Journal of Veterinary Internal Medicine ACVIM

American College of
Veterinary Internal Medicine

STANDARD ARTICLE

Plasma citrulline, arginine, nitric oxide, and blood ammonia levels in neonatal calves with acute diarrhea

Mehmet Gultekin[1] | Huseyin Voyvoda[1] | Kerem Ural[1] | Hasan Erdogan[1] | Canberk Balikci[1] | Gamze Gultekin[2]

[1]Department of Internal Medicine, Faculty of Veterinary Medicine, University of Adnan Menderes, Aydin, Turkey

[2]Department of Biochemistry, Faculty of Veterinary Medicine, University of Adnan Menderes, Aydin, Turkey

Correspondence
Mehmet Gultekin, Department of Internal Medicine, Faculty of Veterinary Medicine, University of Adnan Menderes, Isikli 09017, Aydin, Turkey.
Email: mgultekin@adu.edu.tr

Background: Plasma citrulline (CIT) concentration is considered to be a reliable marker of functional enterocyte mass, primarily in humans. However, information about CIT levels along with related metabolites, arginine (ARG), nitric oxide (NO), and ammonia in neonatal calves are lacking.

Objectives: To compare plasma CIT, ARG, NO, and whole blood ammonia concentrations in neonatal calves with acute diarrhea with those in healthy calves and to assess their possible relationships with diarrhea-related criteria.

Animals: Seventy neonatal calves (60 with acute diarrhea and 10 healthy).

Methods: Observational case-control study. Diarrheic calves were classified into subgroups on the basis of etiology, severity of diarrhea, degree of dehydration, and systemic inflammatory response syndrome (SIRS) status. Plasma CIT and ARG concentrations were measured by liquid chromatography/tandem mass spectrometry.

Results: Plasma CIT (median [range]: 67.5 [61.9-75.4] vs 30.1 [15.0-56.1] μmol/L) and ARG (170.7 [148.5-219.5] vs 106.1 [54.4-190.7] μmol/L) were lower and plasma NO (4.42 [3.29-5.58] vs 6.78 [5.29-8.92] μM) and blood ammonia concentrations (28.7 [26.1-36.9] vs 59.8 [34.6-99.5] μmol/L) were higher in the neonatal calves with diarrhea ($P < .001$). Plasma CIT ($\beta = -0.29$, $P = .02$), ARG ($\beta = -0.33$, $P = .01$), NO ($\beta = 0.55$, $P < .001$), and blood ammonia ($\beta = 0.63$, $P < .001$) were affected by SIRS status. Except for ammonia (0.52), the effects sizes for severity of diarrhea and degree of dehydration were small ($\eta p2 \leq 0.45$) for CIT, ARG, and NO.

Conclusions and Clinical Importance: The changes in these variables might have diagnostic, prognostic, and therapeutic value in diarrheic neonatal calves.

KEYWORDS
amino acids, dehydration, etiology, infectious diseases, systemic inflammatory response, urea cycle

1 | INTRODUCTION

Neonatal calf diarrhea (NCD) is 1 of the most common diseases with serious financial and animal welfare implications in dairy and beef herds worldwide.[1] Apart from calf death, economic losses arise from the cost of treatment as well as impaired calf growth and future performance.[2,3] NCD can be caused by infectious and noninfectious causes. Enterotoxigenic *Escherichia coli* (ETEC) F5, rota- and coronavirus, and *Cryptosporidium parvum* (*C. parvum*) are the 4 most important enteropathogens associated with NCD. In field conditions, the majority of diarrheic calves are concurrently infected with >1 pathogen. These enteropathogens primarily induce damage to the intestinal mucosa, involving villous atrophy and enterocyte mass reduction. The clinical consequences of inflammation and loss of mucosal integrity

Abbreviations: ANOVA, analysis of variance; ARG, arginine; CIT, citrulline; ETEC, enterotoxigenic *Escherichia coli*; IgG, immunoglobulin G; LC/MS/MS, liquid chromatography/tandem mass spectrometry; NCD, neonatal calf diarrhea; NO, nitric oxide; SIRS, systemic inflammatory response syndrome.

primarily in the small bowel include diarrhea, dehydration, malnutrition, potentially systemic inflammatory response syndrome (SIRS) or sepsis, and even death.[1,4,5]

Citrulline (CIT), a nonprotein amino acid, is involved in arginine (ARG) and nitric oxide (NO) metabolism and the urea cycle.[6,7] Synthesized from glutamine primarily in the small intestine, CIT is released from enterocytes into blood.[8,9] For this reason, since the early 2000s, plasma CIT concentration has been used as a biomarker for enterocyte mass and absorptive function in various intestinal diseases[10–15] and sepsis[16,17] in humans, independent of nutritional and inflammatory status.[18] Therefore, it could be useful to predict the health and function of the intestine in calves. NO is known to have various physiological functions, including neurotransmission, immune defense, and organ perfusion and play a key role in various pathological processes. ARG is the only substrate for synthesis of NO.[19] Approximately 80% of CIT is converted to ARG in the kidneys and plays an important role in providing an ARG/NO balance through different mechanisms.[7,16] Ammonia is a toxic compound and excessive blood ammonia concentration might result in respiratory alkalosis, cerebral edema, seizures, coma, and death.[20,21] CIT and ARG play a key role in detoxifying ammonia in the urea cycle.[21] Deficiencies of CIT and ARG might underlie the negative consequences of inflammatory conditions, such as sepsis and endotoxemia.[13,16,17]

In veterinary medicine, plasma CIT concentration has only been investigated to demonstrate acute small bowel injury and measure its prognostic significance in dogs with parvoviral enteritis.[22] Therefore, this study aimed to compare plasma CIT, ARG, and NO and whole blood ammonia concentrations in neonatal calves with acute diarrhea to those in healthy calves and to evaluate their possible associations with diarrhea-related criteria. We hypothesized that neonatal calves with acute diarrhea would have reduced plasma CIT and ARG concentrations and increased plasma NO and blood ammonia, resulting from intestinal damage.

2 | MATERIAL AND METHODS

2.1 | Study design, animals

This observational case-control study was conducted based on approval of the Aydin Adnan Menderes University Animal Experiments Local Ethics Committee (number: 64583101/121, October 27, 2015). Informed written consent was obtained from all owners before enrollment. A total of 70 neonatal Holstein calves (<28 days old) of both genders, including 10 healthy and 60 with acute diarrhea, were used in this study. Sixty calves with acute diarrhea were classified into subgroups as follows: infectious and noninfectious based on etiology; mild, moderate, and severe diarrhea according to fecal consistency scores,[23] mild, moderate, and severe dehydration based on dehydration evaluation criteria[24,25]; SIRS positive and negative based on SIRS criteria.[26,27]

2.1.1 | Healthy calves

Healthy calves were housed at Aydin Adnan Menderes University Faculty of Veterinary Livestock Unit in individual stalls where disinfection was provided by appropriate methods. They received colostrum in 10% of the body weight on each day of the first 2 days of life (first intake within 2 hours after birth). Colostrum immunoglobulin G (IgG) levels were measured by Brix refractometer to assess colostrum quality. On the second day after birth, serum IgG and total protein levels were measured with Brix refractometer to determine the immunity status of the calves. Calves with a serum total protein cut-point of ≤55 g/L were not enrolled in the study. The calves were fed 2 times a day with individual milk bottles. Water was provided *ad libitum*. Access to good quality calf starter and forage was provided from the third week. During the study, no vaccination or medications were applied to the calves. Physical examinations of each calf were performed daily. Hematological, biochemical, and blood gas analyses as well as fecal examination were performed at 2, 7, 14, 21, and 28 days of age. One calf that did not meet the health criteria was excluded from study and a new neonatal calf was enrolled to the study and followed until its 28th day of life.

2.1.2 | Calves with acute diarrhea

Sixty diarrheic calves within the same age range enrolled in this study were obtained from the faculty farm affiliated to the university, the clinic for internal medicine and the 14 different small-to-medium sized enterprises in a total of 20 km² around the faculty between April 2016 and October 2017. The diarrheic calves had not previously received any treatment. Acute diarrhea was assessed by evaluating: (1) fecal consistency, content and color; (2) defecation frequency (>4 times per day); and (3) intensity (abundant aqueous, mucous, bloody) and duration of diarrhea.[28] Calves with congenital malformations or surgical problems were excluded from the study.

2.2 | Etiological evaluation

Stool specimens underwent etiologic evaluation against 5 important enteropathogens in NCD—*C. parvum*, rotavirus, coronavirus, ETEC F5, and *Giardia duodenalis*—by immunochromatography with a rapid commercial test kit (Bovid-5 Ag, Bionote, Korea). The diarrheic calves with 1 or more enteropathogens were assigned to the infectious diarrhea group. Calves with negative results to infectious agents according to laboratory examination findings were enrolled to the noninfectious diarrhea group.

2.3 | Clinical examination

All physical examinations were conducted in accordance with a standardized protocol[24,25] by the same person who was blinded to all laboratory values. Each examination determined rectal temperature, sucking reflex, fecal consistency, capillary refill time, and heart and respiratory rates. Severity of diarrhea was graded according to fecal consistency. Fecal scores (FS) were assigned on a 4 point scale; 0 = normal (retains form/does not flow across a surface), 1 = mild diarrhea (flows slowly across a surface), 2 = moderate diarrhea (fairly watery/flows easily across a surface leaving adherent material), and 3 = severe diarrhea (very watery/leaves no residue when flowing across a surface).[23] Grading of dehydration in diarrheic calves was determined by clinical findings, such as skin tent, eyeball position, mucous membrane dryness,

and mentation, and then these calves were subclassified as mild (6-8%), moderate (8-10%), and severely (10-12%) dehydrated.[3,24,28]

2.4 | Sample collection and analysis

Blood samples were taken at 2, 7, 14, 21, and 28 days of age from the healthy calves to obtain a homogeneous distribution during the neonatal period and were taken only once in the calves with acute diarrhea. Healthy control calves were routinely fasted for at least 8 hours before blood collection. Blood samples were collected aseptically by jugular venipuncture (BD Vacutainer Blood Collection Tube, 5 mL, with K_2EDTA or lithium heparin and without anticoagulant, Becton, Dickinson and Company, Franklin Lakes, New Jersey) for hematologic and biochemical evaluation. At the same time, jugular blood samples were taken anaerobically into lithium-heparinized 2 mL- syringe (BD Vacutainer, Eclips Arterial Blood Syringe) for blood gas analysis. Blood samples collected into lithium heparin-containing tubes and plain tubes were centrifuged at 1000g for 10 minutes for separation of plasma or serum. Complete blood cell count, blood gases, and ammonia assays were performed within 2 hours of sampling. Serum or plasma samples were evaluated visually for hemolysis and stored at −20°C until analysis for other biochemical assays. Stool specimens were collected in sterile stool storage containers in sufficient quantities.

Complete blood cell count was conducted on an automated blood cell counter (Abacus Junior Vet 5, Diatron, Hungary). Blood gas and electrolyte analyses were performed at 37°C with an automated blood gas analyzer (Irma Trupoint, Lifehealth LLC, Littleton, Colorado). All blood gas analyses were corrected for the calf's rectal temperature. Selected serum biochemical tests (aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), urea, creatinine, total protein, and albumin) were performed with an autoanalyzer (Chemray 120 Automated Analyzer, Rayto, China) using commercial test kits (Archem Diagnostics, Turkey). Quantitative analyses of plasma CIT and ARG concentrations were performed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) providing high sensitivity and specificity.[29] Commercial test kits for amino acid analyses (Amino acids, ImmuChrom GmbH, Germany) were used in this regard in accordance with the method of LC/MS/MS (QTRAP 4500, AB Sciex, Framingham, Massachusetts). The method was validated by determining the linearity, and interassay and intraassay precision and reproducibility. The intraassay and interassay coefficient of variation for amino acid concentrations were under 7 and 10%, respectively. Plasma NO concentration was determined by Griess reagent with a commercial test kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemicals, Ann Arbor, Michigan). Whole blood ammonia concentrations were measured with a point-of-care blood ammonia analyzer (Pocketchem BA, Arkray, Japan), which has been used in human and veterinary medicine.[30] The blood ammonia analyzer uses a micro diffusion method based on a colorimetric assay and 20 μL of K_2EDTA whole blood sample.

Systemic inflammatory response syndrome positivity was based on the presence of 2 or more of the following criteria[26,27]: (1) leukopenia or leukocytosis (reference interval, 5-12 × 10^9/L), (2) hyperthermia or hypothermia (reference interval, 38.5-39.5°C), (3) tachycardia (>120 beats/min), and (4) tachypnea (>36 breaths/min).

2.5 | Statistical analysis

Statistical software packages (SPSS 22.0 [SPSS Inc., Chicago, Chicago, Illinois] and GraphPad Prism [GraphPad Software Inc., La Jolla, California]) were used to perform the analyses. The respective distributions of all numerical data within each study group were checked for normality by the Kolmogorov-Smirnov or Shapiro-Wilk tests. Nonnormally distributed variables were log transformed for analysis or analyzed by nonparametric methods. The measurements were analyzed in total and separately by group with the groups formed by diarrhea etiology, severity of diarrhea, degree of dehydration, and SIRS status. Diarrheic subgroups classified by the above mentioned criteria compared with age-matched healthy controls. For normally distributed data with or without transformation, Student's t test was used for comparisons between 2 groups (healthy controls vs all diarrheic calves), and 1-way ANOVA followed by Tukey's test was performed for comparisons among 3 or more groups (healthy controls and diarrheic subgroups). For data not normally distributed even after transformation, comparisons among groups were made by nonparametric tests (2 groups: Mann-Whitney U-test; 3 or more groups: Kruskal-Wallis test). Friedman's test was used to determine for changes in plasma CIT, ARG, NO, and blood ammonia concentrations over time in the healthy control group. Regression analysis was performed to assess the associations among plasma CIT, ARG, and NO, and blood ammonia concentrations and potential explanatory variables, namely age of calf (day), the presence of SIRS (no = 0; yes = 1) and etiology of diarrhea (noninfectious = 0; infectious = 1) in the diarrheic calves. Initially, simple linear regression was performed. A multiple linear regression by backward method was then constructed, which initially included any variables identified as P value <.2 on univariable analysis. One-way analysis of covariance (ANCOVA) was used to examine the influences of severity of diarrhea (mild = 0, moderate = 1, and severe = 2) and degree of dehydration (mild = 0, moderate = 1, and severe = 2) on plasma CIT and its related metabolites, after effect of the covariate, calf age, was controlled. Effect sizes were interpreted by Cohen's guidelines for the partial eta-squared (ηp2) values.[31] Data were expressed as mean (deviation) or median (ranges) based on the results of the normality test. All analyses were considered statistically significant at $P < .05$.

3 | RESULTS

Based on clinical and laboratory findings, 83 and 17% of diarrhea in neonatal calves were infectious (n = 50) and noninfectious (n = 10) in etiology, respectively. According to the degree of dehydration, 60 neonatal calves with acute diarrhea were evaluated in 3 subgroups: mild (n = 15), moderate (n = 25), and severe (n = 20). Based on fecal consistency, the diarrhea was mild (FS 1) in 16 calves, moderate (FS 2) in 20, and severe (FS 3) in 24. According to SIRS criteria, they were assessed in 2 groups: SIRS positive (n = 27) and SIRS negative (n = 33). Of those with acute infectious diarrhea, 10 neonatal calves had mild dehydration; 20, moderate dehydration; and 20, severe dehydration. Furthermore, 27 of these calves were SIRS positive and 23 were SIRS negative. In calves with noninfectious diarrhea, only mild (n = 5) to

TABLE 1 The cause of infectious diarrhea in 50 neonatal calves and the number of calves affected by each agent or combinations of agents

Enteropathogen(s)	Number	%
Cryptosporidium parvum	21	42
C. parvum + rotavirus	11	22
Escherichia coli	5	10
C. parvum + rotavirus + coronavirus	3	6
C. parvum + coronavirus	2	4
C. parvum + *Giardia duodenalis*	2	4
Rotavirus + coronavirus	2	4
Coronavirus	1	2
G. duodenalis	1	2
Rotavirus	1	2
C. parvum + rotavirus + *G. duodenalis*	1	2

moderate (n = 5) dehydration was observed. None of the calves with noninfectious diarrhea were SIRS positive.

The cause of infectious diarrhea and the number of calves affected by each agent or combinations of agents are shown in Table 1. In this study, mono-infection with *C. parvum* (42%) and coinfection with *C. parvum* + rotavirus (22%) were identified as the most common agents in diarrheic calves (Table 1).

Feces were usually characterized as brown to light green or yellow with or without blood and mucus, with a consistency varying from semisolid to very watery and a strong, unpleasant odor. Clinical signs in diarrheic calves ranged from normal to marked changes in posture and behavior, from strong to absent suckling reflex, and from slight to severe delay in skin tenting. The rectal temperature, heart rate, and respiratory rate in diarrheic calves varied from 33.5 to 40.7°C, 60-185 beats/min, and 15-80 breaths/min as minimum and maximum values, respectively, and did not differ from the healthy control calves (Table 2).

Further demographic information and the statistical evaluation of laboratory findings in control and diarrheic calves are summarized in Table 2. Overall, calves with acute diarrhea had higher WBC, K, AST, creatinine and urea, NO and blood ammonia, and lower pH, HCO_3, BE, total protein, CIT, and ARG values than healthy control calves (Table 2), and the differences in plasma CIT and its related metabolites remained important after adjusting for age. There was a significant effect of time on plasma CIT (P = .03), ARG (P < .001), NO (P = .01), and blood ammonia concentrations (P = .008) in the healthy control group during the neonatal period (Supporting information Figure S1).

Kruskal-Wallis test showed important differences in plasma CIT, ARG, NO, and blood ammonia concentrations among the groups (healthy control calves and the diarrheic subgroup calves formed by diarrhea etiology, severity of diarrhea, dehydration grade, and SIRS status) (P < .001 for all variables and criteria). Plasma CIT concentrations were significantly lower in the infectious diarrhea (Figure 1A), moderate (FS 2) and severe (F 3) diarrhea (Figure 2A), severe dehydration (Figure 3A), and SIRS positive and negative subgroups (Figure 4A) than in the healthy control group. Plasma ARG concentrations were

TABLE 2 Demographic parameters, and selected clinical, hematological, and biochemical findings in healthy controls and calves with diarrhea

Parameters	Healthy control (n = 10; 50 measurements) median (range) or mean (SD)	All diarrheic calves (n = 60) median (range) or mean (SD)	P value
Age (day)	14 (7-21)	9 (7-15)	.18
Gender (female/male)	(5/5)	(28/32)	.97
T (°C)	39.0 (38.7-39.2)	38.7 (37.7-39.2)	.07
P (beats/min)	132 (112-145)	129 (110-150)	.82
R (breaths/min)	54.9 (11.3)	50.8 (15.8)	.16
WBC (×10^9/L)	6.3 (5.3-7.1)	9.3 (6.5-14.5)	<.001
HCT (%)	22.0 (19.1-26.2)	24.4 (21.0-29.0)	.06
pH (−log H$^+$)	7.34 (7.32-7.36)	7.25 (7.00-7.34)	<.001
HCO_3 (mmol/L)	30.9 (29.1-32.5)	20.6 (10.7-33.1)	.001
BE (mmol/L)	5.6 (3.9-6.5)	−6.5 (−17.1-+5.0)	<.001
Sodium (mmol/L)	132.1 (4.0)	132.2 (9.0)	.75
Potassium (mmol/L)	4.71 (4.46-5.10)	4.89 (4.58-5.84)	.01
Total Protein (mg/dL)	5.99 (5.65-6.94)	5.63 (4.70-6.42)	.003
Albumin (mg/dL)	3.39 (3.16-3.51)	3.17 (2.75-3.87)	.11
AST (U/L)	40.5 (35.2-47.1)	66.0 (46.7-92.7)	<.001
Creatinine (mg/dL)	0.70 (0.63-0.87)	1.10 (0.70-2.10)	<.001
Urea (mg/dL)	18.5 (14.6-23.3)	40.9 (30.1-84.5)	<.001
CIT (µmol/L)	67.5 (61.9-75.4)	30.1 (15.0-56.1)	<.001
ARG (µmol/L)	170.7 (148.5-219.5)	106.1 (54.4-190.7)	<.001
NO (µM)	4.42 (3.29-5.58)	6.78 (5.29-8.92)	<.001
Ammonia (µmol/L)	28.7 (26.1-36.9)	59.8 (34.6-99.5)	<.001

Abbreviations: ARG, arginine; BE, base excess; CIT, citrulline; HCT, hematocrit; NO, nitric oxide; P, heart rate; R, respiratory rate; T, temperature; WBC, white blood cell.
Median and range are listed for parameters that were not normally distributed and mean and SD are listed for parameters that were normally distributed for both groups. P values obtained from Mann-Whitney U-test and t test according to distribution of data for each parameter.
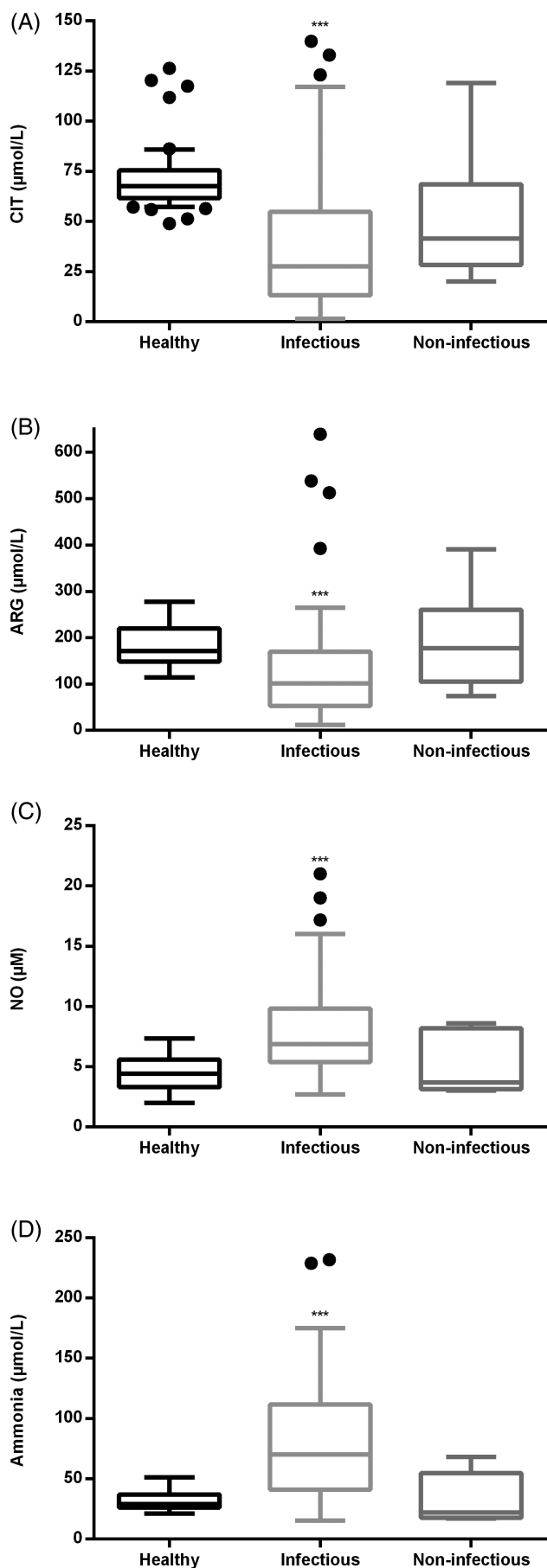
significantly lower in the infectious diarrhea (Figure 1B), in FS 3 (Figure 2B), severe dehydration (Figure 3B), and SIRS-positive subgroups (Figure 4B) than in the healthy control group. In addition, plasma ARG concentrations were significantly lower in the severe dehydrated subgroup than in the mild dehydration subgroup (P = .03) and in the SIRS-positive subgroup than in the SIRS-negative subgroup (P < .001).
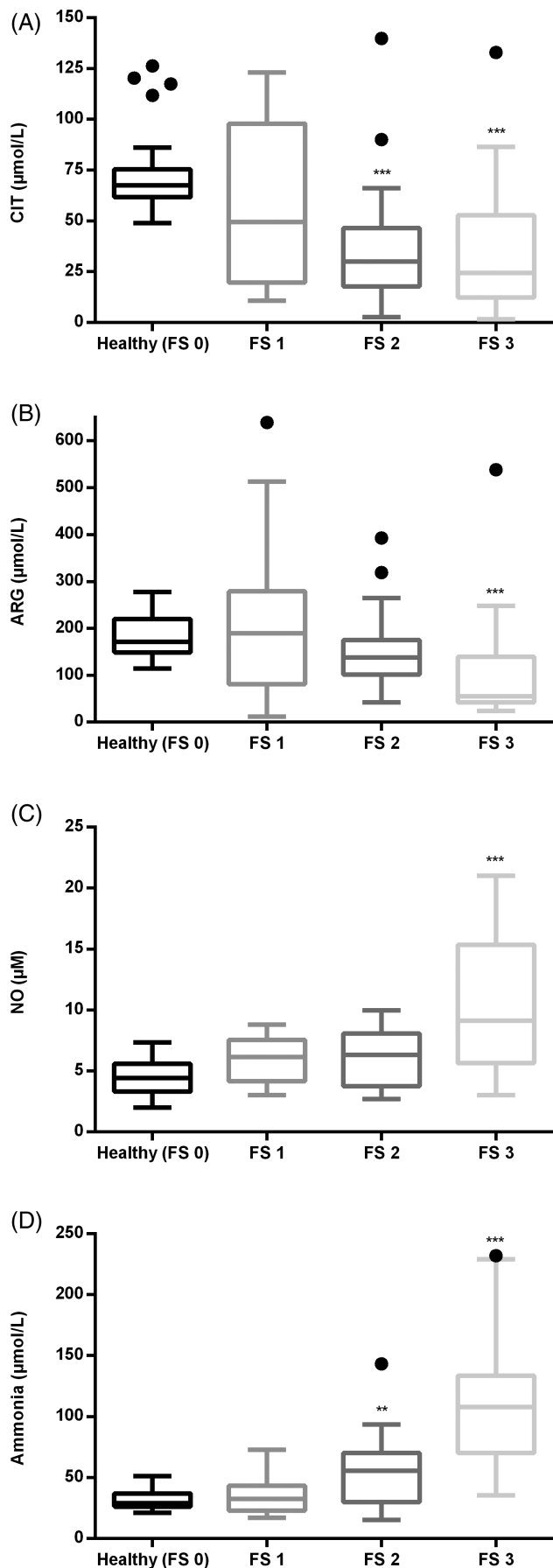
Plasma NO and blood ammonia concentrations were significantly higher in the infectious diarrhea subgroups than in the control group (Figure 1C,D). Plasma NO concentration was higher in severe diarrhea (FS 3) subgroup (Figure 2C) whereas blood ammonia concentration was higher in both moderate (P < .01) and severe diarrhea (P < .001) subgroups (Figure 2D). Both variables were also significantly higher in the severe dehydrated diarrheic subgroup than in the healthy control group (Figure 3C,D), as well as the mild (P < .001) and moderate dehydrated diarrheic subgroups (P < .001). Lastly, plasma NO and blood ammonia concentrations in the SIRS-positive subgroup differed significantly from those in the healthy control group (Figure 4C,D) and SIRS-negative subgroup (P = .001).

The relationships between plasma CIT and its related variables and independent variables (age, diarrhea etiology, SIRS status) by linear regression in diarrheic calves are presented in Table 3. There was no significant effect of age on plasma log-transformed CIT, ARG, and NO as well as blood ammonia concentrations. By contrast, the presence of SIRS was significantly associated with decreased CIT and ARG and with increased NO and ammonia concentrations. In this context, the diarrheic calves with SIRS have plasma log-CIT and ARG concentrations that would be average of 0.58 and 0.55 μmol/L lower than SIRS-negative diarrheic calves, respectively. The slope coefficients for SIRS status were 0.56 and 0.75 so for diarrheic SIRS-positive calves, the predicted plasma log-NO and blood ammonia would be 0.56 and 0.75 units higher than for diarrheic SIRS-negative calves, respectively. Furthermore, diarrhea etiology was found to be positively associated with plasma log-transformed NO and blood ammonia (Table 3).

An ANCOVA revealed no effect of severity of diarrhea (P = .29), degree of dehydration (P = .40) or the covariate calf age (P = .77 for severity of diarrhea, and P = .50 for degree of dehydration) on plasma CIT level. The interactions between severity of diarrhea and age (P = .50) and between degree of dehydration and age (P = .67) were also not significant.

An ANCOVA revealed no effect of the covariate calf age on plasma CIT (P = .77), ARG (P = .14), NO (P = .30), and blood ammonia (P = .09). The interactions between severity of diarrhea and age and between degree of dehydration and age for CIT and its related metabolites were also not significant. However, there were significant effects of severity of diarrhea on plasma CIT (P = .04), ARG (P = .04), NO (P < .001), and

FIGURE 1 Box plot schematic representation of plasma CIT (A), ARG (B), NO (C), and whole blood ammonia (D) concentrations in calves with infectious diarrhea, noninfectious diarrhea, and healthy controls. The line is drawn across the box at the median. The lower line of the box is at the first quartile (Q1) and the upper line is at the third quartile (Q3). The whiskers show 10 and 90% slices. Points represent results outside the slice. Asterisk signs refer to significance in comparison with healthy controls. ***P < .001

blood ammonia ($P < .001$) concentrations after controlling for the effect of calf age. Severity of diarrhea negatively influenced plasma CIT and ARG concentrations whilst it was associated with an increase in NO and ammonia concentrations. Planned contrasts revealed that calves with severe diarrhea had significantly lower plasma CIT and ARG level compared to that in mild diarrheic subgroup ($P = .04$, $P = .03$) and the moderate diarrheic subgroup ($P = .04$, $P = .05$) whereas plasma NO and blood ammonia levels in the severe diarrheic subgroup were significantly higher than the mild diarrheic subgroup ($P = .001$, $P < .001$) and moderate diarrheic subgroup ($P < .001$ for both variables), respectively. The $\eta p2$ values indicated for severity of diarrhea the effect sizes were small in CIT (0.10), ARG (0.12), NO (0.28), and ammonia (0.45).

The effects of degree of dehydration on the plasma concentration of CIT, ARG, NO, and blood ammonia followed a similar pattern to that of severity of diarrhea. There were significant effects of degree of dehydration on plasma CIT ($P = .04$), ARG ($P = .03$), NO ($P < .001$), and blood ammonia ($P < .001$) concentrations after controlling for the effect of calf age. The effect sizes were small in CIT ($\eta p2 = 0.11$), ARG ($\eta p2 = 0.13$), and NO ($\eta p2 = 0.25$), and moderate in ammonia ($\eta p2 = 0.52$). Planned contrasts revealed that calves with severe dehydration had significantly lower plasma CIT and ARG levels compared to those in mild dehydrated subgroup ($P = .02$, $P = .01$) and the moderate dehydrated subgroup ($P = 0.04$) whereas plasma NO and blood ammonia levels in the severe diarrheic subgroup were significantly higher than those of the mild diarrheic subgroup ($P = .01$, $P < .001$) and moderate diarrheic subgroup ($P < .001$ for both variables), respectively.

4 | DISCUSSION

This study described plasma CIT, ARG, NO, and whole blood ammonia concentrations in diarrheic neonatal calves along with healthy controls. The results indicated that neonatal calves with acute diarrhea have lower plasma CIT and ARG and higher plasma NO and blood ammonia concentrations than healthy calves, and that SIRS status, severity of diarrhea, and degree of dehydration significantly affect levels of plasma CIT and its related metabolites.

The etiology of NCD is complex and includes a wide range of factors related to the animal, conditions of the environment and husbandry, and various infectious agents. The majority of NCD cases are caused by rotavirus, coronavirus, *C. parvum* or ETEC F5.[1-4] In field conditions, combinations of these agents frequently occur,[2,4] and the consequences of mixed infections often appear to be more severe

FIGURE 2 Box plot schematic representation of plasma CIT (A), ARG (B), NO (C), and whole blood ammonia (D) concentrations according to severity of diarrhea based on fecal score (FS) in neonatal calves. The line is drawn across the box at the median. The lower line of the box is at the first quartile (Q1) and the upper line is at the third quartile (Q3). The whiskers show 10% and 90% slices. Points represent results outside the slice. Asterisk signs refer to significance in comparison with healthy controls. **$P < .01$, ***$P < .001$; FS 0 = normal, FS 1 = mild diarrhea, FS 2 = moderate diarrhea, and FS 3 = severe diarrhea
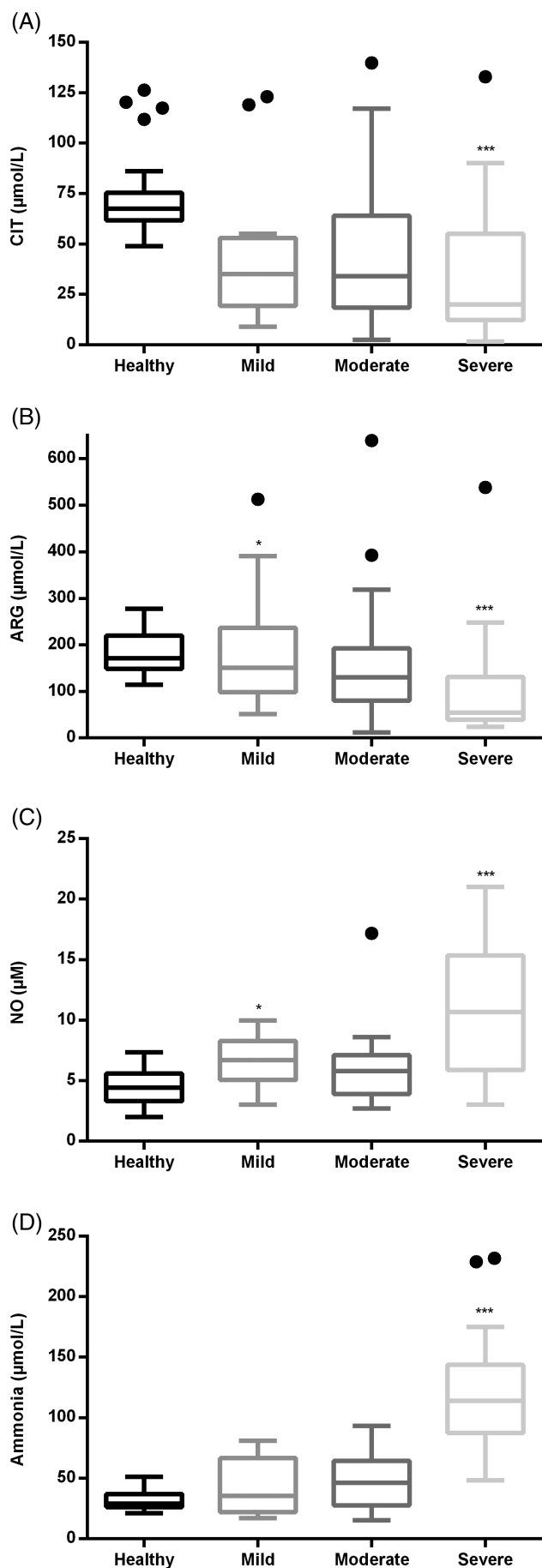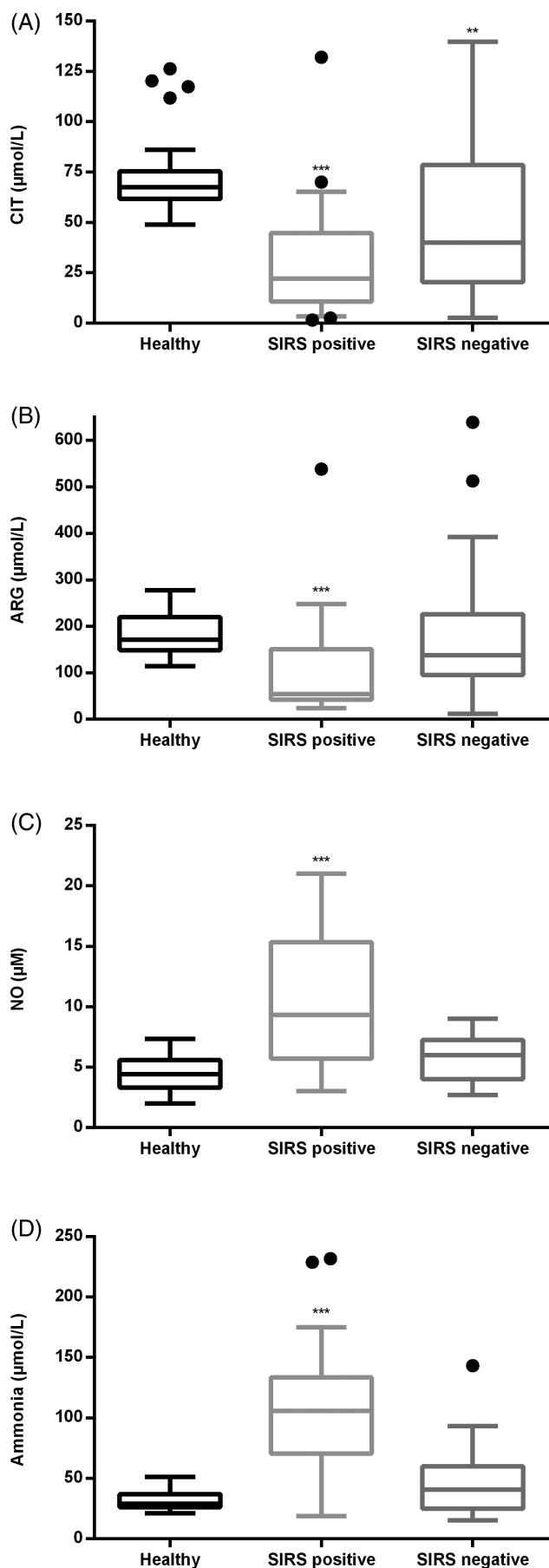
compared to single-agent infections.[5] Similar to other countries, an important proportion of NCD cases in Turkey are caused by *C. parvum* and rotavirus, alone, together, or in combination with other agents. In the present study, 21 (41%) neonatal calves with infectious diarrhea had a single infection of *C. parvum*, and 11 (22%) had concurrent infection of *C. parvum* with rotavirus (Table 1). In concurrently infected calves, clinical signs related to diarrhea were more severe. Rotavirus, coronavirus, and *C. parvum* primarily cause damage to the intestinal mucosa, involving villous atrophy and enterocyte mass reduction. Furthermore, heat-stable toxin production induced by ETEC leads to the upregulation of chloride secretion into the gut.[1] The clinical consequences of inflammation and loss of mucosal integrity in the bowel of the neonatal calf include maldigestion/malabsorption, secretory diarrhea, dehydration, metabolic acidosis, electrolyte imbalance, malnutrition, potentially SIRS or sepsis, and even death.[1–4] In the present study, clinical signs and hematobiochemical results related to dehydration and acid-base imbalance of the diarrheic calves (Table 2) were in agreement with those of other studies[2,5,23–25,27] and are not discussed further.

The tissues of the gastrointestinal tract play an important role in the metabolism of proteins and amino acids in growing animals.[32,33] In contrast to humans, only a few reports describe normal concentrations of CIT, ARG, NO in plasma and blood ammonia, and their role in pathological conditions of calves.[34] Previous studies have reported a range of 190 ± 70-1970 ± 221 μmol/L plasma CIT levels and 60 ± 20-5140 ± 3140 μmol/L plasma ARG levels in healthy calves,[35,36] which differ from our findings of healthy calves (Table 2). Median (range) plasma NO concentrations measured in healthy control calves (Table 2) were higher than 2.50 ± 0.30 μmol/L but lower than the nitrate + nitrite concentrations reflecting NO production of 22.20 ± 4.90 μmol/L reported previously by other studies.[37,38] The reason(s) for these differences are not evident. In contrast to pigs, no previous studies in neonatal calves have reported lower postnatal concentrations of plasma CIT and ARG nor rates of intestinal CIT and ARG synthesis from glutamine.[32,33] Therefore, these factors influencing plasma CIT and ARG levels can be excluded. Another reason for this discordance could be primarily related to different measurement methods. Differences in sample handling and storage procedures might account for differences between NO levels of healthy calves in this study and those reported in the literature.[39] The blood ammonia concentration with a median of 28.7 μmol/L in healthy control calves (Table 2) was in agreement with the values <90 μmol/L reported previously by other investigators.[40]

Veterinary assessment of calves with diarrhea is generally based on clinical examination and routine laboratory variables alone; however,

FIGURE 3 Box plot schematic representation of plasma CIT (A), ARG (B), NO (C), and whole blood ammonia (D) concentrations in diarrheic neonatal calves with mild, moderate, and severe dehydration and healthy controls. The line is drawn across the box at the median. The lower line of the box is at the first quartile (Q1) and the upper line is at the third quartile (Q3). The whiskers show 10% and 90% slices. Points represent results outside the slice. Asterisk signs refer to significance in comparison with healthy controls.
*P < .05, ***P < .001

994 | Journal of Veterinary Internal Medicine ACVIM GULTEKIN ET AL.

Open Access
American College of
Veterinary Internal Medicine

evaluating small bowel function and integrity could provide a more detailed approach to prognostic and therapeutic decisions. Unlike other organs, where easy and reliable laboratory markers of damage or failure are available, no such tests are currently on hand for the bowel. Therefore, daily clinical practice requires a reliable and fast method for the evaluation of intestinal damages in diarrheic calves, regardless of their etiology.

To date, no studies have evaluated plasma CIT in neonatal calves with acute diarrhea, and only 1 study on ARG levels demonstrated lower lysine + ARG concentrations.[41] Therefore, the plasma CIT concentration observed in the present study is not directly comparable. However, our plasma CIT result is in agreement with the findings of several previous reports in humans with enteropathies[10–12,15] and sepsis,[13,16,17] as well as in dogs.[22,42] As previously reported in humans, decreased plasma CIT and ARG in diarrheic calves could be because of impaired intestinal production, different metabolic processes altered in inflammatory conditions, or a combination of both.[9,11,13,33,42] Infectious diarrhea in neonatal calves results in a low to strong inflammatory response.[1,4,43] Approximately 80% of CIT is converted to the amino acid, ARG, in the body to support ARG and NO levels.[6,8,9,44] Conversion of CIT to ARG and converting/recycling ARG to CIT and NO appear to be dependent on the severity of inflammation.[6,7,14] Therefore, the decreases in plasma CIT and ARG levels can especially be based on intestinal damage, dysfunction, or both mechanisms, leading to the impaired production of these amino acids. Although small bowel enteroscopy or histopathological examination was not performed in this study, certain enteropathogens, particularly *C. parvum*, rotavirus, and coronavirus are associated with mucosal injury in calves.[1,4] Considering all of these factors, low plasma CIT and ARG concentrations in neonatal calves with diarrhea (Table 2) could primarily be caused by decreased substrate (glutamine, glutamate) availability in the bowel and impaired gut function because of damage. Because infectious diarrhea might lead more intestinal damage or intestinal dysfunction, we expected plasma CIT levels to be lower in calves with infectious diarrhea than in calves with noninfectious diarrhea. However, differences in CIT and ARG levels between calves with infectious diarrhea and calves with noninfectious diarrhea did not reach statistical significance (27.6 [13.2-54.7] vs 41.5 [28.2-68.5] μmol/L; P = .49) and (101.6 [52.9-169.8] vs 177.5 [104.8-260.5] μmol/L; P = .49), respectively (Figure 1A,B). We cannot explain the exact reasons for these results. Perhaps this result stems from the low number of calves with noninfectious diarrhea enrolled in the study. Another factor affecting plasma CIT concentration might be renal impairment.[6] However, CIT is metabolized into ARG in the intestine instead of

FIGURE 4 Box plot schematic representation of plasma CIT (A), ARG (B), NO (C), and whole blood ammonia (D) concentrations in diarrheic neonatal calves with and without SIRS and healthy controls. The line is drawn across the box at the median. The lower line of the box is at the first quartile (Q1) and the upper line is at the third quartile (Q3). The whiskers show 10% and 90% slices. Points represent results outside the slice. Asterisk signs refer to significance in comparison with healthy controls. **P < .01, ***P < .001

TABLE 3 Results of multiple regression analysis for factors associated with the natural log of plasma CIT, ARG, NO, and blood ammonia (dependent variables) in diarrheic calves

	B	SE B	β	t	P value
Predicting: CIT (μmol/L)					
Constant	3.60	0.17		21.26	<.001
SIRS (no/yes)	−0.58	0.25	−0.28	−2.28	.02
Diarrhea etiology (noninfectious/infectious)	−0.24	0.37	−0.09	−0.64	.51
Age (day)	0.02	0.02	0.11	0.85	.39
Predicting: ARG (μmol/L)					
Constant	4.94	0.14		35.22	<.001
SIRS (no/yes)	−0.55	0.21	−0.33	−2.63	.01
Diarrhea etiology (noninfectious/infectious)	−0.23	0.30	−0.10	−0.76	.44
Age (day)	0.02	0.02	0.19	1.56	.12
Predicting: NO (μM)					
Constant	1.70	0.07		22.70	<.001
SIRS (no/yes)	0.56	0.11	0.55	4.98	<.001
Diarrhea etiology (noninfectious/infectious)	0.23	0.16	0.17	1.43	.16
Age (day)	−0.01	0.01	−0.15	−1.14	.26
Predicting: ammonia (μmol/L)					
Constant	3.89	0.17		22.69	<.001
SIRS (no/yes)	0.75	0.15	0.63	4.86	<.001
Diarrhea etiology (noninfectious/infectious)	0.48	0.20	0.25	2.32	.02
Age (day)	−0.01	0.01	−0.09	−0.72	.47

Abbreviations: ARG, arginine; NO, nitric oxide; SIRS, systemic inflammatory response syndrome.

the kidney in newborn calves, therefore renal failure might have only a very limited effect on plasma CIT concentration in our study.[33]

A significant negative relationship of plasma CIT and ARG with SIRS status (Table 3) and negative and small effects of severity of diarrhea and degree of dehydration on plasma CIT and ARG in neonatal calves with acute diarrhea observed here are in good agreement with the results of a meta-analysis in humans that detected a significant negative correlations (rho = −0.560) between CIT and intestinal disease severity in patients with enteropathies[15] and between CIT and ARG and severity of inflammation in patients with sepsis and trauma or viral disease.[45] Similar to the correlations of plasma CIT and ARG with severity of inflammatory in critically ill dogs,[42] significant relationships between plasma CIT and ARG and SIRS existence (Table 3; Figure 4A,B), and significant main effects of severity of diarrhea and degree of dehydration on these variables were found in the present study. These results could be explained by intestinal damage and increasing mucosal permeability with more severe inflammation in SIRS and diarrhea-induced dehydration by causative agents. Small bowel damage might be central in the development of SIRS and sepsis, thus low levels of CIT and ARG have been reported in adults and children with sepsis and other critical illness[13,19,45,46] as well as in dogs.[22,42] Because SIRS can affect gut function and integrity, alterations in glutamine metabolism might exist and lead to decreased CIT production. Significantly lower plasma CIT concentration in diarrheic calves with SIRS than that in diarrheic calves without SIRS might be in part a result of a defect in the metabolic conversion of glutamine to CIT and decreased uptake of glutamine by the enterocyte.[6] In addition, CIT production is severely reduced during SIRS and sepsis, resulting in diminished ARG de novo production and NO availability.[7,9]

For NO synthesis, inducible NO synthases (iNOS) known as inflammatory NOS, can be expressed by almost all cell types in most tissues.[47] After its induction by certain pro-inflammatory cytokines, lipopolysaccharide, or both, a large amount of NO is produced over a long period of time during the inflammatory process of infectious disease.[48–50] In this study, 50 of 60 diarrheic calves were evaluated as infectious. Similar to previous studies in humans[50] and calves[38] with acute diarrhea, higher plasma NO concentration in diarrheic calves (Table 2) can be related with an increased local NO production in the intestine[49] or by enteropathogens such as rotavirus, *Cryptosporidium* and *Giardia*.[50–52] Additionally, because diarrheic calves had decreased renal perfusion related to hypovolemia as estimated by serum creatinine, decreased urinary excretion of nitrate might have contributed to increased plasma NO concentration of the diarrheic calves in the present study (Table 2). In agreement with the results of previous studies in humans with acute infectious gastroenteritis,[53] plasma NO concentrations were significantly higher in calves with acute infectious diarrhea than in calves with noninfectious diarrhea and healthy calves (Figure 1C). The increases in plasma NO concentrations were greater in severe diarrhea (FS 3), severe dehydrated and SIRS positive subgroups, indicating also that severity of diarrhea, degree of dehydration, and SIRS status influence the blood levels of NO (Figures 2C and 4C). Besides intestinal damage because of enteropathogens, dehydration might play a role in the acute phase response, thus increasing the induction of iNOS.[47] Therefore, significantly higher plasma NO concentration might be possible in severely dehydrated diarrheic calves than those in mildly or moderately dehydrated calves, as well as healthy calves. In addition, increased plasma NO concentration is related to SIRS positivity. Increased nitrate levels in serum or plasma,

indicating NO formation, are well documented in patients with SIRS or sepsis, and this alteration contributes to intestinal damage and its consequences.[9] In the present study, significantly higher plasma NO concentration in diarrheic calves with SIRS can also be explained by a similar process (Figure 4C).

Ammonia is a toxic compound to many organ systems, especially the brain. Excessive blood ammonia concentration might result in respiratory alkalosis, cerebral edema, mental disorders, seizures, coma, and death. Increases in blood ammonia concentration are generally considered to only be a result of liver-related disorders, but other underlying causes should be assessed as well.[20] High blood ammonia levels can develop from several different mechanisms, including (1) hepatic insufficiency, (2) reduced activity of urea cycle enzymes, (3) metabolic disorders of organic acids, and (4) portosystemic shunt.[20] In the present study, blood ammonia concentration with a median of 59.8 μmol/L in diarrheic calves was significantly higher than that in healthy calves (Table 2). Although these median levels remained within the reference range (<90 μmol/L) reported for cattle,[40] median blood ammonia levels in the severe diarrhea (105.7 μmol/L), severe dehydration (113.9 μmol/L), and SIRS positive (105.7 μmol/L) subgroups were above the reference limits (Figures 2D, 3D, and 4D). This increase in these subgroups can be considered to be hyperammonemia and might be related to the loss of liver or kidney function because of circulatory failure in the result of diarrhea, as well as the decrease of plasma citrulline and arginine concentrations which decreases the efficiency of the urea cycle. In this content, hyperammonemia has also been reported in a human case of short bowel syndrome along with decreased concentrations of arginine and citrulline[54] and in systemic infections with bacteria or virus.[55] The moderate and positive relationship of blood ammonia with SIRS status (Table 3) and the effects of severity of diarrhea and degree of dehydration) might be based on the factors affecting urea cycle and ammonia metabolism.[20]

The results of this study are subject to some limitations. This study included a variety of mono or combined causative agents (Table 1) in neonatal calves with acute diarrhea of various degree and duration. First, because we did not perform an endoscopy, histopathological examination, or both modalities, it was not possible to determine the localization and extension of the inflammatory process in the intestine of diarrheic calves. Therefore, it is difficult to directly explain significant decreases in plasma CIT and ARG and increases in plasma NO and blood ammonia concentrations in neonatal calves. Second, measurements of plasma CIT, ARG, NO, and blood ammonia levels were only performed at 1 point in time. Serial measurements of these variables during the diarrhea course would provide additional important information. Third, the absence of all enteropathogens associated with NCD in calves with noninfectious diarrhea cannot be determined because only the antigens of the most common enteropathogens in feces were evaluated. Additionally, the duration of disease is a confounding factor which could cause difficulty in diagnosis of noninfectious diarrhea. Lastly, the sample size of this study was small, especially in neonatal calves with noninfectious diarrhea.

In conclusion, the present study reported that neonatal calves with acute diarrhea have significantly lower plasma concentrations of CIT and ARG, as well as higher plasma NO and blood ammonia levels, and these changes were associated with SIRS status, severity of diarrhea, and degree of dehydration. Plasma CIT concentrations could be a useful marker to evaluate the presence and degree of intestinal damage and gut barrier dysfunction in neonatal calves with acute diarrhea. However, further studies are needed to determine the importance of decreased plasma CIT and ARG levels and increased levels of plasma NO and blood ammonia with regard to diagnosis, prognosis, and therapy in NCD.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Animal Experiments Local Ethics Committee of Adnan Menderes University with the date October 27, 2015 and number 64583101/121.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Mehmet Gultekin https://orcid.org/0000-0002-5197-2403
Hasan Erdogan https://orcid.org/0000-0001-5141-5108

REFERENCES

1. Foster DM, Smith GW. Pathophysiology of diarrhea in calves. *Vet Clin North Am Food Anim Pract*. 2009;25(1):13-36.
2. Smith DR. Field disease diagnostic investigation of neonatal calf diarrhea. *Vet Clin North Am Food Anim Pract*. 2012;28:465-481.
3. Smith GW. Treatment of calf diarrhea: oral fluid therapy. *Vet Clin North Am Food Anim*. 2009;25:55-72.
4. Cho YI, Yoon KJ. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. *J Vet Sci*. 2014;15:1-17.
5. Trefz FM, Lorenz I, Lorch A, Constable PD. Clinical signs, profound acidemia, hypoglycemia, and hypernatremia are predictive of mortality in 1,400 critically ill neonatal calves with diarrhea. *PLoS One*. 2017;12 (8):1-27.
6. Curis E, Nicolis I, Moinard C, et al. Almost all about citrulline in mammals. *Amino Acids*. 2005;29:177-205.
7. Breuillard C, Cynober L, Moinard C. Citrulline and nitrogen homeostasis: an overview. *Amino Acids*. 2015;47:685-691.

8. Sultana H, Kitano A, Wadud S, Takahashi T, Morita T, Onodera R. Synthesis of citrulline from ornithine by the small intestinal mucosa of cattle. *Animal Sci J*. 2003;74:283-287.

9. de Betue CT, Deutz NE. Changes in arginine metabolism during sepsis and critical illness in children. *Nestle Nutr Inst Workshop Ser*. 2013;77:17-28.

10. Crenn P, Coudray-Lucas C, Thuillier F, et al. Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. *Gastroenterology*. 2000;119:1496-1505.

11. Crenn P, Vahedi K, Lavergne-Slove A, Cynober L, Matuchansky C, Messing B. Plasma citrulline: a marker of enterocyte mass in villous atrophy-associated small bowel disease. *Gastroenterology*. 2003;124:1210-1219.

12. Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. *Clin Nutr*. 2008;27:328-339.

13. Crenn P, Neveux N, Chevret S, et al. Plasma L-citrulline concentrations and its relationship with inflammation at the onset of septic shock: a pilot study. *J Crit Care*. 2014;29:315e1-315e6.

14. Curis E, Crenn P, Cynober L. Citrulline and the gut. *Curr Opin Clin Nutr Metab Care*. 2007;10:620-626.

15. Fragkos KC, Forbes A. Citrulline as a marker of intestinal function and absorption in clinical settings: a systematic review and meta-analysis. *United European Gastroenterol J*. 2018;6:181-191.

16. Kao CC, Bandi V, Guntupalli KK, Wu M, Castillo L, Jahoor F. Arginine, citrulline and nitric oxide metabolism in sepsis. *Clin Sci*. 2009;117:23-30.

17. Wijnands KA, Castermans TM, Hommen MP, Meesters DM, Poeze M. Arginine and citrulline and the immune response in sepsis. *Nutrients*. 2015;7:1426-1463.

18. Papadia C, Sherwood RA, Kalantzis C, et al. Plasma citrulline concentration: a reliable marker of small bowel absorptive capacity independent of intestinal inflammation. *Am J Gastroenterol*. 2007;102:1474-1482.

19. Luiking YC, Poeze M, Ramsay G, Deutz NE. The role of arginine in infection and sepsis. *J Parenter Enteral Nutr*. 2005;29(suppl 1):70-74.

20. Kumar S, Asrani SK. Non-cirrhotic hyperammonemia—when high ammonia is not always from cirrhosis. *Curr Hepatol Rep*. 2015;14:25-31.

21. Dams K, Meersseman W, Wilmer A. Hyperammonemia in the Adult Critical Care Setting. In: Vincent JL, ed. *Yearbook of Intensive Care and Emergency Medicine*. Berlin, Heidelberg: Springer; 2008:481-490.

22. Dossin O, Rupassara SI, Weng HY, Williams DA, Garlick PJ, Schoeman JP. Effect of parvoviral enteritis on plasma citrulline concentration in dogs. *J Vet Intern Med*. 2011;25:215-221.

23. McGuirk SM. Disease management of dairy calves and heifers. *Vet Clin North Am Food Anim Pract*. 2008;24(1):139-153.

24. Constable PD, Walker PG, Morın DE, Foreman JH. Clinical and laboratory assessment of hydration status of neonatal calves with diarrhea. *J Am Vet Med Assoc*. 1998;212:991-996.

25. Trefz FM, Lorch A, Feist M, Sauter-Louis C, Lorenz I. Metabolic acidosis in neonatal calf diarrhea—clinical findings and theoretical assessment of a simple treatment protocol. *J Vet Intern Med*. 2012;26:162-170.

26. Bone RC, Balk RA, Cerra FB, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Chest*. 1992;101:1644-1655.

27. Trefz FM, Feist M, Lorenz I. Hypoglycaemia in hospitalised neonatal calves: prevalence, associated conditions and impact on prognosis. *Vet J*. 2016;217:103-108.

28. Radostits OM, Gay CC, Hinchcliff KW, Constable PD. Diseases associated with bacteria. In: Radostits OM, Gay CC, Hinchcliff KW, Constable PD, eds. *Veterinary Medicine. A Textbook of the Diseases of the Cattle, Horses, Sheep, Pigs and Goats*. 10th ed. Saunders; 2007:847-876.

29. Le A, Ng A, Kwan T, Cusmano-Ozog K, Cowan TM. A rapid, sensitive method for quantitative analysis of underivatized amino acids by liquid chromatography–tandem mass spectrometry (LC–MS/MS). *J Chromatogr B*. 2014;944:166-174.

30. Goggs R, Serrano S, Szladovits B, Keir I, Ong R, Hughes D. Clinical investigation of a point-of-care blood ammonia analyzer. *Vet Clin Pathol*. 2008;37(2):198-206.

31. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*. 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.

32. Burrin DG. Gastrointestinal protein and amino acid metabolism in growing animals. In: Zabielski R, Gregory PC, Weström B, Salek E, eds. *Biology of growing animals*. Amsterdam; Boston: Elsevier; 2002:695-725.

33. Wu G, Bazer FW, Davis TA, et al. Arginine metabolism and nutrition in growth, health and disease. *Amino Acids*. 2009;37(1):153-168.

34. Hill TM, Bateman HG, Aldrich JM, Schlotterbeck RL, Tanan KG. Optimal concentrations of lysine, methionine, and threonine in milk replacers for calves less than five weeks of age. *J Dairy Sci*. 2008;91:2433-2442.

35. Healy PJ, Harper PA, Dennia JA. Bovine citrullinaemia: a clinical. pathological, biochemical and genetic study. *Aust Vet J*. 1990;67:255-258.

36. Tsukano K, Suzuki K, Shimamori T, et al. Profiles of serum amino acids to screen for catabolic and inflammation status in calves with *Mycoplasma bronchopneumonia*. *J Vet Med Sci*. 2015;77:67-73.

37. Ozkan C, Altuğ N, Yüksek N, Kaya A, Akgul Y. Assessment of electrocardiographic findings, serum nitric oxide, cardiac troponins and some enzymes in calves with hyperkaliemia related to neonatal diarrhoea. *Revue Méd Vét*. 2011;162:171-176.

38. Kojouri GA, Hassanpour H, Taghavi N, Taghadosi C. Nitric oxide metabolites status in calves with acute and chronic diarrhea. *Comp Clin Path*. 2012;21(6):1257-1261.

39. Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J Chromatogr B*. 2007;851:51-70.

40. Craig AM, Pearson EG, Rowe K. Serum bile acid concentrations in clinically normal cattle: comparison by type, age, and stage of lactation. *Am J Vet Res*. 1992;53:1784-1786.

41. Basoglu A, Baspinar N, Tenori L, Hu X, Yildiz R. NMR based metabolomics evaluation in neonatal calves with acute diarrhea and suspected sepsis: a new approach for biomarker/S. *Metabolomics*. 2014;4:1-12.

42. Chan DL, Rozanski EA, Freeman LM. Relationship among plasma amino acids, C-reactive protein, illness severity, and outcome in critically ill dogs. *J Vet Intern Med*. 2009;23:559-563.

43. Pourjafar M, Badiei K, Nazifi S, Naghib SM. Acute phase response in Holstein dairy calves affected with diarrhoea. *Bulg J Vet Med*. 2011;14:142-149.

44. Sultana H, Inada M, Wadud S, et al. A quantitative study on arginine synthesis from argininosuccinic acid and citrulline by crude enzymes of cattle kidney. *Animal Sci J*. 2003;74:289-294.

45. van Waardenburg DA, de Betue CT, Luiking YC, Engel M, Deutz NE. Plasma arginine and citrulline concentrations in critically ill children: strong relation with inflammation. *Am J Clin Nutr*. 2007;86:1438-1444.

46. Su L, Li H, Xie A, et al. Dynamic changes in amino acid concentration profiles in patients with sepsis. *PLoS One*. 2015;10:1-15.

47. Chokshi NK, Guner YS, Hunter CJ, Upperman JS, Grishin A, Ford HR. The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. *Semin Perinatol*. 2008;32:92-99.

48. Sowmyanarayanan TV, Natarajan SK, Ramachandran A, et al. Nitric oxide production in acute gastroenteritis in Indian children. *Trans R Soc Trop Med Hyg*. 2009;103:849-851.

49. Herulf M, Svenungsson B, Lagergren Å, et al. Increased nitric oxide in infective gastroenteritis. *J Infect Dis*. 1999;180:542-545.

50. Kukuruzovic R, Robins-Browne RM, Anstey NM, Brewster DR. Enteric pathogens, intestinal permeability and nitric oxide production in acute gastroenteritis. *Pediatr Infect Dis J*. 2002;21:730-739.

51. Rodriguez-Diaz J, Banasaz M, Istrate C, et al. Role of nitric oxide during rotavirus infection. *J Med Virol*. 2006;78:979-985.

52. Zarebavani M, Dargahi D, Einollahi N, Dashti N, Safari F, Rezaeian M. Significance of nitric oxide level in giardiasis. *Clin Lab*. 2017;63(1):47-52.

53. Forte P, Dykhuizen RS, Milne E, McKenzie A, Smith CC, Benjamin N. Nitric oxide synthesis in patients with infective gastroenteritis. *Gut*. 1999;45(3):355-361.

54. Yokoyama K, Ogura Y, Kawabata M, et al. Hyperammonemia in a patient with short bowel syndrome and chronic renal failure. *Nephron*. 1996;72:693-695.

55. Upadhyay R, Bleck TP, Busl KM. Hyperammonemia: what urea-lly need to know: case report of severe noncirrhotic hyperammonemic encephalopathy and review of the literature. *Case Rep Med*. 2016; 2016:1-10.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.