Effects of low dietary cation-anion difference induced by ruminal ammonium chloride infusion on performance, serum, and urine metabolites of lactating dairy cows

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Objective: The objective of the present study was to determine ammonium chloride tolerance of lactating dairy cows, by examining effects of negative dietary cation anion difference (DCAD) induced by ruminal ammonium chloride infusion on performance, serum and urine minerals, serum metabolites and enzymes of lactating dairy cows.

Methods: Four primiparous lactating Chinese Holstein cows fitted with ruminal cannulas were infused with increasing amounts (0, 150, 300, or 450 g/d) of ammonium chloride in a crossover design. The DCAD of the base diet was 279 mEq/kg dry matter (DM) using the DCAD formula (Na + K − Cl − S)/kg of DM. Ammonium chloride infusion added the equivalent of 0, 128, 330, and 536 mEq/kg DM of Cl in treatments. According to the different dry matter intakes (DMI), the resulting actual DCAD of the four treatments was 279, 151, –51, and –257 mEq/kg DM, respectively.

Results: DMI decreased linearly as DCAD decreased. Yields of milk, 4% fat-corrected milk, energy-corrected milk, milk fat, and milk protein decreased linearly as DCAD decreased. Concentrations of milk protein and milk urea nitrogen increased linearly with decreasing DCAD. Concentration of Cl− in serum increased linearly and concentration of PO₄³⁻ in serum increased quadratically as DCAD decreased. Urine pH decreased linearly and calculated urine volume increased linearly with decreasing DCAD. Linear increases in daily urinary excretion of Cl−, Ca²⁺, PO₄³⁻, urea N, and ammonium were observed as DCAD decreased. Activities of alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transferase in serum and urea N concentration in serum increased linearly as DCAD decreased.

Conclusion: In conclusion, negative DCAD induced by ruminal ammonium chloride infusion resulted in a metabolic acidosis, had a negative influence on performance, and increased serum enzymes indicating potential liver and kidney damage in lactating dairy cows. Daily ammonium chloride intake by lactating dairy cows should not exceed 300 g, and 150 g/d per cow may be better.

Keywords: Lactating Dairy Cow; Dietary Cation Anion Difference; Ammonium Chloride; Serum and Urine Metabolites

INTRODUCTION

In dairy cows, the concept of negative dietary cation anion difference (DCAD, defined as milliequivalents of Na + K – Cl – S per kg of feed dry matter [DM]) has been used in dry cow nutrition to reduce the incidence of parturient paresis [1,2]. Interest has also grown in the potential effects of DCAD on lactating dairy cows. Tucker et al [3] were the first to evaluate DCAD in lactating dairy cows, and their results reported that milk yield was 8.6% higher when a diet with DCAD of 20 vs –10 mEq (Na + K –Cl)/100 g of DM was fed. Similar re-
The main objective of this study was to determine NH₄Cl tolerance of lactating dairy cows, by examining effects of negative DCAD induced by ruminal NH₄Cl infusion on performance, serum and urine minerals, serum metabolites and enzymes of lactating dairy cows. The second objective of this study was to determine whether NH₄Cl could pose risk of ammonia toxicity under the conditions of the present study.

In China, regulatory authorities require demonstrations of product safety to animals before new products can be registered. Such demonstrations may include whether a feed approved for one use, such as for creating a negative DCAD in non-lactating cows, might have detrimental effects if accidentally used in another application within that species, such as feeding ammonium chloride (NH₄Cl) to lactating cows. Therefore, further efforts need to be made to examine effects of NH₄Cl on milk performance, serum, and urine metabolites of lactating dairy cows under Chinese feeding conditions in order to determine NH₄Cl tolerance of lactating dairy cows.

NH₄Cl, as a feed additive, has been widely used in livestock production. Since Leoschke and Elvehjem [8] reported prevention of urinary calculi formation in mink by decreasing urinary pH with addition of NH₄Cl, it has been applied as an acidity regulator in feed for bovines [9,10], goats [11-13], sheep [14], dogs [15], cats [16], and horses [17]. Administration of NH₄Cl also has been widely used to create a model of metabolic acidosis in both humans and animals [18,19]. Common chloride salts used to decrease DCAD for dry cows include MgCl₂, CaCl₂, and NH₄Cl. Compared to Mg²⁺ and Ca²⁺, NH₄⁺ does not result in overload of essential elements to the cow or environment. However, as a source of nonprotein nitrogen (NPN), NH₄⁺ poses the risk of ammonia toxicity. However, Grookshank et al [20] reported that NH₄Cl can be used as a source of NPN without clinical signs of ammonia toxicity when added up to 1% of the total ration. Bartley et al [21] showed that rumen pH was more clearly associated with ammonia toxicity than rumen ammonia. NH₄Cl dissociates into NH₄⁺ and Cl⁻ ions in the rumen without increasing rumen pH as occurs when urea is hydrolyzed to ammonia [22]. Kertz et al [23] found that low rumen pH traps ammonia within the rumen, in which case high rumen ammonia should not produce sublethal or lethal ammonia toxicity.

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Materials and methods

Animals, diets, and experimental design

All animals involved in this study were cared for according to principles of the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China). Four primiparous Chinese Holstein cows (body weight [BW] = 556±39 kg, days in milk = 357±2 d) that had been fitted previously with ruminal cannulas (10-cm center diameter; Bar Diamond, Parma, ID, USA) were housed in a free-stall barn equipped with a computerized monitoring system (RIC system, Insentec B.V., Marknesse, the Netherlands). The system automatically identified individual cows by ear tags and recorded their feeding time and duration, as well as the quantity of feed intake at each meal. The basal diet (Table 1) was formulated to meet or exceed nutrient requirements for energy, protein, minerals, and vitamins according to the Feeding Standards of Dairy Cattle (Ministry of Agriculture of P. R. China recommendations, 2004). The diet was fed as a total mixed ration (TMR) 3 times daily (0730, 1330, and 2000 h) to ensure ad libitum intake, allowing for 5% orts, along with free access to water. Cows were milked 3 times daily, at 0800, 1400, and 2030 h.

The experimental design (Table 2) was as used previously [24,25]. Cows were administered the 2 treatments in a crossover design: an NH₄Cl solution at varying concentrations versus water as control. Each experimental period of the crossover design lasted 4 wk (Table 2). A cow in each period was thus considered a main plot. To prepare the cows for the first period, there was an initial 1-wk adaptation during which all cows were infused with water. During the 4-wk period, graded amounts of NH₄Cl (0, 150, 300, and 450 g/d) were administered to the treated cows with each amount coinciding with 1 of the 4 wk (i.e., the sub-plots of the split plot). Control cows continued to be infused with the same amount of water. For the treated cows, we determined based on preliminary experiments that it was not desirable physiologically to randomize the administration of these 4 amounts. Therefore, as in the study by Drackley et al [25], we allowed the cows to adapt to each amount of NH₄Cl before receiving a higher amount. After period 1, all cows were returned to water infusion for a 2-wk washout period. Then period 2 was initiated for another 4 wk with the same 4 cows in opposite treatment groups. With this design, the effects of week of administration and amount of NH₄Cl were confounded. Because control cows received water infusion each week, against which the treated cows were compared, the confounding is not relevant (see description of statistical analysis).

During the experiment periods, cows were fed with a TMR 3 times daily (0730, 1330, and 2000 h) to ensure ad libitum intake and infused 3 times daily after feeding 30 min. The infused NH₄Cl solution was freshly prepared before each infusion by dissolving one-third of the daily dose of NH₄Cl for each

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treatment amount in 600 mL of distilled water. Solutions were infused into the rumen manually by opening the ruminal can and administering the solutions into the rumen.

### Sampling and measurements

The TMR and orts from individual cows were sampled on the last 3 d of each week and analyzed for DM content by drying samples at 50°C for 48 h in a forced-air oven [26]. The samples were ground to pass through a 6-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA), and composited by cow. Subsamples of TMR and orts were ground to pass through a 1-mm screen to analyze crude protein (CP), ether extract, acid detergent fiber, and ash according to AOAC International [27]. The contents of neutral detergent fiber were obtained according to Van Soest et al [28], with α-amylase and without sodium sulfite. The dietary contents of Ca, P, Mg, Na, K, Cl, and S were determined at an official laboratory (National Food Safety Supervision and Inspection Center, Beijing, China) by Inductively Coupled Plasma-Optical Emission Spectrometer (Optima 8000DV, Perkin-Elmer, Shanghai, China).

Milk production was recorded daily and milk samples were collected on the last 3 d of each experimental week. Milk samples were collected at each milking of every sampling day, and the 3 samples from each day were pooled in a proportion of 4:3:3 by volume (this ratio reflecting the milk yield of morning, afternoon, and night) into 50-mL subsamples to which was added 1 milk preservative tablet (Bromopol, D and F Control Systems, San Ramon, CA, USA). This subsample was then stored at 4°C for future analysis of milk composition by infrared analysis [29] with a Foss-MilkoScan TM Minor (MilkoScan FT120, Foss Electric A/S, Hillerod, Denmark). Milk urea N was measured by an assay kit (urease method) purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Blood was sampled from the coccygeal vein on d 7 of each experimental week 3 h after the a.m. feeding. Blood samples collected in serum separator tubes (Serum Clot Activator, Greiner Bioone GmbH, A-4550 Kremsmunster, Austria) were allowed to clot for 30 min at room temperature and stored in the refrigerator overnight, and serum was harvested by centrifugation at 3,000×g for 15 min at 4°C [30]. Serum was stored at −20°C for future analysis of alanine aminotransferase (ALT; Reitman-Frankel colorimetric method), aspartate aminotransferase (AST; Reitman-Frankel colorimetric method), γ-glutamyl transferase (GGT; Szasz method), urea N (urease method), creatinine (picric acid colorimetric method), and uric acid (phosphotungstic acid colorimetric method), using an automated chemistry analyzer (Hitachi 7080, Beijing CIC Clinical Laboratory, Beijing, China). Assay kits for ALT, AST, GGT, urea N, creatinine, uric acid, and serum ammonium (protein precipitation method) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Urine samples were collected from each cow on the last 3 d of each experimental week [9]. Grab samples of midstream urine were collected between 1100 and 1145 h from each cow after eliciting micturition by manual stimulation of the vulva.
Table 2. Schematic of experimental design and application of treatments

| Cow     | Preliminary | Period 1 | Week | Period 2 |
|---------|-------------|----------|------|----------|
|         |             | 1       | 2   | 3       | 4   | 5 | 6 | 7 | 8 | 9 | 10 |
| 10,658  | W           | 0       | 150 | 300     | 450 | W | W | 0 | 0 | 0 | 0 |
| 10,662  | W           | 0       | 150 | 300     | 450 | W | W | 0 | 0 | 0 | 0 |
| 10,725  | W           | 0       | 0   | 0       | 0   | W | W | 150 | 300 | 450 |
| 10,714  | W           | 0       | 0   | 0       | 0   | W | W | 150 | 300 | 450 |

W, cows were infused with water only. For 0 g/d of NH₄Cl, cows were infused with water, too.

RESULTS

DCAD, DMI, and health

The DCAD of the base diet was 279 mEq/kg DM using the DCAD formula (Na + K – Cl – S)/kg of DM. NH₄Cl infusion added the equivalent of 0, 128, 330, and 536 mEq/kg DM of Cl from the 0, 150, 300, and 450 g/d treatments. Accordingly, based on actual DMI, the DCAD of the 4 treatments were 279, 151, –51, and –257 mEq/kg DM, respectively. Data for measured variables are reported and discussed in terms of these DCAD values.

Mean DMI decreased linearly as DCAD decreased (Table 3). During both periods 1 and 2, when infusion amount came to 300 g/d, the treatment cows began to have severely depressed DMI. By the last day of period 2, the 2 treatment cows were deemed too sick to complete the trial. Blood samples were
Table 3. Effects of low DCAD on dry matter intake and performance of lactating dairy cows

| Item                  | DCAD (mEq/kg DM) | SEM | Treatment by amount, p value |
|-----------------------|------------------|-----|-----------------------------|
|                       | 279              | 151 | –51                        | –257                        |
| DMI (kg/d)            | 21.6             | 21.9| 17.0                       | 15.7                       | 1.58 | 0.0001 | 0.376 |
| Milk (kg/d)           | 30.2             | 30.1| 27.6                       | 17.8                       | 2.89 | 0.001  | 0.060 |
| 4% FCM (kg/d)         | 29.4             | 30.1| 26.2                       | 17.4                       | 2.92 | 0.002  | 0.080 |
| ECM (kg/d)            | 32.2             | 32.6| 28.9                       | 19.4                       | 3.07 | 0.002  | 0.084 |
| Fat (%)               | 3.80             | 4.00| 3.66                       | 3.77                       | 0.071| 0.374  | 0.512 |
| Fat (kg/d)            | 1.16             | 1.20| 1.01                       | 0.68                       | 0.118| 0.003  | 0.120 |
| Protein (%)           | 3.40             | 3.26| 3.43                       | 3.94                       | 0.149| 0.002  | 0.022 |
| Protein (kg/d)        | 1.02             | 0.98| 0.95                       | 0.66                       | 0.082| 0.008  | 0.141 |
| Lactose (%)           | 4.86             | 4.90| 4.85                       | 4.41                       | 0.116| <0.0001| 0.004 |
| Lactose (kg/d)        | 1.48             | 1.48a| 1.34                       | 0.80                       | 0.162| 0.0004 | 0.039 |
| MUN (mg/dL)           | 21.04            | 22.77| 22.69                      | 27.03                      | 1.280| 0.002  | 0.703 |
| Total solids (%)      | 11.98            | 12.10| 11.88                      | 12.18                      | 2.797| 0.611  | 0.683 |
| Solids-not-fat (%)    | 8.54             | 8.45 | 8.55                       | 8.84                       | 0.085| 0.258  | 0.336 |

DCAD, dietary cation anion difference; DM, dry matter; SEM, standard error of the mean; DMI, dry matter intake; FCM, fat-corrected milk; ECM, energy-corrected milk; MUN, milk urea nitrogen.

1) 4% FCM (kg/d) = 0.4 × milk (kg/d) + 15 × fat (kg/d), (NRC [52]).
2) ECM (kg/d) = 0.327 × milk (kg/d) + 12.95 × fat (kg/d) + 7.65 × protein (kg/d).

obtained and cows received medical attention. After receiving therapy for 1 wk, the 2 sick cows had recovered.

Rumen pH (6.06, 6.16, 5.97, 5.88) tended to decrease linearly (p = 0.084) and concentration of ammonia (14.97, 20.81, 25.54, 39.30 mg/L) in the rumen contents increased linearly (p<0.0001) as infusion increased [35].

**Milk yield and composition**

Effects of decreasing DCAD on milk yield and composition are shown in Table 3. Yields of milk, 4% fat-corrected milk (FCM), and energy-corrected milk (ECM) decreased linearly. The quadratic effects approached significance, indicating that the magnitude of decrease tended to be greater when DCAD was from –51 to –257 mEq/kg DM. Similarly, milk fat yield and milk protein yield decreased linearly. Concentrations of milk protein and milk urea nitrogen (MUN) increased linearly with decreasing DCAD. Milk lactose concentration decreased as DCAD decreased, with decrease being greater as DCAD was from –51 mEq/kg DM or greater (quadratic effect, p = 0.004). Yield of milk lactose decreased in a quadratic fashion similar to milk lactose concentration, with the largest decrease when DCAD was –257 mEq/kg DM. No effects were observed on concentrations of milk fat, total solids, and solids-not-fat.

**Serum and urine minerals**

Table 4 presents mean values of serum ions, urine ions and urine metabolites of dairy cows as DCAD decreased. Urine pH decreased linearly and the quadratic effects approached significance, indicating that the magnitude of decreases tended to increase when DCAD was –51 or –257 mEq/kg DM. Concentration of Cl⁻ in serum increased linearly; the quadratic effect approached significance because the magnitude of increase tended to be greater when DCAD was –257 mEq/kg DM. Concentration of Ca²⁺ in serum tended to decrease linearly, but Mg²⁺ in serum tended to increase linearly with decreasing DCAD. Concentration of PO₄³⁻ in serum increased quadratically as DCAD decreased, with the largest increases when DCAD was –257 mEq/kg DM. Calculated urine volume increased linearly as DCAD decreased. Daily excretion of Cl⁻, Ca²⁺, PO₄³⁻, UUN, and urine ammonium increased linearly as DCAD decreased, whereas daily excretion of K⁺ and urine acid in urine decreased linearly.

**Serum metabolites and enzymes**

Blood biochemical indices of dairy cows as DCAD decreased are shown in Table 5. Linear increases were observed in serum activities of ALT, AST, and GGT, as well as the concentration of blood urea nitrogen, as DCAD decreased. However, the concentration of uric acid in serum decreased linearly with decreasing DCAD. Concentration of creatinine in serum tended to increase linearly while concentration of serum ammonium was unaffected with decreasing DCAD.

**DISCUSSION**

Urea and NH₄Cl, as sources of NPN, undergo different dissociation processes in the rumen. When urea is hydrolyzed by microbial ureases it forms NH₃, but NH₄Cl dissociates into NH₃ and Cl⁻. The NH₃ is converted to NH₄⁺ with the addition of a hydrogen ion, and rumen pH is unchanged or elevated [22] and vice versa. Bartley et al [21] reported that rumen pH was more related to ammonia toxicity than was rumen ammonia. Visek [36] showed that elevated pH facilitates greater absorption of ammonia across the rumen wall. In our study,
although ruminal ammonia concentration increased mark-
edly, ruminal pH decreased as infusion increased. Lower rumen
pH can trap ammonia within the rumen, and high rumen
ammonia does not produce sublethal or lethal ammonia toxici-
ty [23]. Rumen ammonia dissociated from NH\(_4\)Cl would be
absorbed gradually through ruminal epithelium cell to blood
and then excreted into urine. Therefore, the concentration of
ammonia in serum was not affected by the infusion of NH\(_4\)Cl,
and signs consistent with ammonia toxicity did not appear.
Therefore, we attribute most of the negative effects of increasing
NH\(_4\)Cl infusion to the negative DCAD and resulting uncom-
pensated metabolic acidosis.

Decreasing dietary DCAD had negative effects on the late-
lactating cows in this study. The linear decreases of milk yield,
4% FCM, ECM, milk fat yield, milk protein yield, and milk
lactose yield likely were attributable in large part to the de-
crease of DMI. Because NH\(_4\)Cl was infused into the rumen of
lactating dairy cows, the decrease of DMI could not have been
carried by palatability issues. Instead, decreased DMI likely was
debt to the metabolic acidosis that was induced by low DCAD. Cows
infused with the highest amount of NH\(_4\)Cl showed obvious
signs of distress due to the negative DCAD.

Dietary cation-anion balance has well-known effects on
acid-base status and production of cows and other animals. The
optimum DCAD in ruminants depends on their pro-
duction status. Block [1] reported that the incidence of milk
fever was reduced from 47.4% in cows fed a cationic diet to
0% when cows received an anionic diet during the dry period.
Conversely, Tucker et al [3] showed that a cationic diet (200

### Table 4. Effects of low DCAD on serum ions, urine ions and urine metabolites of lactating dairy cows

| Item                                      | DCAD (mEq/kg DM) | SEM | Treatment by amount, p value
|-------------------------------------------|------------------|-----|-----------------------------|
|                                           | 279              | 151 | –51 | –257 | Linear | Quadratic |
| Serum ions (mmol/L)                       |                  |     |     |      |        |           |
| K\(^+\)                                   | 4.23             | 4.34 | 4.32 | 4.08 | 0.059  |           |
| Na\(^+\)                                  | 137.5            | 137.8 | 138.4 | 142.4 | 1.14   |           |
| Cl\(^-\)                                  | 95.6             | 98.0 | 99.1 | 109.6 | 3.10   |           |
| Ca\(^{2+}\)                               | 2.30             | 2.30 | 2.28 | 2.19 | 0.026  |           |
| PO\(_4\)^{3-}\)                           | 1.68             | 1.67 | 1.78 | 2.25 | 0.137  |           |
| Mg\(^{2+}\)                               | 0.99             | 0.98 | 0.98 | 1.01 | 0.007  |           |
| Urine pH                                  | 7.92             | 7.54 | 5.83 | 5.70 | 0.573  | < 0.0001 |
| Urine ions (mmol/d)                       |                  |     |     |      |        |           |
| K\(^+\)                                   | 5,710            | 4,932 | 5,723 | 4,047 | 397.5  | 0.028    |
| Na\(^+\)                                  | 3,001            | 2,325 | 3,168 | 3,704 | 284.4  | 0.441    |
| Cl\(^-\)                                  | 3,356            | 5,232 | 7,854 | 7,716 | 1,078.32 | 0.0003   |
| Ca\(^{2+}\)                               | 14               | 100  | 214  | 731  | 160.8  | 0.0009   |
| PO\(_4\)^{3-}\)                           | 14.2             | 11.0 | 27.8 | 190.0 | 43.2   | 0.016    |
| Mg\(^{2+}\)                               | 114.5            | 123.1 | 151.2 | 154.4 | 10.0   | 0.790    |
| Urine volume (kg/d)                       | 42.7             | 44.8 | 92.5 | 127.9 | 20.5   | 0.025    |
| Creatinine clearance (L/min)              | 1.86             | 1.72 | 1.76 | 1.52 | 0.071  | 0.104    |
| UUN (mmol/d)                              | 4,465            | 4,541 | 5,274 | 6,451 | 562.8  | 0.001    |
| Urine ammonium (mmol/d)                   | 30               | 80   | 826  | 2541 | 586.1  | 0.004    |
| Allantoin (mmol/d)                        | 167              | 246  | 465  | 367  | 65.8   | 0.287    |
| Uric acid (mmol/d)                        | 46.3             | 48.9 | 49.4 | 29.8 | 4.64   | 0.015    |

DCAD, dietary cation anion difference; DM, dry matter; SEM, standard error of the mean; UUN, urine urea nitrogen.

### Table 5. Effects of low DCAD on biochemical indices of blood of lactating dairy cows

| Item                                      | DCAD (mEq/kg DM) | SEM | Treatment by amount, p value
|-------------------------------------------|------------------|-----|-----------------------------|
|                                           | 279              | 151 | –51 | –257 | Linear | Quadratic |
| Alanine aminotransferase (U/L)            | 26               | 28  | 28  | 46   | 4.7    |           |
| Aspartate aminotransferase (U/L)          | 79               | 85  | 95  | 114  | 7.7    |           |
| γ-Glutamyl transferase (mmol/L)           | 31.0             | 30.9 | 31.9 | 36.2 | 1.25   |           |
| Urea N (mmol/L)                           | 4.88             | 5.54 | 5.40 | 6.36 | 0.307  |           |
| Uric acid (umol/L)                        | 37.8             | 32.7 | 25.6 | 20.6 | 3.80   |           |
| Creatinine (umol/L)                       | 53               | 57  | 56  | 67   | 3.0    |           |
| Ammonia (umol/L)                          | 123.6            | 122.1 | 111.1 | 141  | 6.2    |           |

DCAD, dietary cation anion difference; DM, dry matter; SEM, standard error of the mean.
mEq/kg DM) resulted in greater milk yield than when diets containing −100, 0, or 100 mEq/kg were fed. In a meta-analysis, Hu and Murphy [37] reported that milk yield was greatest when the DCAD (Na + K – Cl) was 340 mEq/kg of DM, 4.0% FCM production was highest at 490 mEq/kg of DM DCAD, and DMI was maximized at 400 mEq/kg of DM DCAD. In our study, DMI, milk yield, ECM yield, and 4% FCM yield did not decrease when DCAD decreased from 279 to 151 mEq/kg DM, but decreased markedly when DCAD was −51 mEq/kg DM. The optimum DCAD may relate to stage of lactation and milk production, so lack of production change when DCAD was 151 mEq/kg DM may be attributable to the late stage of lactation (days in milk = 357±2 d) of our cows.

The concentration of milk protein measured by infrared analysis in our study represents CP or total nitrogen rather than true protein. Although the concentration of milk total protein increased with the higher infusion amounts, the concentration of MUN also increased with decreasing DCAD. So, the concentration of milk true protein likely did not increase with increasing amount of NH₄Cl infused. It seemed that NH₄⁺ as a source of NPN had little influence on the synthesis of milk protein under conditions of our study.

In general, responses of mineral ions in serum and ion excretion in urine followed well described patterns. Increased serum Cl⁻ concentration at the highest infusion rate may have resulted from the inability of the kidneys to excrete additional Cl⁻, as the amounts excreted were similar for DCAD of −151 and −257. The kidneys can efficiently eliminate excess anions from the blood, thus infusion of NH₄Cl induced a sharp reduction in urinary pH. The effect of increased dietary anions (Cl and S) to decrease urine pH is well documented [38-40]. Monitoring the pH of urine is considered a sensitive method for assessing the acid-base balance of animals [41].

Urinary excretion of Ca²⁺ increased sharply with decreasing DCAD, with serum Ca²⁺ concentration decreasing at the lowest DCAD. Previous studies revealed that decreased DCAD increased urinary Ca²⁺ excretion in lactating [42], nonlactating [43], and close-up prepartum [44,45] dairy cows. The metabolic acidosis induced by the negative DCAD diets in this study likely decreased Ca²⁺ reabsorption via the kidney tubules, so more Ca²⁺ was excreted into the urine. Although negative DCAD diets prepartum are believed to increase serum Ca²⁺ in newly calved cows by increasing Ca mobilization from bone to maintain blood Ca²⁺ [46], the extreme acidosis induced in this study likely created more urinary excretion of Ca²⁺ than could be maintained in the blood.

Because bone hydroxyapatite is the source of increased Ca²⁺ concentrations excreted in urine, increased concentration of PO₄³⁻ in serum and increased urinary PO₄³⁻ excretion was expected. Block [1] observed an increase in concentration of serum PO₄³⁻ during the peripartum period for cows receiving anionic diets. Metabolic acidosis induced by low DCAD diets may result in an increase in parathyroid hormone release, which in turn would increase phosphorus mobilized from bone, increase serum PO₄³⁻, and result in excess PO₄³⁻ being lost in urine. In contrast, other studies [40,47,48] reported no significant effect of prepartum DCAD on serum PO₄³⁻. The different results may be attributable to the different lactation stage of test cows and differences in the degree of acidosis induced.

The liver as a main organ in ruminant metabolism is sensitive to nutritional modifications. Serum ALT, AST, and GGT are frequently used as markers of liver damage resulting from metabolic disease or stressors [49,50]. Activities of ALT, AST, and GGT in serum are increased when liver is damaged, resulting in liberation of these cellular enzymes into the serum. In our trial, ALT, AST, and GGT activities increased linearly with decreasing DCAD, suggesting that the cows incurred some degree of liver damage. Renal function indices such as serum urea and creatinine are used to evaluate the functional integrity of the kidney, with elevated values being an indication of defective functional state [51]. Linearily increased serum urea and the tendency to linearly increase creatinine concentrations observed in this study also may imply some degree of renal toxicity imposed by negative DCAD. However, increasing ruminal infusion of ammonium ions and decreased DMI may have resulted in excess ammonia and more urea formation, which confounds the interpretation.

**CONCLUSION**

Negative DCAD caused by acute changes in the amount of NH₄Cl infused into the rumen had negative influence on performance of lactating dairy cows. In particular, a DCAD of −51 mEq/kg DM as induced by infusion of 300 g/d of NH₄Cl caused severe metabolic acidosis, decreased DMI, and decreased milk yield. The most negative DCAD also caused some indications of liver and kidney damage. Infusion of NH₄Cl did not affect concentration of milk true protein and did not seem to cause ammonia toxicity under the conditions of the present study. While a small amount of NH₄Cl that does not create a negative DCAD can be tolerated, short-term ingestion of amounts that create a negative DCAD could be detrimental in lactating dairy cows. As a result, we recommended that daily NH₄Cl intake by lactating dairy cows should not exceed 300 g, and a more appropriate daily intake may be 150 g.

**CONFLICT OF INTEREST**

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript. Liu S is an employee of China Feed Industry Association, and Zhang K is an employee of Beijing Sino Farm.
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