Evaluation of Serum Neutrophil Gelatinase-Associated Lipocalin, Cystatin C, and Clusterin Concentrations in Neonatal Calf Diarrhea

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SUMMARY

Neonatal diarrhea adversely affects calf growth, overall health, and productivity. Many factors such as poor environmental conditions, less colostrum intake, infectious and parasitic factors contribute to the development of calf diarrhea. Diarrhea, which is one of the most important causes of calf deaths all over the world, causes significant economic losses if it is not correctly diagnosed and appropriate treatment is not initiated. Systemic inflammatory response and kidney dysfunction are crucial components associated with the pathogenesis and progression of neonatal diarrhea. Early detection and management of these conditions are essential for minimizing their detrimental effects and improving calf health outcomes. However, traditional biomarkers such as haptoglobin and creatinine have limitations in terms of sensitivity, specificity, and accuracy. This study aimed to investigate the potential of emerging markers, including neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, and clusterin, in assessing inflammation and kidney injury in neonatal calves with diarrhea. The levels of NGAL, cystatin C, clusterin, haptoglobin, and creatinine were measured and compared among the groups. The results showed no significant differences in NGAL and cystatin C concentrations between the diarrheic calves and healthy calves. However, there was a significant increase in serum clusterin concentration in calves infected with *Escherichia coli* (*E. coli*) compared to other pathogen-infected calves and healthy calves. Moreover, a moderate positive correlation was observed between clusterin and creatinine concentrations, suggesting a potential association between clusterin and kidney injury. These findings highlight the potential of clusterin as a marker for kidney dysfunction in neonatal calves with diarrhea, particularly in cases of *E. coli* infection. This study will contribute to the diagnosis and treatment in field conditions, while shedding light on the parameters used in the early diagnosis of calf diarrhea. Further research is needed to investigate the underlying mechanisms and evaluate the diagnostic and prognostic value of clusterin in assessing the severity and progression of kidney dysfunction in affected calves.

KEY WORDS

Calf; clusterin; cystatin C; neonatal diarrhea; neutrophil gelatinase-associated lipocalin.

INTRODUCTION

Neonatal diarrhea is a common and economically significant health issue in calves, causing substantial morbidity and mortality rates worldwide. The condition is often multifactorial, resulting from various infectious agents such as viruses, bacteria, and protozoa (1). Neonatal diarrhea also adversely affects calf growth, overall health and productivity (2).

Systemic inflammatory response and renal dysfunction associated with decreased renal perfusion are important components associated with the pathogenesis and progression of neonatal diarrhea (3, 4). Early detection and appropriate management of systemic inflammatory response and kidney dysfunction are crucial for minimizing the detrimental effects of these conditions and improving calf health outcomes. Traditionally, haptoglobin, a major acute-phase protein in cattle, has been utilized as a standard marker of inflammation in bovine medicine. It serves as a sensitive indicator of systemic inflammation and is primarily synthesized by the liver in response to pro-inflammatory cytokines. (5). However, the routine use of serum haptoglobin is limited because its measurement requires laboratory facilities and new acute phase proteins capable of detecting systemic inflammation are required. Furthermore, serum creatinine has long been used as a conventional marker for assessing kidney function (6). However, serum creatinine fails to detect acute kidney injury (AKI) early enough and is influenced by nonrenal factors such as age, sex, muscle mass, hydration status and tubular secretion (7). For this reason new and more reliable biomarkers are needed in clinical practice to diagnose and monitor AKI at an earlier stage.

Recent investigations have explored the potential of neutrophil gelatinase-associated lipocalin (NGAL), cystatin C (CysC), and clusterin as emerging markers of inflammation and kidney injury in various pathological condition (8, 9, 10, 11). NGAL, an iron-transporting protein, is released by neutrophils and renal tubular epithelial cells in response to inflammation and kidney injury (7). It has demonstrated sig-

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nificant diagnostic value as an inflammatory and kidney injury marker in both human and veterinary medicine (12, 13). CysC, a cysteine protease inhibitor, has also shown promise as a sensitive indicator of renal dysfunction (14). Previous studies have shown that CysC is superior to serum creatinine for the detection of early-stage kidney injury (15). Clusterin, a multifunctional glycoprotein, has been implicated in various biological processes, including tissue repair, apoptosis, and immune modulation (16).

The aim of this study was to investigate the levels of NGAL, CysC, and clusterin in the serum of neonatal calves with diarrhea and compare them with haptoglobin and creatinine which are recognized as reference biomarkers for inflammation and kidney dysfunction, respectively. We hypothesized that these biomarkers could be useful for early diagnosis of renal impairment and inflammation in neonatal calves with diarrhea.

MATERIALS AND METHODS

Animals

Out of the 53 neonatal calves presented at the Fırat University Veterinary Teaching Hospital for diarrhea diagnosis and treatment, a selection of 29 calves was included in this study to form study groups. All of the 53 calves were tested by using the calf scours test (Rainbow Calf Scours-Bio K 306, Bio X Diagnostics, Rochefort, Belgium) to identify the etiological agents responsible for the diarrhea. This calf scours test was developed to detect *Escherichia coli (E. coli)* F5 attachment factor, rotavirus, coronavirus, Cryptosporidium parvum (C. parvum) and *Clostridium perfringens* in fecal samples. The calves infected with two or more etiological agents were excluded based on the test results. The remaining 29 calves infected with only one etiological agent were divided into four groups: *E. coli* infected group (n = 8), rotavirus infected group (n = 8), coronavirus infected group (n = 6), C. parvum infected group (n = 7). To serve as a control, a separate group consisting of seven clinically healthy calves raised on a dairy farm was also included in the study. The median age of the calves was as follows: *E. coli* infected group was 2 days (min: 2 days, max: 4 days), rotavirus infected group was 8 days (min: 5 days, max: 15 days), coronavirus infected group was 8.5 days (min: 3 days, max: 12 days), C. parvum infected group was 7 days (min: 4 days, max: 12 days), control group was 1 day (min: 1 day, max: 3 days). Fluid and electrolyte therapy was initiated in diarrheic calves using a 0.9% sodium chloride solution (Polifleks, Polifarma, Türkiye) and hypertonic sodium bicarbonate solution (Bikarvil 8.4% infusion solution, Vilsan, Türkiye), guided by blood gas analysis conducted with a benchtop blood-gas analyzer (ABL80, Radiometer, Brønshøj, Denmark). Furthermore, the diarrheic calves were administered enrofloxacin (5 mg/kg BW intramuscularly once daily, Baytril 10% injectable solution, Bayer Animal Health, Germany), meloxicam (0.5 mg/kg BW intravenously, BAVET Meloxicam injectable solution, BAVET, Türkiye), or halofuginone lactate (8-12 ml/calf orally once daily, Halocur, MSD Animal Health, Türkiye) based on the outcomes of the scours test. The study protocol was approved by the Firat University Local Ethics Committee of Experimental Animals (24.11.2021, Decision No: 2021/19).

Blood Collection and Biochemical Analysis

Before receiving treatment, blood samples were obtained from the calves and collected into clot-activated tubes (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The tubes were then subjected to centrifugation to separate the serum. After centrifugation, the obtained serum samples were divided into three portions, containing 0.5 ml of serum. These portions were stored at a temperature of -20 °C until further analysis.

Bovine-specific ELISA kits were used to measure the serum levels of NGAL, cystatin C, clusterin, and haptoglobin (Bioassay Technology Laboratory, Shanghai, China). The ELISA assays were carried out following the manufacturer's instructions, including appropriate sample dilutions and incubation periods. Optical densities of the samples were determined using a microplate reader set to the specified wavelength as recommended by the ELISA kit guidelines. Additionally, serum creatinine concentrations were measured using a dry chemistry analyzer (Fuji Dri-Chem NX500i, Fujifilm Corporation, Tokyo, Japan).

Statistical evaluations were conducted using SPSS version 21 software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Descriptive statistics, including mean, standard deviation, median, minimum and maximum values, were calculated for each marker within the different groups.To determine the normality of the data, the Shapiro-Wilk test was used. Depending on the normality assessment, the differences between the groups were analyzed using appropriate statistical tests. Specifically, if the data followed a normal distribution, ANOVA with posthoc Tukey test was applied. For non-normally distributed data, the Kruskal-Wallis test with posthoc Dunn test was utilized. A significance level of greater than 0.05 was deemed statistically significant.

RESULTS

Table 1 summarizes the mean, median, standard deviation, minimum and maximum values for the variables, along with the corresponding p-values. Additionally, all of the results were displayed in Figure 1. No significant differences were found between the groups in terms of serum NGAL and CysC concentrations. However, significant differences were observed between the groups in serum clusterin, haptoglobin, and creatinine concentrations. Specifically, the serum clusterin concentration was significantly higher in *E. coli* infected calves compared to those infected with other pathogens and healthy calves ($p < 0.001$). On the other hand, the serum haptoglobin concentration was significantly higher in the C. parvum, rotavirus, and coronavirus infected groups compared to the *E. coli* infected group and healthy calves ($p < 0.001$).

Furthermore, the Spearman correlation test indicated a moderate positive correlation between serum creatinine and clusterin concentrations ($r = 0.481$, $p = 0.003$). Moreover, serum haptoglobin demonstrated a weak positive correlation with NGAL ($r = 0.365$, $p = 0.029$) and a moderate positive correlation with CysC ($r = 0.433$, $p = 0.008$).

DISCUSSION

The aim of this study was to investigate the serum concentra-

Table 1 - The mean, median, standard deviation, minimum and maximum values for the variables in the study groups, along with the corresponding p-values.

Variable	Group	Mean	Standard Deviation	Median	Min-Max	p-value
NGAL (ng/mL)	C. parvum	42.6	5.4	41.8	36.1-50.5	0.617
	E. coli	37.5	10.2	37.5	21.3-53.8	
	Control	42.9	2.6	41.8	39.9-47.0	
	Coronavirus	40.6	8.2	41.9	30.6-50.4	
	Rotavirus	44.3	13.2	39.1	31.8-69.5	
CysC (mg/L)	C. parvum	1.64	0.27	1.72	1.33-2.15	0.430
	E. coli	2.00	0.68	1.45	0.55-2.65	
	Control	1.62	0.12	1.70	1.50-1.78	
	Coronavirus	1.74	0.33	1.72	1.10-1.94	
	Rotavirus	1.67	0.51	1.87	1.52-2.95	
Clusterin (ng/mL)	C. parvum[b]	225	116	181	103-456	<0.001
	E. coli[a]	610	217	637	321-933	
	Control[b]	256	117	251	33-372	
	Coronavirus[b]	247	62.7	262	152-319	
	Rotavirus[b]	387	140	404	146-592	
Creatinine (mg/dL)	C. parvum[b]	1.42	0.521	1.41	0.6-2.0	0.0013
	E. coli[a]	6.25	2.60	6.24	3.6-11.9	
	Control[b]	2.72	1.57	2.24	1.3-2.9	
	Coronavirus[a,b]	4.37	3.25	3.42	1.3-9.9	
	Rotavirus[b]	2.18	1.59	1.78	0.8-5.4	
Haptoglobin (ng/mL)	C. parvum[a]	111	16.4	105	94-133	<0.001
	E. coli[b]	71	28.0	70.8	16-103	
	Control[b]	53	11.3	51.5	43-77	
	Coronavirus[a]	113	18.5	118	80-131	
	Rotavirus[a]	116	28.1	109	88-166	

[a,b] Different letters in each column show the statistical difference between groups. NGAL = Neutrophil Gelatinase Associated Lipocalin; CysC = Cystatin C.

tions of NGAL, CysC and clusterin in neonatal calves with diarrhea and compare them with serum haptoglobin and creatinine concentrations, which are considered as traditional markers of systemic inflammation and kidney dysfunction, respectively. Additionally, the study aimed to explore whether these markers can be used as potential markers of systemic inflammation and kidney injury in calves with neonatal diarrhea.

While serum creatinine is widely acknowledged as a measure of overall kidney function, it does not effectively indicate the presence of kidney injury (17). It's crucial to recognize that serum creatinine may lack sensitivity in the early detection of kidney injury (7). This becomes particularly relevant in cases like neonatal calf diarrhea, where azotemia may result from hypovolemia (4). The fundamental question that emerges is whether kidney injury occur in neonatal calf diarrhea and, if so, to what extent they have developed. Additionally, it's worth mentioning that even though appropriate fluid and electrolyte therapy can often restore kidney function in many hypovolemic calves (18), uncertainty remains regarding whether kidney injuries have indeed occurred or persist following treatment. Based on the results of previous studies (10, 19, 20), the concentration of NGAL, CysC and clusterin in serum may be indicators of kidney injury in neonatal calf diarrhea.

Our findings revealed that there were no significant differences in serum NGAL and CysC concentrations between neonatal calves with diarrhea and healthy calves. This suggests that these markers may not be directly associated with the presence of diarrhea in this specific population. The discrepancy between the findings of the present study, which showed no increase in serum NGAL and CysC concentrations in diarrheic calves, and previous studies indicating increased levels of these biomarkers in populations with impaired kidney function can be attributed to several factors (9, 21). It is possible that other factors, such as the severity or duration of diarrhea, may have a greater impact on these markers than the mere presence of diarrhea itself. Additionally, it is important to consider that NGAL and CysC are not specific to the gastrointestinal system and may be influenced by other physiological processes in the body.

Interestingly, we observed a significant increase in serum clusterin concentration in calves infected with *E. coli* compared to both other pathogen-infected calves and healthy calves. This suggests that clusterin may play a role in the inflammatory response associated specifically with *E. coli* infection in neonatal calves. Clusterin, also known as apolipoprotein J, is a multifunctional glycoprotein that has been implicated in various physiological processes, including inflammation and immune response regulation (16). Previous studies have shown that clusterin can be upregulated in response to infection and inflammation, acting as a chaperone protein that helps in the clearance of cellular debris and apoptotic cells (22). The significant increase in serum clusterin concentration in calves infected with *E. coli* suggests that this pathogen may trigger a specific immune response that involves the upregulation of clusterin. Furthermore, our study revealed a moderate positive correlation between serum clusterin and creatinine concentrations. This suggests that the increased levels of clusterin may be associated with kidney injury, as indicated by elevated creatinine levels. The correlation between these markers supports the notion that clusterin may serve as an indicator of kidney injury in neonatal calves with diarrhea. However, further investigations are

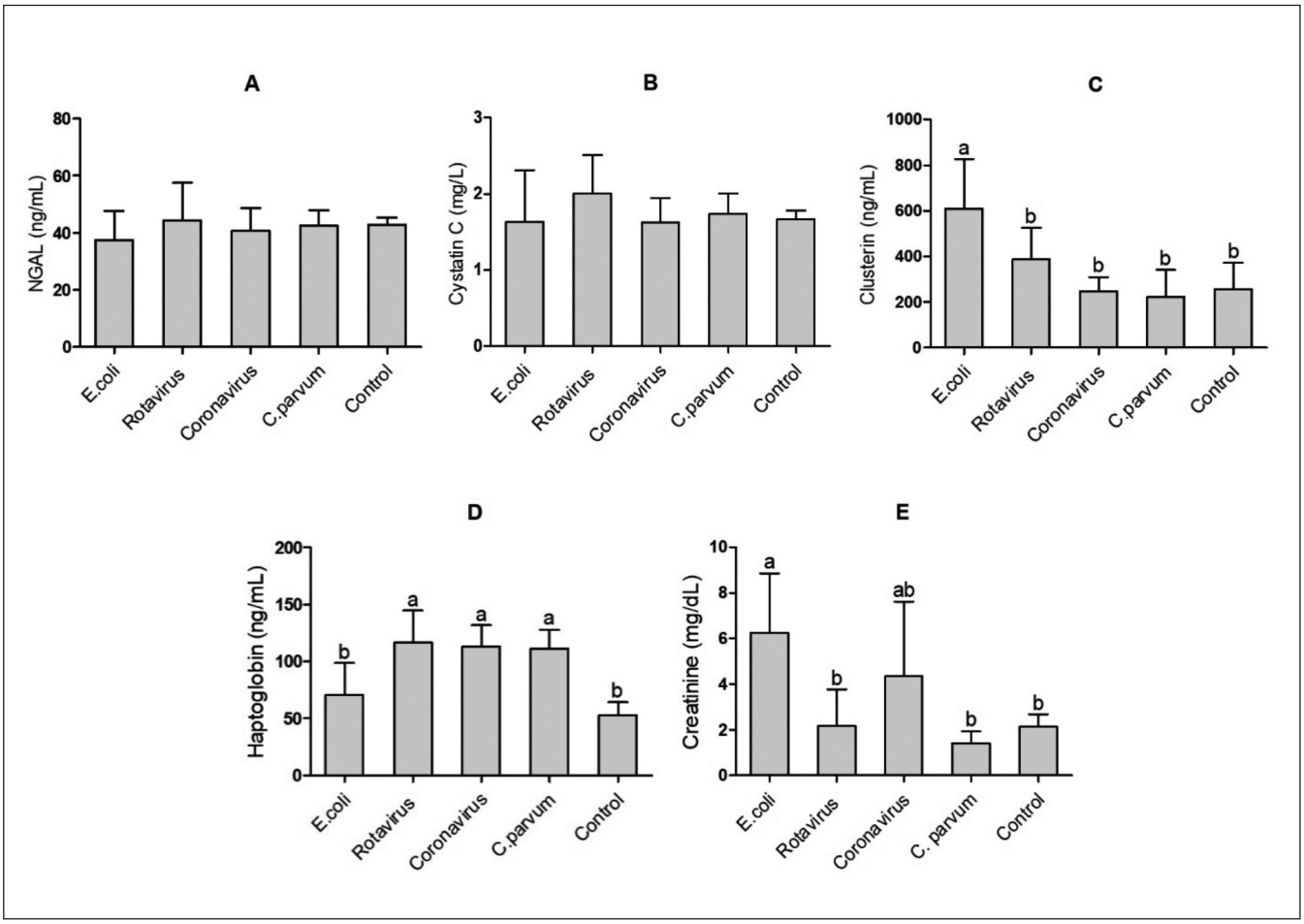

Figure 1 - Graphical visualization of the differences in serum concentrations of NGAL, cystatin C, clusterin, haptoglobin and creatinine in calves infected with *E. coli*, Rotavirus, Coronavirus, C. parvum and healthy control calves. Different letters on the boxes indicate differences between groups.

needed to elucidate the underlying mechanisms linking clusterin and kidney injury in this specific context.

CONCLUSION

Overall, these findings shed light on the potential role of clusterin as a marker of kidney injury in neonatal calves with diarrhea, particularly in cases of *E. coli* infection. Future studies could investigate the underlying mechanisms linking clusterin and kidney injury, as well as explore its diagnostic and prognostic value in assessing the severity and progression of kidney injury in affected calves.

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Author contributions
KCT and PFPD conceived and designed the study. KCT executed the study and analyzed the serum samples. KCT and PFPD analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Declaration of Competing Interest
The authors declare no conflict of interest.

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