Original Article

Evaluation and Comparison of Stool Calprotectin Level in Necrotizing Enterocolitis Infected and NonInfected Neonates of <1500 g

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ABSTRACT

Background: Necrotizing enterocolitis (NEC) is one of the most prevalent digestive emergencies in neonates, leading to mortality and morbidity in premature neonates. Since there is no specific test for NEC diagnosis, only clinical symptoms and radiological findings are used for diagnosis. Therefore, if a reliable biomarker found to detect NEC, it can help to reduce the mortality and morbidity; hence, the present study evaluated the stool calprotectin levels in the infected and noninfected neonates weighing <1500 g. **Methods:** This case-control study was performed on 35 neonates with NEC and 35 healthy neonates. Demographic information and calprotectin levels were measured, and the values were recorded and compared between the two groups. **Results:** The results of this study revealed that the level of stool calprotectin in the case group with the mean of 459.66 ± 172.63 was significantly higher than the control group with the mean of 103.03 ± 30.97 ($P < 0.001$). Furthermore, the level of calprotectin had a significant relation with the severity of the disease. Moreover, this biomarker can be a good diagnostic criterion for detecting NEC (cutoff >176 μg/g, sensitivity = 97.14%, Specificity = 100%, $P < 0.0001$). **Conclusion:** Given that the level of calprotectin in the stool was not associated with factors such as the sex of neonate, gestational age, birth weight, and type of delivery, it appears that stool calprotectin level can be used as a reliable biomarker for NEC diagnosis.

Keywords: *Calprotectin, necrotizing enterocolitis, neonate, preterm*

Introduction

Necrotizing enterocolitis (NEC) is the most common digestive emergencies in neonates. It is characterized by various degrees of mucosal necrosis or the entire intestinal wall, leading to mortality and morbidity of premature neonates, especially those under 32 weeks, and <1500 g.[1] Although the exact pathophysiology of the NEC is not clear, it seems that prematurity and intestinal immaturity, feeding with formulas and bacterial agents cause inflammatory response to the intestinal wall, and following the inflammation, the intestinal epithelial barrier would be damaged. NEC is likely to occur as a result of the interaction between mucosal damage and the host's response that leads to affected area necrosis which its progress causes rupture of the intestine, peritonitis, sepsis, and death.[2] The range of symptoms is wide and varies from mild disease without specific symptoms to severe illness or rupture of the intestine and even death. The first symptoms of disease onset are nonspecific that makes it difficult to differentiate NEC from sepsis and other digestive diseases. Since there is no specific test for NEC diagnosis, only clinical symptoms and radiological findings are used in this regard. In addition, simple abdominal radiography is required for diagnosis. The presence of intestinal pneumatosis has diagnostic value, and the presence of air in the portal vein and the pneumoperitoneum is a sign of the deterioration of the disease.[3]

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Nowadays, none of the laboratory findings for NEC is decisive, and there is no reliable biomarker for its detection, and that most surveyed biomarkers do not have the ability to differentiate NEC from other diseases.

On the other hand, there is no definitive cure for this disease. Supportive therapies include regulating water and electrolyte, stop feeding, gastric decompression, modification of acidosis, anemia and thrombocytopenia, and maintaining blood pressure, and initiating an appropriate antibiotic to cover intestinal pathogens. In mild cases, supportive measures result in treatment, whereas in severe cases (30%–50%), surgery is required. It should be noted that the exact time of surgical intervention is still a matter of controversy. Medical cures failed in about 20% to 40% of the cases and 10% to 30% of them will die.[4] However, finding a reliable biomarker to diagnose NEC can reduce the mortality and morbidity, as well as unnecessary intervention in neonates without NEC and surgical intervention in NEC patients, can be done at appropriate times. Furthermore, the severity of the disease and even the response to treatment can be monitored well.[5]

Calprotectin is a protein bound to calcium and zinc with a molecular weight of 36.5 kD, accounting for 60% of the protein in the neutrophil cytosol. There are also macrophages and monocytes in the epithelial cells, and they are not subject to degradation due to their stable structure. *In vitro* studies have shown that calprotectin inhibits the growth of a variety of microorganisms by apoptosis induction and also regulates inflammatory processes. The level of fecal calprotectin (FC) increases due to neutrophil migration to the damaged intestinal wall in the NEC and its amount is directly proportional to the inflammation severity. FC levels in the healthy neonates are about five times higher than for older children and adults.[6] Furthermore, some studies have reported the association between calprotectin excretion rates and NEC and have identified this marker as an appropriate biomarker for NEC diagnosis but have not yet reached a specific cutoff point for diagnosis by this marker.[7-10] Accordingly, the purpose of this study was to evaluate the value of noninvasive stool calprotectin level measurements in the early diagnosis of NEC neonates to reduce the complications and mortality of this disease through timely diagnosis.

Methods

This case-control study was conducted on all preterm neonates with the birth weight of <1500 g in Al-Zahra Hospital from April 2017 to April 2018. Based on the sample size formula, 35 neonates were selected in each group to compare the means at 95% confidence level, the test power of 80%, the standard deviation of stool calprotectin level in neonates, estimated to be 1.17, and the least significant difference between the two groups were equal to 0.8.

The inclusion criteria included preterm neonates weighing <1500 g with NEC digestive and radiological symptoms, born with cesarean section, and fed through the breast. If a neonate received NSAID during admission or for any reason, unable to take a stool sample or died before the stool was sampled, they were excluded from the study and another alternative was replaced to prevent the loss of the sample. In addition, 35 preterm neonates with the weight of <1500 g and noninfectious NECs who were born with cesarean section and fed with breast were also included in the study as the control group.

After obtaining written consent from the parents, the neonates eligible for admission to the study were hospitalized. First, the information such as sex, gestational age, birth weight, and stage of the disease (in terms of Bell's criteria) were recorded. Then, with the development of NEC symptoms, including abdominal distention, intolerance to nutrition, lavage, etc., and after confirmation of diagnosis by abdominal radiography, stool samples were sent for the measurement of calprotectin levels, whereas other routine measures including antibiotic initiation and other supportive measures performed according to the Neonatal Department protocol. In the control group, stool samples were also sent for the measurement of calprotectin levels.

It should be noted that to measure calprotectin, about 3–5 g of neonate stool were sent to the laboratory in fecal transfer containers. Given the fact that stool calprotectin is stable at room temperature, it is possible to delay the test. Then using a stool tube, which consists of three parts, blue lid and yellow sampler, proceed to the sample preparation according to the following steps.

First, the yellow sampler was removed from the tube with a blue lid, and 750 λ extracting solution was added to the tube. Then, the yellow sampler was closed to the tube with a blue lid, and only the yellow sampler was wrapped in the clockwise direction from the blue and tube, and the yellow sampler was inserted into the stool to enter the sample into the sampler end grooves. Then, the yellow sampler containing the stool was entered in the blue lid through the hole and was rotated clockwise to close firmly. In the next step, the tube was vortexed for 30 s at a rate of 1800 bpm, and the sample was introduced into the extraction solution from the yellow sampler, then it was shaken for 15 min at high speed to smooth out. The smooth sample was transferred to centrifugal tube and centrifuged at a rate of 3000 times/min to create a clear solution over the sediment. The solution would be

stable for 5 days at 2–8°C and up to 4 months at −20°C. Eventually, 10 λ was poured out of a clear solution into a calprotectin well port, and the strip was put in a fully automated Alegria machine. Using the Elisa method, the amount of calprotectin with a sensitivity of 97.6% and a specificity of 93.8% based on micrograms per gram of stool was obtained.

Finally, the collected data were entered into SPSS software (version 20; SPSS Inc., Chicago, Ill., USA). According to the Kolmogorov–Smirnov test, given the normal distribution of data, Chi-square, independent *t*-test, one-way ANOVA, and Pearson correlation coefficient were used. Moreover, the receiver operating characteristic (ROC) analysis was used to evaluate the calprotectin diagnostic value, and in all analyses, the significance level was considered to be <0.05.

Results

In this study, 18 (51.4%) boys and 17 (48.6%) girls with a mean birth weight of 1.15 ± 0.16 kg were in the group of neonates with NEC and 16 (45.7%) boys and 19 (54.3%) girls with the mean birth weight of 1.13 ± 0.16 kg (P = 0.632) in the group of neonates without NEC. The two groups also were similar in terms of gestational age, and fecal sampling age (day) (P > 0.05). In addition, of 35 neonates with NEC, 33 cases (94.35) were in Stage II (bell's Stage IIa or IIb) and two ones (5.7%) in Stage III, which eventually led to the death of both neonates in Stage III [Table 1].

On the other hand, the level of calprotectin in the NEC group was significantly (459.66 ± 172.63) higher than that control group with a mean of 103.03 ± 30.97 (P < 0.001) [Figure 1].

In addition, the result of linear regression to identify effective factors on the level of calprotectin suggests that only the stage of the disease can play a significant role in calprotectin levels (β =148.430, P = 0.003). For this purpose, Table 2 compares the level of calprotectin in different stages of NEC. The results showed that with an increase in disease severity, the level of calprotectin was also significantly increased (P = 0.015). Furthermore, the mean level of calprotectin in the NEC group was increased with the neonate's age and on the day of sampling; although, this association was statistically direct and very weak in both groups [Figure 2].

Finally, the results of the ROC analysis to assess the diagnostic value of calprotectin level in NEC showed that this marker could diagnose the disease in the value

Table 1: Demographic data of the two groups

Characteristics	NEC (n=35)	Control (n=35)	P
Gender, *n* (%)			
Boy	18 (51.4)	16 (45.7)	0.632
Girl	17 (48.6)	19 (54.3)	
Gestational age, week	29.41±2.20	28.99±1.28	0.342
Birth weight, kg	1.15±0.16	1.13±0.16	0.622
Sampling age (days)	13.54±3.30	14.60±2.19	0.119
Staging, *n* (%)			
IIa	28 (80)	-	-
IIb	5 (14.3)		
IIIa	2 (5.7)	-	

NEC – Necrotizing enterocolitis

Table 2: Comparison of the calprotectin level to the severity of necrotizing enterocolitis

Factors	Calprotectin (μg/g)	P
Staging		
IIa	421.54±138.81	0.015
IIb	569.40±206.29	
IIIa	719.00±295.57	
Sequel		
Improved	443.94±156.45	0.026
Died	719.00±295.57	

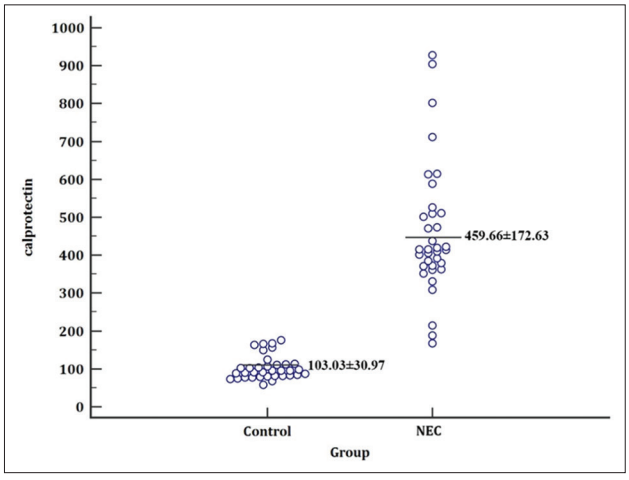

Figure 1: Dot plot of the calprotectin level in the two groups studied

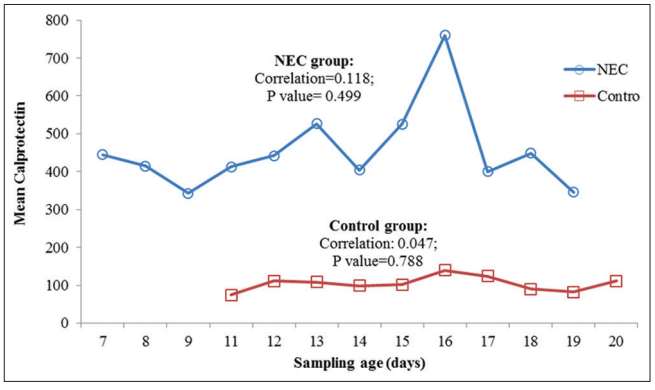

Figure 2: The mean of calprotectin level in the sampling age (day) in two groups

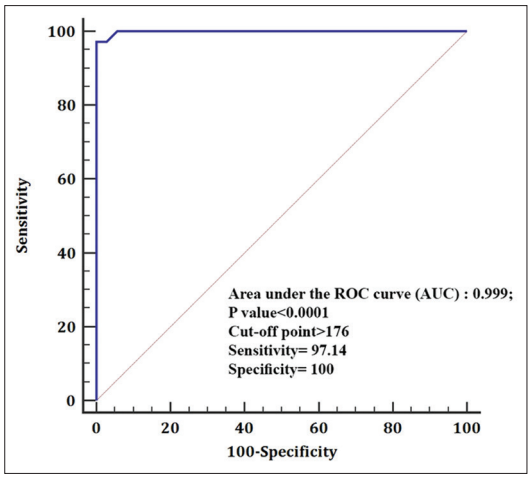

Figure 3: Rock chart for the diagnostic value of calprotectin level in detecting necrotizing enterocolitis

of 176 μg/g (cutoff) with a sensitivity of 97.14% and specificity of 100% ($P < 0.0001$) [Figure 3].

Discussion

The present study was conducted on two groups of preterm neonates with and without NEC with a birth weight of <1500 g who were born by cesarean section. The two groups were matched for age, sex, and birth weight. Neonates with NEC have also been diagnosed with Stage II/III according to Bell's criteria; both patients with Stage III had died.

According to the results of this study, the calprotectin level was significantly higher in NEC neonates than healthy ones. It should be mentioned that if the delay taken for sampling (the day after birth) was more, calprotectin level was higher in neonates with NEC; however, due to the small size of the study, this association was not significant.

In line with the present study, the results of many previous studies have indicated that calprotectin level was significantly higher in the NEC patients than healthy controls.[7-12] For example, Thuijls *et al*. in the Netherlands and Carroll *et al*., as well as Yoon *et al*. (2014) in Korea showed that calprotectin level was significantly higher in the neonates with NEC than healthy ones.[7,9,13] A case study by Niemarkt *et al*. (2015) suggested that calprotectin as a specific biomarker of inflammatory bowel disease (IBD) increases in neonates with NEC.[14]

In this regard, it can be stated that calprotectin as an antimicrobial protein is composed of one light chain (MRP8) and two heavy chains (MRP14) and can be found in the cytoplasm of neutrophils.[15] This marker contains His-x-x-x-His motif, which justifies the anti-bacterial role of it.[16] Previous studies have proved that FC is an exact marker of neutrophil migration toward the gastrointestinal tract in IBD and its measurement in feces is considered to be a reliable marker for IBD diagnosis.[17,18]

In addition, the stage is the only significant factor affecting calprotectin level, and hence that with increasing the severity of disease, calprotectin level was significantly elevated in neonates ($P = 0.015$). However, there was no correlation between calprotectin level and other factors, including sex, gestational age, and mode of delivery, birth weight, and sampling day. In line with the present study, in their study, Reisinger *et al*., also found FC level was increased in the NEC cases and its level was directly associated with the severity of the disease.[11] Moreover, in another study conducted by Baldassarre *et al*., the mode of delivery was not found to affect FC level,[19] but in their study, Josefsson *et al*., the level of the marker was higher in neonates born by cesarean delivery.[12] Some studies also found no difference between FC level in breastfeeding neonates compared with formula-fed ones,[19,20] whereas Golden *et al*. showed that FC level was lower in breastfed neonates,[21] and conflicting results from Dorosko *et al*. suggested that level of this marker was higher in breastfed neonates.[22]

Therefore, considering the contradictory results from previous studies on the presence or absence of correlation between calprotectin level and factors, including mode of delivery, age, birth weight and sex, it can be said that one of the most important strengths of our study is paid attention to these factors, and hence that neonates in this study were all preterm with a birth weight of <1500 g and born by cesarean delivery.

On the other hand, the measurement of FC level is a useful method for differentiating constitutive epithelial disorders of IBD in the 1st month after birth,[23] so that the high levels of calprotectin include the inflammatory process and is normalized by antibiotic and anti-inflammatory therapies. This marker can tolerate proteolysis and intestinal bacterial degradation, which allows the use of fecal sample even after a week of storage at room temperature without changing significantly in its calprotectin level.[9,24] Therefore, measuring this marker would facilitate detection of intestinal inflammatory process such as NEC, without the need for aggressive action.[25,26]

In this regard, the results of the study by Albanna *et al*. (2014) in Egypt showed that the measurement of FC level had a diagnostic value for NEC, and its level is higher in patients with advanced disease and dead cases.[4]

In this study, values of the biomarker >176 μg/g with 97.14% sensitivity, and 100% had a good diagnostic, predictive value for NEC. According to another study in Turkey in 2012, FC level was reliable laboratory test to assess newborns at risk for NEC, and FC value of 792 μg/g was found to be 76% sensitive and 92% specific for the diagnosis of NEC.[10] In other studies, the appropriate diagnostic value of this marker was indicated, and its cutoff values were 350 μg/g,[8] 200 μg/g,[9] 633 μg/g,[27] 286.2 μg/g,[7] and 32.5 μg/g.[28] All studies conducted so far have shown the appropriateness of this criterion for disease diagnosis, but the difference between the cutoff values of the studies was due to different types of laboratory kit. By increasing the number of studied samples and controlling the confounding factors, a more accurate cutoff point can be obtained.

Although potential confounding factors have been controlled in our study and considered as the strength of this study, the lack of evaluation of other factors such as white blood cell count, hemoglobin and platelet count, lymphocytes, and C-reactive protein may be regarded as the weakness of this study. Therefore, it is suggested that future researchers evaluate these factors along with this marker.

Moreover, a lower incidence of the disease required a long time to access to more cases, but in the limited course of study, we hardly found cases. Thus, further studies with larger sample sizes are still required to generalize results to the population.

Conclusion

According to the results of the present study, calprotectin as a biologic marker was higher in NEC neonates than healthy neonates and a cutoff point of >176 μg/g had a good diagnostic, predictive value for NEC.

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Conflicts of interest

There are no conflicts of interest.

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