2 production (Ochoa et al., 2001); furthermore, the level of TNF- α , a macrophage derived peptide in burned mice, also increased in response to dietary supplementation of Arg (Tsai et al., 2002). However, studies in lactating cows found that

infusion of Arg decreased IL-1 β and TNF- α in cows with inflammatory stress triggered by LPS (Zhao et al., 2018). The different effects of Arg on cytokines in these studies might have been caused by the different species or status of the experimental animals.

Arginine has a number of roles that are crucial to the immune response (Satriano, 2004). In mammals, there are 2 well-investigated pathways of Arg catabolism associated with immunity (Cook et al., 1994): the conversion of Arg to NO and the degradation of Arg to Orn. Based on previous studies in animals and humans, Arg regulates immune function mainly by affecting the production of NO mediated by NOS (Choi et al., 2009; Hamani et al., 2010). As described by Wu et al. (2016), the addition of Arg increased the content of inducible NOS and NO and attenuated the inflammatory response in BMEC incubated with LPS. In addition, Arg is a potent secretagogue for insulin, prolactin, growth hormone, and IGF-1 (Newsholme et al., 2005), which mediate a NO-independent effect on immune function.

In the present study, no treatment effects were detected for most biomarkers, but the concentrations of

Table 7. Effects of *N*²-hydroxy-nor-L-arginine (nor-NOHA) infusion on the utilization efficiency of nitrogen in dairy cows

Item	Treatment ¹			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
Feces N/Dietary N intake	0.26	0.25	0.25	0.01	0.40
Urine N/Total N intake ²	0.39	0.43	0.40	0.04	0.22
Milk protein N/Total N intake	0.25 ^{ab}	0.23 ^b	0.28 ^a	0.01	0.02
Retained N/Total N intake	0.08	0.07	0.10	0.02	0.78

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Control = saline; Nor-NOHA = saline + 125 mg/L of nor-NOHA; Nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

²Total N intake = dietary N intake + N in perfusate (12.13 g/d).

IL-1 β and TNF- α increased along with nitrate (the indicator of NO) in response to nor-NOHA + Arg. Although IL-1 β , IL-6, and TNF- α are normally regarded as proinflammatory cytokines, IL-6 (Scheller et al., 2011) and TNF- α (Liu et al., 1998) also can elicit anti-inflammatory responses. Furthermore, the increased IgA concentration, which was reported to be related to an anti-inflammatory response (Ben Mkaddem et al., 2013; Monteiro, 2014), suggested that these cytokines might exert an anti-inflammatory effect in the cows infused with Arg and nor-NOHA. Furthermore, a previous study from our laboratories (L. Y. Ding, Y. Z. Shen, G. Zhou, X. Zhang, M. Z. Wang, J. Zhang, Y. F. Wang, and J. J. Looor, unpublished data) found that the infusion of Arg through jugular vein increased the concentrations of IgM and albumin but decreased haptoglobin level in blood, reflecting a relatively reduced inflammatory reaction. Together, the results reported previously and in the present study lead us to speculate that the Arg-Orn pathway might affect the immune function of cows to a small degree.

Effects of nor-NOHA Infusion on Plasma Parameters

Growing evidence indicates that Arg has positive effects on liver function in growing finishing pigs (Tan et al., 2014) and broiler chickens (Fouad et al., 2013). The effects of Arg on liver function include a reduction of plasma triglycerides and cholesterol concentrations and an increase in insulin sensitivity in obese rats (Fu et al., 2005; Jobgen, 2007). Further studies conclusively showed that Arg effects on liver function involve multiple NO-dependent pathways (Jobgen et al., 2006). Nitric oxide stimulates the oxidation of energy substrates in liver, such as fatty acids and glucose, by regulating mitochondrial biogenesis (Kwiterovich, 2000).

In our study, the levels of glucose, triglyceride, and LDL were not affected by different treatments, whereas the concentration of HDL level in nor-NOHA + Arg increased compared with that in control. A similar result was also reported by Nagasaka et al. (2006), where the concentration of HDL (indicated by the detection of HDL cholesterol) in serum of children with ornithine transcarbamylase deficiency was increased after oral administration of Arg via a dose of 55 to 70 mg/kg twice daily for 3 mo. A previous study from our laboratories (L. Y. Ding, Y. Z. Shen, G. Zhou, X. Zhang, M. Z. Wang, J. Zhang, Y. F. Wang, and J. J. Looor, unpublished data) also found an increase of HDL level in dairy cattle infused with Arg. Furthermore, HDL has been closely associated with the decreasing accumulation of fat molecules such as cholesterol, triglycerides, and phospholipids (Kwiterovich, 2000). The increase of HDL level should have contributed to the decrease

of cholesterol in the nor-NOHA + Arg compared with that in the control group in our study. As described in a study with rats (Méndez and Balderas, 2001), the injection of Arg to diabetic animals induced by alloxan reduced the concentration of total cholesterol in serum together with an increase in level of HDL in serum.

Effects of nor-NOHA Infusion on DMI and Apparent Digestibility of Nutrients

Arginine was first reported to elicit a positive effect on food intake in growing kittens (Costello et al., 1980). Morley and Flood (1991) also found that insufficiency of Arg inhibited food intake and daily BW gain in mice. However, some studies also report that the addition of Arg to diets had no effect on food intake in normal or diabetic rats and neonatal pigs (Kohli et al., 2004; Yao et al., 2008). Research has indicated that NO is the main factor responsible for the regulation of food intake in response to Arg through its effects within the central nervous system (Toda et al., 2005). Han et al. (2013) reported that NO had positive effects on the food intake and daily weight gain of mice. The lack of effect on food intake by the inhibition of the Arg-Orn pathway through infusion of nor-NOHA casts doubt on this pathway as being responsible for the regulation of food intake in dairy cows. The lack of effect on DMI with the addition of Arg to nor-NOHA perfusate was similar to result found by Tian et al. (2017) in cows. The supplementation of substrate (Arg) of arginase and NOS should have activated the Arg-NO pathway to increase the production of NO while arginase was inhibited; the lack of effect seems to suggest a species-specific response.

Effects of nor-NOHA Infusion on Digestion, Absorption, and Utilization of N

Arginine increases in vitro casein protein synthesis in BMEC by regulating the transcription of casein genes and mTOR-related genes (Wang et al., 2014). Recent studies in BMEC also revealed that Arg regulates the synthesis of casein via its catabolic product polyamines (Wang et al., 2017). In our experiment, the inhibition of the Arg-Orn pathway by infusing nor-NOHA did not affect milk protein N production and the ratio of milk protein N to total N intake compared with the control. These results were in line with our previous study (Ding et al., 2018), where the inhibition of the Arg-Orn pathway reduced the concentration of total protein in milk but had no effects on total milk protein yield. However, with the addition of Arg to cows infused with nor-NOHA, the production of NO (detected by nitrate) increased while the Arg-Orn pathway was inhibited and

milk protein N production and the milk protein-to-total N intake ratio increased.

The infusion of nor-NOHA or nor-NOHA + Arg had no effects on dietary N intake and feces N, which meant the inhibition of the Arg-Orn pathway and the supplementation of Arg had no effects on the digestibility of N. The retained N index and the ratio of retained N to total N intake was also not affected by different treatments. Although we found no difference in the ratio of retained N to total N intake between the nor-NOHA and nor-NOHA + Arg groups, the ratio of milk protein N to total N intake of cows increased in nor-NOHA + Arg. The nitrogen utilization efficiency (NUE) should have been better with the nor-NOHA + Arg compared with the nor-NOHA group. Previous studies have verified that urinary N was negatively correlated with N utilization efficiency (Marini and Van Amburgh, 2005; Burke et al., 2008). In our study, the urine N production and urine N-to-total N intake ratio decreased with nor-NOHA and increased with nor-NOHA + Arg compared with control, but the difference was not significant. Furthermore, the output of urea N in milk was also reported to be another NUE indicator closely related to urinary N in animals fed concentrate diets containing different concentrations of CP (Hynes et al., 2016). In present study, the output of MUN also had a nonsignificant tendency to decrease in nor-NOHA + Arg compared with nor-NOHA alone, which partly supported the increased NUE in nor-NOHA + Arg compared with that in nor-NOHA alone. Combined with the results that the infusion of Arg increased milk yield, milk protein concentration, and production together with NUE in a previous study from our laboratories (L. Y. Ding, Y. Z. Shen, G. Zhou, X. Zhang, M. Z. Wang, J. Zhang, Y. F. Wang, and J. J. Looor, unpublished data), it appears that milk production in the mammary gland is partly regulated by the Arg-Orn and the Arg-NO pathways. The exact mechanisms should be addressed in future studies, in particular during the periparturient period when a multitude of adaptations in key organs of the cow may be responsive to Arg supply (Looor et al., 2013).

CONCLUSIONS

As described, the injection of nor-NOHA had no effects on the levels of cholesterol, HDL, IgA, IL-1 β , TNF- α , and alanine transaminase compared with the control. In conclusion, the lack of effect on immune and liver function when the Arg-Orn pathway was inhibited by nor-NOHA infusion suggests a minor role of Arg-Orn in these physiologic processes. However the concentrations of nitrate (the indicator of NO), HDL, IgA, IL-1 β , and TNF- α in nor-NOHA + Arg increased,

together with a decrease in the content of cholesterol in serum compared with control, indicating important roles of Arg in immune function. Furthermore, the nor-NOHA infusion had no effects on food intake, the apparent digestibility of N, and the concentration of milk protein N. However, the addition of Arg combined with nor-NOHA increased milk protein N production by improving milk yield, leading to the increase of the NUE, which partly supported the results reported before (Ding et al., 2018).

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