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Hepcidin as a biomarker of neonatal infections

Хепцидин као биомаркер неонаталних инфекција

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SUMMARY

Introduction/Objective Nonspecific clinical signs of neonatal infection dictate routinely determination of C-reactive protein (CRP) and procalcitonin levels in order to confirm the diagnosis. As hepcidin is an acute phase reactant, the aim of our study was to analyze its significance in diagnosis of neonatal infections.

Methods The prospective study included 71 term neonates, 37 with signs of infection in the absence of other pathological conditions and 34 healthy neonates. After standard bacteriological examination, at the time of diagnosis and after 6 days of antibiotic therapy complete blood count, serum CRP, procalcitonin and hepcidin were determined.

Results There was no difference in serum hepcidin levels between the control (55.17 ± 21.22 ng/ml) and infection group (59.72 ± 59.70 ng/ml) on the first day. Hepcidin values in neonates with infection up to 72 hours were significantly lower (30.20 ng/ml, IQ: 25.9–39.9 ng/ml) than in older neonates (82.20 ng/ml, IQ: 39.70–128.10 ng/ml). In neonates up to 72 hours, after 6 days of antibiotics, the hepcidin values show a significant increase (36.68 ng/ml, IQ; 31.23–50.30 ng/ml). High hepcidin values (128.05 ng/ml, IQ: 60.95–201.00 ng/ml) were recorded in neonates with CRP over 100 mg/l.

Conclusion. Our results shows that the determination of serum hepcidin as a marker of neonatal infection is not relevant in neonates up to 72 hours of life. After 6 days of antibiotic therapy, the neonates of this group reacted with an increase in hepcidin, while the parallel determined values of CRP and procalcitonin showed a significant decrease.

Keywords: neonates; infection; hepcidin; CRP; procalcitonin

САЖЕТАК

Увод/Циљ Неспецифичност клиничких знакова инфекције код новорођенчади налаже рутинско одређивања серумског нивоа *C* реактивног протеина (*CRP*) и прокалцитонина у циљу потврде дијагнозе. Будући да је хепцидин реактант акутне фазе запаљења, циљ нашег рада је био испитати значај одређивања хепцидина у дијагностици неонаталних инфекција.

Методе Проспективним студијом обухваћено је 71 терминско новорођенче, 37 са клиничким и лабораторијским знацима инфекције без других патолошких стања и 34 здрава новорођенчета. Након стандардне бактериолошке обраде, у време дијагнозе и након 6 дана антибиотске терапије одређиване су вредности параметара у комплетној крвној слици, серумских нивоа *CRP*-а, прокалцитонина и хепцидина.

Резултати Није утврђена разлика у серумској вредности хепцидина између контролне групе (55.17 ± 21.22 *ng*/*ml*) и групе новорођенчади са инфекцијом (59.72 ± 59.7 *ng*/*ml*) у првом дану. Вредности хепцидина код новорођенчади са инфекцијом унутар 72 сата (30.2 *ng*/*ml*, *IQ*: 25.9–39.9 *ng*/*ml*) су статистички значајно ниже у односу на вредности новорођенчади старије од 72 сата (82.20 *ng*/*ml*, *IQ*: 39.7–128.1 *ng*/*ml*). Након шест дана антибиотске терапије вредности хепцидина код новорођенчади са инфекцијом узраста до 72 сата показују статистички значајан раст (36.68 *ng*/*ml*, *IQ*; 31.23–50.3 *ng*/*ml*). Високе вредности хепцидина (128.05 *ng*/*ml*; *IQ*: 60.95–201 *ng*/*ml*) забележене су само код новорођенчади са *CRP* преко 100 *ng*/*l*.

Закључак Према резултатима нашег истраживања, одређивање серумског хепцидина као маркера неонаталне инфекције није релевантно код новорођенчади са инфекцијом до 72 сата живота. Након шест дана антибиотске терапије новорођенчад ове групе су реаговала порастом хепцидина при чему су паралелно одређиване вредности *CRP* и прокалцитонина показале сигнификантан пад.

Кључне речи: новорођенчад, инфекције, хепцидин, *CRP*, прокалцитонин

INTRODUCTION

Despite progress in the diagnosis and treatment of infections, neonatal infections remain a global challenge in perinatal medicine and treatment of neonates [1].

Neonatal infections are defined as all infections occurring during the first 28 days of life. Due to the possibility of rapid progression and unfavorable outcome of infection in neonates,

antibiotic therapy must be started before obtaining positive microbiological culture. Prompt diagnosis is one of the most delicate challenges in neonatology. Clinical (Tolner) and hematological (Rodwell) scoring systems, as well as determination of inflammatory biomarkers such as C-reactive protein (CRP) and procalcitonin (PCT) are of great importance in the diagnostic process [2, 3, 4].

It is known that the specificity of CRP and PCT in differentiating infectious from non-infectious causes of the inflammatory response is limited [5]. Also, unreliable values of CRP and PCT in premature neonates limits their diagnostic value [4]. In order to overcome these problems, it is necessary to find new biomarkers of infection that would enable well-timed diagnosis, monitor disease progression and evaluating the effect of treatment.

It has been proven that proinflammatory cytokines cause increased expression of hepcidin (a 25-amino acid peptide containing eight cysteine residues), which is now considered as a acute phase reactant type II [6, 7]. Several studies have demonstrated the antibacterial activity of hepcidin-25 and hepcidin-20 against a large number of Gram-positive and Gram-negative bacteria [8, 9, 10]. The role of hepcidin in innate immunity is not only significant in infection; it can also have antitumor effects [7, 10, 11]. Hepcidin leads to the retention of iron in macrophages and a decrease its absorption in the intestines, which results in hypoferremia and contributes to the host's defense against pathogens [7, 11, 12, 13]. Based on the present knowledge it will be expected that neonates with infection would have significantly higher serum hepcidin levels than healthy ones.

The aim of our study was to examine the potential significance of hepcidin as a marker of neonatal infection, compared with the serum levels of CRP and procalcitonin as standard diagnostic markers.

METHODS

A prospective study was conducted in the Clinic for Children's Diseases and the Clinic for Gynecology and Obstetrics, University Clinical Center of the Republika Srpska (UKC RS). The research was approved by the Ethics Committee of University clinical centre of Banja Luka and was conducted in accordance with the principles of the Helsinki Declaration. For all neonates before inclusion in the study, the parents signed the informed consent for the child's participation. The study included 71 term neonates: 37 with clinical and laboratory signs of

infection in the absence of other pathological conditions and 34 healthy neonates with matched demografic characteristics.

In addition to the clinical evaluation (collection of anamnestic data on risk factors for neonatal infections, clinical examination, monitoring of the course of treatment and outcome), capillary and venous blood was taken from all subjects.

The clinical examination consisted of an assessment of the child's general condition, assessment of skin color, vital parameters, body temperature, physical findings by organs and systems, and determination of the Tolner sepsis score [2].

In laboratory analyses, in addition to the complete blood count, peripheral blood smear, C-reactive protein, procalcitonin, hepcidin concentration were performed for all subjects. The hematological sepsis score (HSS) according to Rodwell [3] was determined for all neonates.

In neonates with infection, blood was taken at two time points: at the time of clinical diagnosis of infection and blood collection for culture, and after 6 days of antibiotic therapy, as part of venous blood collection for routine tests.

In both time points 3 ml of peripheral blood was drawn in a tube without anticoagulant. Upon separating the serum by centrifugating the samples at 3000 × g, part of the serum was kept frozen at -70°C until the time of measurement of hepcidin levels. Other parameters were measured immediately.

The complete blood count was obtained by routine methods on Advia 2120, Siemens, Germany. Serum concentration of C-reactive protein and prokalcitonin were measured by routine methods on Roche/Hitachi Cobas 6000 using commercial Roche's kits. Values of CRP below 10mg/l were considered physiological [14]. According to personal experiance values of 10–40 mg/l were considered as a low risk for bacterial infection, from 40 mg/l to 100 mg/l as a moderate risk, and values above 100 mg/l as a high risk for bacterial infection.

Concentrations of procalcitonin below 0.5 ng/mL were considered the physiological. Values from 0.5 ng/ml to 2 ng/ml defined a moderate risk for sepsis, while values above 2 ng/ml represented a high risk for sepsis.

The level of hepcidin in the serum was measured by the by enzyme-linked immunosorbent assay intended strictly for research (DRG Hepcidin 25 ELISA; EIA 5258) according to the manufacturer's instructions on an open system device (BP 2000, Siemens). The results were expressesd in ng/ml.

Data distribution was tested using the Kolmogorov-Smirnov test. Depending on the type of variables and the normality of the distribution Student's t-test and Mann-Whitney U test were used to test data differences between two independent samples. Categorical variables were tested by the Chi-square test. Wilcoxon signed-rank test and Student-t test for dependent data were used to test changes between two repeated observations in the same group. Data are shown as mean ± standard deviation for normally distributed variables. The median for independent data or the median of difference for dependent data with interquartile range were presented for non-normally distributed variables. Relative or absolute frequencies are shown for categorical variables.

Statistical analysis and presentation of results were performed using Statistical Package for Social Sciences – SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

The study included 71 neonates, divided into two groups: a control group of 34 healthy neonates and a group of 37 neonates with signs of infection and no other pathological conditions. The groups were similar in sex, age, gestational age, birth weight, birth length, and Apgar score (Table 1).

The Tolner sepsis score was positive in 14 (37.84%) neonates of the infection group, while score was negative in all neonates of the control group. Rodwell score was positive in 18 (48.65%) neonates in the infection group on the 1st day, while all neonates in the control group had a negative Rodwell score. The stated difference between the neonates of the control and infection group was statistically significant ($p < 0.001$).

Laboratory findings of our patients are shown in Table 2. The mean value of hepcidin in neonates with infection was 59.72 ± 59.70 ng/ml (95% CI: 40.48–8.96 ng/ml) and no significant differences in this parameter were observed compared to the control group (55.17 ± 21.22 ng/ml; 95% CI: 48.04–2.30 ng/ml).

In the group of neonates with infection overall, regardless of whether they were up to 72 hours old (early infections) or older (late infections), no statistically differences were observed in hepcidin values obtained on 1st day (34.79 ng/ml; IQ: 26.67–70.60 ng/ml) compared to the 6th day (36.73 ng/ml; IQ: 30.39–56.61 ng/ml). The values obtained in the group of neonates with early infections (median 30.20 ng/ml, IQ: 25.90–39.90 ng/ml), were significantly lower

compared to the values in neonates with late infections (82.20 ng/ ml, IQ: 39.70–128.10 ng/ml). In neonates with early infections, hepcidin values on the 1st day (median 30.19 ng/ml, IQ: 25.90–39.88 ng/ml) were significantly lower than on the 6th day (36.68 ng/ml, IQ: 31.23–50.30 ng/ml). In neonates with late infections, hepcidin values on the 1st day (median 82.20 ng/ml, IQ: 39.70–128.10 ng/ml) were significantly higher than on the 6th day (median 44.88 ng/ml, IQ: 27.16–66.20 ng/ml) (Table 3).

In early infections, a slight increase in hepcidin values from the 1st to the 6th day (9.21 ng/ml; IQ: 1.12 - 22.03 ng/ml) is noticeable, while in late infections, there was a marked decrease in hepcidin values from the 1st to the 6th day (52.20 ng/ml, IQ: 10.44–69.95 ng/ml). This difference between early and late infections was significant (p = 0.001).

Our results showed statistically higher values of hepcidin (median 128.05 ng/ml, IQ: 60.95 - 201.00 ng/ml) in the group of neonates with CRP values over 100 mg/l (high risk of sepsis) compared to neonates with moderate (median 32.68 ng/ml, IQ: 24.69–43.00 ng/ml) and low (median 28.00 ng/ml, IQ: 27.14–29.56 ng/ml) risk of sepsis according to CRP (p=0.012).

DISCUSSION

Our study included neonates born after 37 weeks of gestation, average postnatal age 4.27 days in the group of neonates with infection, or 3.21 days in the control group of healthy neonates. Premature babies were not tested, due to the underdevelopment of their immune system [15, 16].

Although positive culture is the "golden" standard in the diagnosis of infection, the percentage of positive culture results in different studies ranges from 8% to 73% [17]. In our study, a positive blood culture was obtained in 10.72% of neonates. The frequency of positive urine cultures was 29.73%, and there was only one case with a positive cerebrospinal fluid culture (data not shown).

Prompt antibiotic therapy is required in order to prevent the development of sepsis, but time required to obtain culture results is at least 48 hours [18]. Due to the non-specificity of clinical sings and long time to microbiological results, acute phase reactants have great importance in therapeutic decision. One widely used biomarker of neonatal infection is C-reactive protein. According to CRP values, all neonates in the control group were without signs of inflammation, while the mean level of CRP in the group of neonates with infection was 65.50 mg/l. The mean level of procalcitonin in the group of neonates with infection was

41.44 ± 76.34 ng/ml. In addition to these standard parameters of systemic inflammatory response that are used in clinical practice, we determined the values of hepcidin, an acute phase reactant synthesized in the infection.

Studies in children have not provided uniform reference range of hepcidin values. The median (interquartile range) of hepcidin in the plasma of boys is 21.89 ng/mL (16.50–51.70 ng/mL), and that of girls is 21.95 ng/mL (19.20–47.70 ng/mL) [19]. The levels of hepcidin in cord blood of term neonates (19.40 ± 4.40 ng/ml) were similar to the values in preterm neonates (20.90 ± 13.80 ng/ml) [20]. In the Kulik-Recherberger study, hepcidin values of healthy neonates on the 3rd day of life were 66.79 ± 22.85 ng/ml [21]. The median value of hepcidin in the serum of preterm neonates who required erythrocyte transfusions, in the study by Muller *et al.* was 52.40 ng/ml, about 30% lower than the hepcidin values detected in the umbilical blood of term neonates in previous studies [22, 23]. Lower hepcidin values in premature neonates are thought to be the result of reduced iron reserves due to a shorter gestation [22].

In their study, Koukoulas *et al.* found significantly increased hepcidin levels in patients with bacteremia on day 0 and day 7, compared to healthy controls, and a significant reduction of serum hepcidin after a 7-day treatment [24]. In a prospective study, Olinder *et al*. have shown that hepcidin values are significantly higher in septic than in non-septic patients [25].

In the study of Sherbiny *et al.*, hepcidin values in the serum of premature neonates with late-onset sepsis (288 ± 81.3 ng/ml) were significantly higher compared to the control group (66.0 ± 12 ng/ml). They have also shown a significant decrease of hepcidin (to 98.3 ± 31ng/ml) after a 7-day antibiotic therapy to 98.3 ± 31 ng/ml [26].

In their research, Wu *et al.* found four times higher hepcidin values in neonates with late-onset sepsis compared to healthy neonates (26.80–67.70 ng/mL) and neonates with infection who did not develop sepsis (5.30–89.80 ng/ml) [27]. The results of the study by Cizmeci and colleagues show elevated values of hepcidin in the umbilical cord blood of neonates who developed early neonatal sepsis [28]. Similar results were obtained by Motalib and colleagues among neonates with early neonatal sepsis [5]. Wu and Motalib have also proven a significant correlation between hepcidin and CRP [5, 27]. In the study of Delaby *et al.* in adult patients, subjects with CRP below 10 mg/dl had a mean hepcidin values of 4.64 ng/ml, while those whose CRP level was higher than 10 mg/dl, had a mean hepcidin level of 55.85 ng/ml [29].

In our study, hepcidin values in neonates with infection on the 1st day were not significantly different from hepcidin values in healthy neonates. However, hepcidin values in

neonates with late infections were significantly higher compared to neonates with early infections (p=0.012). In the group of neonates with early infections, hepcidin values on the 6th day were significantly higher compared to the values on the 1st day (p=0.040). In contrast to this, in the group of neonates with late infections, the values obtained on the 6th day were significantly lower compared to the 1st day (p=0.005). The values of hepcidin in neonates with a high risk of sepsis according to the CRP value were 131.52 ± 84.37 ng/ml, which was higher than the values in the low- and moderate-risk group, indicating that the severity and intensity of inflammatory response in severe bacterial infections lead to a significant increase in hepcidin, in line with other studies [5, 27, 29].

It remains questionable whether the observed differences in hepcidin values are a consequence of maturation and a better response to various stimuli with increasing postnatal age, or a consequence of the longer time from the onset of infection to blood sampling for analysis, considering the fact that in our study the "older" neonates were hospitalized from home, while neonates with early infections had not been discharged and the signs of infection being recognized earlier. According to our results, early infection (from 1st to 6th day) is associated with an increase in hepcidin levels while late infections are followed by a decrease in hepcidin. Thus the relationship between observed hepcidin levels and the time of infection recognition and blood sampling in our study appear to be in agreement with the expected kinetics of hepcidin synthesis during the inflammatory response.

Even though our study has certain limitations, due to the small number of patients, the diversity of severity and time of infection onset, based on the results obtained, we summarized that hepcidin values are dependent on postnatal age. The neonatal period is also characterized by numerous changes in hematopoiesis and iron metabolism in which hepcidin plays a significant role.

CONCLUSION

According to our results serum hepcidin may not be a relevant marker of neonatal infection in the first 72 hours of life. After 6 days of antibiotic therapy all investigated neonates had increased hepcidin levels while their CRP and procalcitonin levels were decreased. Despite the significant increase in hepcidin found in neonates with CRP above 100 mg/l, we conclude that hepcidin has no significant advantages as a tool to ascertain neonatal infection compared to CRP and procalcitonin and could be used only as an additional biomarker of serious neonatal

infections. Additional studies of hepcidin level alterations are, however, clearly warranted, primarily in the healthy neonates, and also under pathological conditions.

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Table 1. Demographic characteristics of the study population

Parameter		Experimental group	Control group	p
Sex N (%)	Male	20 (*54.05)*	19 (*55.88)*	0.877*
	Female	17 (*45.95)*	15 (*44.12)*	
Age on admission (days) (median; IQ)		2 (2–5)	3 (3–4)	0.293**
Gestational age (days) (median; IQ)		279 (274–286)	279 (271–281)	0.185**
Apgar score 1st minute (median; IQ)		9 (9–9)	9 (9–9)	0.098**
Apgar score 5th minute (median; IQ)		10 (10–10)	10 (10–10)	0.054**
Birth weight (grams) (mean ± SD; 95%CI)		3637.84 ± 392.34 (3511.42–3764.26)	3605.88 ± 338.5 (3492.1–2790.66)	0.716***
Birth length (cm) (median; IQ)		53 (52–55)	53 (53–55)	0.480**

*Pearson's χ^2 test;

**Mann–Whitney U test;

***Student's t-test

Table 2. Laboratory characteristics of the study population

Parameter	Group	N	Mean ± SD (95%CI)	Minimum–maximum	Median (interquartile interval)	p
Erythrocytes ($\times 10^{12}$)	Control	34	4.68 ± 0.55 (4.5–4.86)	3.63–6.55	4.70 (4.38–4.95)	0.155*
	Experimental	37	4.51 ± 0.46 (4.36–4.66)	3.63–5.65	4.59 (4.22–4.71)	
Hemoglobin (g/l)	Control	34	164.79 ± 14.15 (159.43–170.16)	144–218	163.5 (149–173)	**0.025***
	Experimental	37	156.32 ± 15.18 (151.43–161.21)	122–193	158.0 (145–162)	
Hematocrit (l/l)	Control	34	0.49 ± 0.06 (0.47–0.51)	0.37–0.65	0.49 (0.46–0.52)	0.164*
	Experimental	37	0.47 ± 0.05 (0.43–0.49)	0.37–0.55	0.48 (0.45–0.5)	
Thrombocytes ($\times 10^{9}$)	Control	34	330.34 ± 6.843 (307.23–353.24)	226–493	321.5 (284–347)	0.613**
	Experimental	37	309.86 ± 91.48 (280.39–339.34)	127–478	321 (253–363)	
Leukocytes ($\times 10^{9}$)	Control	34	14.41 ± 4.8 (12.79–16.02)	5.66–26	14.0 (11.39–16.2)	0.102*
	Experimental	37	16.56 ± 6.02 (14.62–18.5)	6.01–29.32	15.99 (12.64–18.93)	
CRP (g/l)	Control	34	3 ± 1.86 (2.38–3.63)	0.4–7.6	2.5 (1.8–3.9)	**< 0.001****
	Experimental	37	80.11 ± 44.1 (65.9–94.32)	29.1–241	65.5 (52.8–96.7)	
PCT (ng/ml)	Control	34	0.43 ± 0.3 (0.33–0.53)	0.05–0.97	0.34 (0.15–0.7)	**< 0.001****
	Experimental	37	41.44 ± 76.34 (16.84–66.83)	0.24–408.65	15.83 (3.23–35.86)	
Hepcidin (ng/ml)	Control	34	55.17 ± 21.22 (48.04–2.3)	17.99–13.3	57.07 (36.80–1.3)	0.061*
	Experimental	37	59.72 ± 59.7 (40.48–8.96)	86.51–252.1	34.79 (26.67–0.6)	

CRP – C-reactive protein; PCT – procalcitonin;

*Student's t test;

**Mann-Whitney U test

Table 3. Hepcidin value in group of neonates with infection

Group	Parameter	n	Hepcidin (ng/ml)			p
			Mean ± SD (95%CI)	Minimum–Maximum	Median (interquartile interval)	
All infections	1st day	37	59.72 ± 59.7 (40.48–78.96)	86.51–252.1	34.79 (26.67–70.6)	0.428*
	6th day	37	44.72 ± 22.04 (37.62–51.82)	9.1–105	36.73 (30.39–56.61)	
Infections 1st day	early onset infections	24	40.07 ± 43.5 (22.67–57.47)	13.30–235	30.19 (25.9–39.88)	**0.012****
	late onset infections	13	95.99 ± 69.78 (58.06–133.92)	19.54–252.1	82.20 (39.7–128.1)	
Infections 6th day	early onset infections	24	44.05 ± 19.34 (36.32–51.79)	25.12–105	36.68 (31.23–50.3)	1.000**
	late onset infections	13	45.94 ± 27.16 (31.18–60.7)	9.1–86.68	44.88 (27.16–66.2)	
Early onset infections	1st day	24	40.07 ± 43.5 (22.67–57.47)	13.3–235	30.19 (25.9–39.88)	**0.040***
	6th day	24	44.05 ± 19.34 (36.32–51.79)	25.12–105	36.68 (31.23–50.3)	
Late onset infections	1st day	13	95.99 ± 69.78 (58.06–133.92)	19.54–252.1	82.2 (39.7–128.1)	**0.005***
	6th day	13	45.94 ± 27.16 (31.18–60.7)	9.1–86.68	44.88 (27.16–66.2)	

*Wilcoxon W test;

**Mann–Whitney U test

