Serum iron, copper and zinc concentrations in neonatal lambs naturally infected with *Cryptosporidium parvum*

Research Article

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ABSTRACT

The aim of this study was to evaluate the serum concentration of iron, copper and zinc in neonatal lambs naturally infected with *Cryptosporidium parvum*, and to determine the relations between these trace elements and the clinical severity of the disease. For this purpose, neonatal lambs infected with *Cryptosporidium parvum* (n=27) and healthy control lambs (n=10) were evaluated. Faecal consistency, willingness to rise, stance when up, appetite, attitude, and hydration status were scored within the scope of clinical examination in infected lambs. Thus, clinical health scores were calculated for each lamb to quantify the clinical severity of the disease. Iron, copper and zinc concentrations were measured from serum samples of lambs in both groups. Compared to the healthy control lambs, serum iron and zinc concentrations were significantly lower in infected lambs ($p < 0.001$ and $p < 0.05$, respectively), while there was no difference between the two groups in terms of serum copper concentrations. Additionally, a significant negative correlation (r=-0.60; p<0.001) was observed between serum zinc concentration and clinical health score, whereas the serum iron (r= -0.359; p= 0.066) and copper (r=0.322; p = 0.102) concentrations did not significantly correlate with clinical health score. In conclusion, the obtained results of the study provide valuable information about trace elements status in lambs with cryptosporidiosis.

Keywords: *Cryptosporidium parvum*, lambs, iron, copper, zinc

INTRODUCTION

Cryptosporidium species are protozoan parasites that infect a broad range of hosts including humans and animals worldwide, causing asymptomatic or mild-to-severe gastrointestinal disease in their host species (Robertson et al., 2014; Ryan et al., 2016). *Cryptosporidium parvum (C. parvum)* was detected as a primary etiological agent in neonatal diarrhoea of calves, lambs and kids (de Graaf et al., 1999). This protozoon invades epithelial cells primarily in the distal small intestine, and it destroys intestinal epithelia resulting in a reduction of enzymatic activity and a decrease in the absorptive surface, finally leading to maldigestion and malabsorption followed by osmotic diarrhoea (Certad et al., 2017; de Graaf et al., 1999). In particular, the extent and severity of the small intestine damage affect the clinical findings and clinical severity of the disease (Angus et al., 1982; de Graaf et al., 1999).

Trace elements as iron (Fe), copper (Cu) and zinc (Zn) have a significant role in metabolic function and tissue maintenance (Yatoo et al., 2013). These essential elements have important positions in continuing vital metabolic function like oxygen transport and storage, electron transport, in functions of several enzymes, oxidative metabolism, cell growth and proliferation (Beisel, 1976; Yatoo et al., 2013).

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It’s known that the metabolism of trace elements is significantly affected by varying degree of intestinal degenerations (Beisel, 1976). It is also reported that during the acute phase reaction, serum concentrations of these trace elements change, especially Fe and Zn serum concentrations decrease while serum Cu concentration increases (Gruys et al., 2005). Considering both the intestinal damage and acute-phase reaction caused by *Cryptosporidium*, changes in serum concentrations of these trace elements are expected. According to our knowledge, there is no study evaluating the effect of cryptosporidiosis on these trace elements in lambs.

The aim of this study was to specify the serum Fe, Cu and Zn concentrations in lambs with cryptosporidiosis, and to determine whether a relation between these elements and the clinical severity of the disease.

MATERIAL and METHOD

The animal material of this study consisted of 27 neonatal lambs naturally infected with *C. parvum* (INF group) and 10 healthy control lambs (CON group) from a commercial sheep farm in Aydın province. Lambs in the INF group were 3-20 days old and of both sexes (17 females, 10 males). The healthy CON group was selected as 6 females and 4 males at the age of 3-20 days, similar to the INF group.

Stool samples were taken directly from the rectum for diagnostic purposes from 3-20 days old neonatal lambs with varying degrees of diarrhea. Stool specimens underwent etiologic evaluation against 5 important enteropathogens (rotavirus, coronavirus, ETEC F5, *Giardia duodenalis* and *C. parvum*,) with a rapid commercial test kit (Bovid-5 Ag, Bionote, Korea). Fresh faecal smears were prepared from stool samples that test positive for *C. parvum* with the rapid test kit. It was stained with modified Ziehl Neelsen (Henriksen and Pohlenz, 1981) and carbol-fuchsin (Heine, 1982), and examined at 100 x magnifications by microscopy. The certain diagnosis was made with recognize of *Cryptosporidium* oocysts. The lambs mono-infected with only *Cryptosporidium* and who had not received any treatment before were included in the INF group of the study. Neonatal lambs who appeared clinically healthy and who did not have any agent in the stool examination both with the rapid test kit and microscopically were included in the CON group. All lambs were reared under the same husbandry conditions.

Faecal consistency, willingness to rise, stance when up, appetite, attitude, and hydration status were evaluated within the scope of clinical examination. A scale from 0 to 3 was assigned to each parameter as shown in Table 1, and thus the clinical health score (CHS) was calculated in each lamb in the INF group. The CHS applied for experimentally infected calves in previous studies (Imboden et al., 2012; Riggs and Schaefer, 2020; Schaefer et al., 2016) was slightly modified.

Blood samples collected into tubes without an anticoagulant were allowed to clot at room temperature for 1 h and then centrifuged at 3000 x g for 10 min for obtaining sera. The sera samples were stored at −20 °C until analysis for serum Fe, Cu and Zn concentrations. The serum concentrations of Fe, Cu and Zn were measured using commercially available test kits (Archem Diagnostics Ind. Ltd., Turkey) based on colorimetric methods (ferrozine, 3.5 Di-Br-PAESA and Nitro-PAPS, respectively) with autoanalyzer device (Rayto Chemray 120, China).

Statistical Analysis

Statistical analyses were performed using the software Statistical Package for the Social Sciences (SPSS) version 22.0 (IBM Corporation, Armonk, USA). Arithmetic means and standard error of the mean (SEM), for each evaluated parameters were calculated using descriptive statistic. The Shapiro-Wilk test was used to assess the respective data distributions for Fe, Cu and Zn concentrations within each study group. It was determined that all evaluated trace elements showed normal distribution. Therefore, the independent sample t-test was used to assess the statistical significance of these trace elements between INF and CON groups. Also, the Pearson correlation coefficient (*r*) was calculated to determine the correlations between CHS and trace elements concentrations. All analyses were considered statistically significant at $p < 0.05$. All the graphics were drawn using GraphPad 8.0.2 (San Diego, CA, USA).

Table 1. Clinical health parameter evaluation and scoring modified from Imboden et al (2012), Schaefer et al (2016) and Riggs and Schaefer (2020).

Score	Fecal consistency	Willingness to rise	Stance when up	Appetite	Attitude	Hydration status
0	Normal-solid faeces	Normal-eager to rise	Normal-stable	Ravenous, consumes full basal diet, wants more	Normal bright, alert, responsive	Normal-euhydrated, asymptomatic
1	Pasty faeces	Willing to rise on own but needs prodding	Somewhat unstable	Normal-consumes basal diet, reaches satiety	Somewhat depressed, dull	Mild dehydration (~5%)
2	Liquid faeces	Reluctant to rise but able with assistance	Clearly unstable, loses balance or sways	Decreased, but if coaxed will suckle	Clearly depressed, minimally responsive	Moderate dehydration (~6–8%)
3	Watery and profuse faeces	Unwilling or unable to rise on own, requires lifting	Unable to stand on own if lifted	Anorectic, will not suckle		Severe dehydration (~9–12%)

RESULTS

The clinical examination and CHS calculation of the lambs in the INF group were performed as indicated in Table 1. In this context, it was determined that CHS ranged between 5 and 14 (9.59±0.55) in naturally infected lambs with *C. parvum* included in the study.

Figure 1 demonstrates the mean ± SEM of Fe (A), Cu (B) and Zn (C) concentrations of the lambs in the CON and INF groups. In this context; serum Fe and Zn concentrations of the INF group were statistically lower (p=0.000 and p=0.024, respectively) than those of the CON group. Also, there was no statistical difference (p=0.761) between the two groups in terms of serum Cu concentrations.

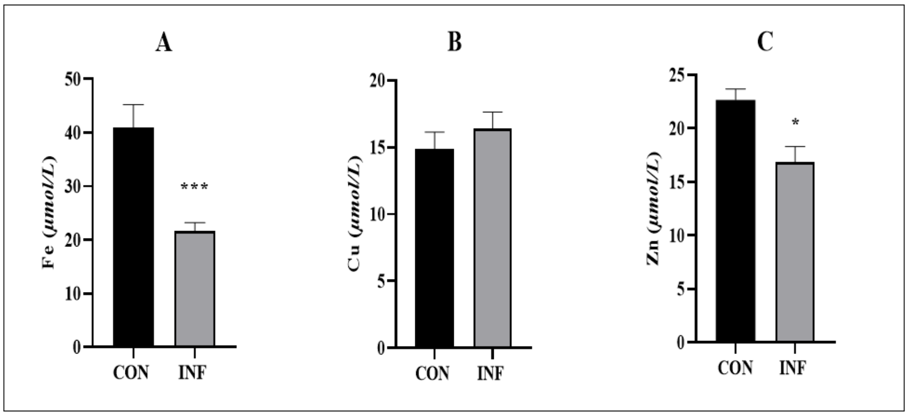

Figure 1. Mean (± SEM) of serum concentrations of iron (A), copper (B) and zinc (C) in healthy control (CON) and infected (INF) neonatal lambs. * $p<0.05$, *** $p<0.001$

Table 2 presents the result of correlation analysis between CHS and the trace elements in infected lambs. The Pearson correlation coefficient (*r*) results showed a significant moderate negative relation between CHS and Zn concentration (*r*= -0.60; *p*=0.001). Whereas, CHS did not significantly correlate with serum concentration of Fe (*r*= -0.359; *p*= 0.066) and Cu (*r* =0.322; *p*= 0.102).

Table 2. Pearson correlation coefficients (*r*) between serum iron (Fe), copper (Cu) and zinc (Zn) concentrations and clinical health score (CHS) in lambs infected with *Cryptosporidium parvum*.

	Trace Element	*r*	*p*
CHS	**Fe**	-0.359	0.066
	Cu	0.322	0.102
	Zn	-0.600	**0.001**

DISCUSSION

Information about the levels of serum Fe, Cu and Zn and their relationship with clinical severity of the disease in lambs naturally infected with *C. parvum* are lacking. In this study, it was shown for the first time that the serum Fe and Zn concentrations of lambs naturally infected with *C. parvum* were significantly lower than in the healthy control lambs, and serum Zn concentration correlated significantly (*r*= -0.60; *p*=0.001) with CHS.

Several laboratory variables like trace elements are influenced by many factors such as animal species, age, gender, nutrition content etc. (Hidiroglou, 1980; Soetan et al., 2010). Variables especially in the neonatal period differ from those of adults, therefore; they should be interpreted under consideration of the animal's age. In this study, in order to eliminate these factors, the lambs in the CON group were selected to be similar to the lambs in the INF group in terms of age, gender and maintenance feeding conditions.

It is known that lambs and kids are born with adequate Fe reserve (Hidiroglou, 1980; Oliveira et al., 2012). Fe mobilization is increased to need the physiological requirement such as growth and sufficient blood production, and replace of foetal red blood cells with adult cells (Kojouri and Shirazi, 2007) in the neonatal period. On the other hand, Fe is not only essential for the organism but also important for many bacteria, viruses, and parasites. Several studies reported that serum concentration of Fe decreases in various infections due to nutritional immunity (Beisel, 1976). In addition, intestinal damage adversely affects the absorption of Fe, and decreases in serum Fe values develop abruptly during the incubation period of most systemic infectious processes, in some instances several days before the onset of fever or any symptoms of clinical illness (Beisel, 1976). In our study, the mean serum iron concentration of the INF group (41.02±4.2 µmol/L) was significantly (*p*=0.001) lower than that in the CON group (21.72±1.51 µmol/L). Consistent with our findings, previous studies indicated lower serum concentration of Fe in diarrheic yaks (Han et al., 2016), in diarrheic sheep with coccidiosis (Davoodi and Kojouri 2015) and, in calves experimentally infected with *C. parvum* (Babaç, 2014) than those of the healthy subjects. Lower serum Fe concentration in the INF group in this study could be explained by the intestinal changes caused by *C. parvum* and their consequences such as systemic inflammation.

Copper is absorbed through the small intestine and, to a lesser extent, through the large intestine in sheep as other species (Turner et al., 1987). In cases of malabsorption-causing diseases such as cryptosporidiosis, Cu absorption may be expected to decrease (Turner et al., 1987). On the other hand, an increased serum level of copper due to undulating levels of copper-containing ceruloplasmin and other erythrocytic antioxidant enzymes during infection was reported (Abou-Shady et al. 2011; Ranjan et al., 2006). The lack of statistical difference between the INF group and the CON group in terms of serum Cu concentrations in this study may be related to the single or simultaneous effect of the factors mentioned above.

Zinc has a direct effect on intestinal villus, brush-border disaccharidase activity, and intestinal transport of water and electrolytes (Arora et al., 2006). This effect involves both the maintenance of the gut structure and gut immune function (Koski et al., 2003). In a study of children with enteric parasitic infections like Giardia lamblia and *Cryptosporidium* sp., the serum concentrations of Zn in infected children were found to be significantly lower than non-infected children (Yones et al., 2015). Additionally, serum Zn concentration of acute diarrhoeic children with unknown aetiology was 13.1% lower than healthy ones (Arora et al., 2006). Similar to researches on humans, the serum Zn levels of the yaks in the diarrheic group were found that significantly lower than those in the healthy control group (Han et al., 2016). The serum Zn concentrations of lambs with coccidiosis-associated diarrhoea were also lower than the after-treatment levels (Davoodi and Kojouri 2015). The decrease in serum/plasma Zn concentrations in these studies is based on loss of Zn from the gastrointestinal tract, weak Zn absorption throughout intestines, increased Zn requirement in the immunization system, and use of Zn for the synthesis of antioxidant enzymes. (Arora et al., 2006; Han et al., 2016; Yones et al., 2015). In the present study (Fig. 1C), the lower serum Zn concentration in the INF group can also be associated with the above-mentioned mechanisms. On the other hand, it was reported that there was no significant difference between healthy control calves and experimentally infected calves with *C. parvum* in terms of serum Zn concentrations (Babaç 2014). This disparity may be related to differences in the severity of the infection, except for other reasons. Because, we also revealed a significant negative correlation (r= -0.60; p<0.001) between serum Zn concentration and CHS, different from serum Fe and Cu concentration. This indicates a relatively lower serum Zn concentration in lambs with clinically more severe cryptosporidiosis than in those with clinically milder. This situation may be associated with the increase in excretion and consumption of Zn as a result of the morphological and inflammatory changes caused by *Cryptosporidium* in the intestines. Strand et al. (2004) report a strong association between inflammation and plasma Zn concentration in children with acute diarrhea. From another point of view, considering the direct positive effects of Zn on the intestine, low serum zinc levels of lambs with clinically severe cryptosporidiosis may have contributed to this severity of the disease, among other factors.

CONCLUSION

These alterations in serum trace elements concentrations in infected lambs may depend on the direct effect on the gastrointestinal system of *C. parvum* infection and/or its consequences. Additionally, considering that serum Zn concentration shows a negative correlation with the clinical severity of the disease in this study; the controlled studies to investigate the effect of Zn supplementation on clinical improvement in neonatal lambs with *C. parvum*-associated diarrhoea would be beneficial.

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