



Increased Levels of Plasma Extracellular Heat-Shock Proteins 60 and 70 kDa Characterized Early-Onset Neonatal Sepsis

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Background: Extracellular heat-shock proteins (eHsp) are highly conserved molecules that play an important role in inflammatory diseases and have been quantified in plasma from patients with infectious diseases, including sepsis. There is a constant search for dependable biochemical markers that, in combination with conventional methods, could deliver a prompt and reliable diagnosis of early-onset neonatal sepsis.

Objective: We sought to assess the level of eHsp-27, eHsp-60, eHsp-70, and tumor necrosis factor-alpha (TNF α) in plasma of healthy neonates at term and infants with early-onset neonatal sepsis.

Methods: This study included 34 newborns that were classified as healthy neonates at term (blood samples from the umbilical cord, $n = 23$) or infants with early-onset neonatal sepsis (blood samples obtained from umbilical artery by standard sterile procedures before starting a systemic antibiotic intervention, $n = 11$). All blood samples were centrifuged, and the plasma recovered to determine eHsp-27, eHsp-60, eHsp-70, and TNF α levels by ELISA.

Results: Our results indicate that the level of eHsp-27 in healthy neonates at term was 0.045 ± 0.024 pg/ml. This value decreased 2.5-fold in infants with early-onset neonate sepsis (0.019 ± 0.006 pg/ml, $p = 0.004$). In contrast, the levels of eHsp-60 and eHsp-70 in healthy neonates at term were 13.69 ± 5.3 and 4.03 ± 2.6 pg/ml, respectively. These protein levels increased significantly 1.8- and 1.9-fold in the plasma of infants with early-onset neonatal sepsis ($p \leq 0.001$). The level of TNF α in healthy neonates at term was 2.94 ± 0.46 pg/ml, with a 3.0-fold increase in infants with early-onset neonatal sepsis (8.96 ± 0.72 pm/ml, $p \leq 0.001$). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of eHsp compared with that of C-reactive protein were 73.3, 60.0, 47.8, and 33.3%, respectively.

Conclusion: This study demonstrated a consistent increase of eHsp-60 and eHsp-70 in the plasma of infants diagnosed with early-onset neonatal sepsis. These proteins showed higher sensitivity and specificity than C-reactive protein and blood culture test.

Keywords: early-neonatal sepsis, extracellular heat-shock protein, neonatal intensive care unit, tumor necrosis factor alpha, neonatal infection

INTRODUCTION

Extracellular heat-shock proteins (eHsp) are highly conserved molecules that regulate cellular homeostasis (1, 2), proliferation, and differentiation of the professional immune system cells and are modulated by temperature (3–5). The eHsp have been classified in high molecular weight of 60, 70, 90, and 100 kDa and low molecular weight of 20 and 27 kDa (6, 7). When released into the extracellular space, eHsp function as cell-to-cell mediators (8, 9). eHsp-60 (HSPD1; heat shock protein family D member 1) and eHsp-70 (HSPA1A; heat shock protein family A member 1A) can stimulate pro-inflammatory cytokines (10, 11), whereas eHsp-27 (HSPB1; heat shock protein family B (small) member 1) has an important anti-inflammatory function (12–14). Their presence has also been shown and their levels quantified in serum and plasma of patients with severe trauma (15, 16), chronic obstructive pulmonary disease (17, 18), inflammatory processes induced by multiple sclerosis (19), and sepsis (20, 21). Therefore, eHsp have been used as sensible indicators of the physiological status during the onset and resolution of different human pathological conditions (19, 22, 23).

Neonatal sepsis is a common and serious disease that affects a large number of newborns around the world. Although its incidence is low (one to eight cases for every 1,000 live births) (24), the risk of morbidity and mortality is high, affecting 15–50% of reported cases (24, 25). In developed countries, the estimated prevalence is 2–8% (24). The Department of Neonatal Intensive Care of the National Institute of Perinatology “Isidro Espinosa de los Reyes” (INPerIER) in Mexico City has reported an incidence of 2.3% in the total number of births attended during a 5-year period (26).

The clinical diagnosis of early-onset neonatal sepsis poses challenges due to the subtlety of signs and symptoms, which are often concealed with other transient medical conditions such as hypothermia, delayed transition from fetal to neonatal life, tachypnea, and metabolic alterations (25). The clinical identification and diagnosis of neonatal sepsis is confirmed by blood culture (27, 28), and the assessment of acute phase reactants includes C-reactive protein (CRP) (29, 30), procalcitonin (30, 31), presepsin (32, 33), and inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF α) (34, 35). It has been shown that, when two or more tests are combined, the accuracy of a prompt diagnosis of early-onset neonatal sepsis increases (36, 37). At INPerIER, some of these acute phase reactants or biological markers have not provided the accuracy and sensitive to support clinical data in the diagnosis of early-onset neonatal sepsis. Interestingly, several reports have shown that eHsp are reliable and practical biomarkers to identify sepsis in children (21, 38).

In this study, we set out to quantify the eHsp and TNF α in plasma of healthy neonates at term and infants with early-neonatal sepsis.

MATERIALS AND METHODS

Ethics Statements

This study was reviewed and approved by the National Institute of Perinatology Ethics and Research Committees (registration number 212250-3210101). All patients were informed about the purpose of the study and a maternal informed consent obtained in all cases.

Study Design and Patients

From July 2018 to June 2019, a cross-sectional study was carried out in the Neonatal Intermediate Therapy Unit for the newborn. A total of 34 newborns were included. The neonates were divided into two groups: (1) healthy neonates at term (blood samples obtained at birth from the umbilical cord, $n = 23$) and (2) neonates with visible signs of early-onset neonatal sepsis (blood samples obtained from umbilical artery by standard sterile procedures before starting a systemic antibiotic intervention; $n = 11$).

Clinical Definitions and Inclusion Criteria

Healthy neonates consisted of neonates at term, gestational age ≥ 37 weeks, delivery without obstetric complications of labor and/or signs of maternal sepsis. Neonates with early-onset neonatal sepsis consisted of infants with visible signs and symptoms (feeding intolerance, lethargic or tachypnic, poor perfusion, seizures, respiratory distress, bradycardia, abdominal distension, or vomiting) normally associated with suspected sepsis as defined according to the guidelines for the management of newborns with suspected sepsis (39–41).

Some of the maternal patients presented the following conditions: preterm rupture of fetal membranes (pPROM), which was diagnosed by discharge of amniotic fluid through the vaginal canal or by a positive nitrazine test (42); clinical chorioamnionitis (CAM) was diagnosed by the presence of fever ($>38^{\circ}\text{C}$), accompanied by two or more of the following signs: tachycardia (heart rate >100 beats per min), uterine pain or tenderness, fetid or purulent amniotic fluid, leukocytosis $>15,000/\text{mm}^3$, CRP (>2 mg/dl), and fetal tachycardia (heart rate >160 beats per minute) (43–45).

Exclusion Criteria

Neonatal sepsis cases were excluded from the study when (1) the amount of blood collected for the

TABLE 1 | Demographic and clinical characteristics of maternal and neonatal patients.

Characteristics	Healthy neonates at term (n = 23)	Neonates with early-onset sepsis (n = 11)	p-value
Maternal conditions			
Age, year	28.3 ± 7.5	26.2 ± 7.1	0.423
Body mass index, kg/cm ²	25.7 ± 6.5	26.3 ± 8.2	0.927
Number of previous pregnancies alive			
0 n (%)	11 (48)	5 (46)	0.887
1 n (%)	4 (17)	2 (18)	0.851
2 n (%)	5 (22)	4 (36)	0.207
3 n (%)	1 (4)	0 (0)	0.214
4 n (%)	2 (9)	0 (0)	0.013
CAM, n (%)	0 (0)	4 (36)	0.013
pPROM, n (%)	0 (0)	3 (27)	0.001
CAM + pPROM, n (%)	0 (0)	1 (9)	0.013
PE, n (%)	0 (0)	3 (27)	0.001
Severe PE, n (%)	0 (0)	2 (18)	0.001
Fever >38°C, n (%)	0 (0)	0 (0)	
Histological inflammation			
Fetal membranes, n (%)	0 (0)	1 (9)	0.013
Umbilical, n (%)	0 (0)	0 (0)	1.0
Placental, n (%)	0 (0)	0 (0)	1.0
Neonatal conditions			
Gender			
Male, n (%)	10 (43)	5 (45)	0.886
Female, n (%)	13 (56)	6 (54)	
Gestational age (weeks)	38.6 ± 1.1	33.0 ± 3.3	0.001
Birth weight (g)	2,970.5 ± 441.0	1,380 ± 804.8	0.006
Irritability	0 (0)	0 (0)	
APGAR at 5 min <8, n (%)	0 (0)	9 (82)	0.001
Fever >38°C, n (%)	0 (0)	0 (0)	

CAM, Chorioamnionitis; pPROM, preterm-prelabor rupture of membranes; PE, pre-eclampsia.

quantification of eHsp and TNF α was insufficient (plasma < 1,200) and (2) antibiotic treatments started prior to blood collection.

Blood Sample Collection

Two milliliters of blood were obtained by trained medical staff. The blood samples were collected in K₂-EDTA vacutainer tubes (Becton-Dickinson, NJ, USA) and centrifuged at 329 g (Beckman, GS-6R Centrifuge) for 5 min. The plasma was recovered in Eppendorf tubes and stored at -80°C until quantification of the eHsp-27, eHsp-60, eHsp-70, and TNF α by enzyme-linked immunosorbent assay (ELISA).

Biochemical Assays

Commercial ELISA kits were used to quantify the levels of eHsp-27 (DYC-1580, R&D Systems, Minneapolis, MN, USA), eHsp-60 (DYC1800-2, R&D Systems), eHsp-70 (DYC1663-2, R&D System), and TNF α (DY210, R&D System). The plasma used for the quantified was not diluted. Standard curves were calculated from 31.3 to 2,000 ng/ml, 1.25 to 80 ng/ml, 312.5 to 20,000

pg/ml, and 15.0 to 960 pg/ml, respectively, according to the manufacturer's instructions and the protocol previously reported by our research group (46). The following sensitivity values for each protein were calculated 50, 0.70, 150.0, and 5.0 pg/ml, respectively. The CRP levels were determined a few hours after the blood was obtaining. CRP was measured by nephelometry using a MININEPH PLUS System (Birmingham, UK) and with commercial kit (ZK044.L.R, Birmingham, UK) according to the manufacturer's instructions. CRP assay has a detection range of 6 to 1,232 mg/L and Inter- and Intra-Assay Coefficients of Variability <4% both at low and high concentration. CRP was processed at INPerIER core facility.

Microbiological Analysis

Bacterial culture analysis and identification test for aerobic and anaerobic microorganisms were performed at the Department of Infectología e Inmunología at the INPerIER. The procedure was performed with the Bact/Alert 3D (Dirham, BioMerieux, NC, USA) as previously reported by Thorpe et al. (47).

TABLE 2 | Clinical diagnosis of early-onset neonatal sepsis with bacterial detections.

Neonatal blood sample	Sex	Gestational age (w)	Birth weight (g)	Bacteria detected	Clinical diagnosis and maternal condition
1	F	32	3,302	ND	CAM
2	F	33	1,170	<i>E. coli</i>	CAM
3	F	31	1,060	ND	7 days of pPROM
4	M	34	2,485	ND	8 days of pPROM, without clinical data of CAM
5	F	30	1,300	ND	Severe PE and RCIU
6	M	35	1,245	<i>S. epidermidis</i>	Without clinical pathological data
7	M	28	1,085	ND	Severe PE
8	M	37	2,700	<i>S. dysgalactiae</i>	PE
9	F	39	2,640	ND	Without clinical pathological data
10	F	30	1,380	ND	CAM
11	M	33	2,094	ND	2 days of pPROM

CAM, chorioamnionitis; pPROM, preterm-prelabor rupture of membranes; PE, pre-eclampsia; ND, not detected.

Statistical Analysis

Chi-square test was used to compare maternal and neonatal clinical data. eHsp and TNF α levels in healthy neonates at term and infants with early-onset neonatal sepsis were analyzed using one-way ANOVA and significant difference between groups were determined by the Tukey's test. All assays were independently replicated at least three times, and the data are presented as mean \pm SEM. Statistical analyses were carried out using SigmaStat software (version 3.0). A significant difference was accepted at $p \leq 0.05$.

RESULTS

Demographic Data of the Study Population

Table 1 shows the demographic and clinical characteristics of maternal and neonatal patients included in this study. In maternal patients, no significant difference between groups was detected in age ($p = 0.423$), body mass index ($p = 0.927$), and number of neonates delivered alive during the first three pregnancies, but a significant difference during the fourth pregnancy (9.0 vs. 0.0%, $p = 0.013$) was found.

Maternal patients who delivered infants with early-onset neonatal sepsis developed clinical CAM in 36.0% of cases, whereas pPROM occurred in 27.0%. Only 9.0% presented CAM and pPROM simultaneously. Furthermore, 27.0% of maternal patients showed clinical preeclampsia (PE) and 18.0% of them had severe PE (Table 1) and 8.3% of newborns had intrauterine growth restriction (Sample 5, Table 2).

In neonatal patients, we found significant differences between groups. Infants with early-onset neonatal sepsis showed 1.2-fold decrease in gestational age compared with healthy neonates at term (38.6 ± 1.1 weeks; $p \leq 0.001$); 2.15-fold decreased in body

weight at birth ($2,970.5 \pm 441.0$ vs. $1,380 \pm 804.8$ kg; $p = 0.006$), and 82% of infants with early-onset neonatal sepsis showed APGAR < 8 at 5 min. No gender-based difference was found (Table 1).

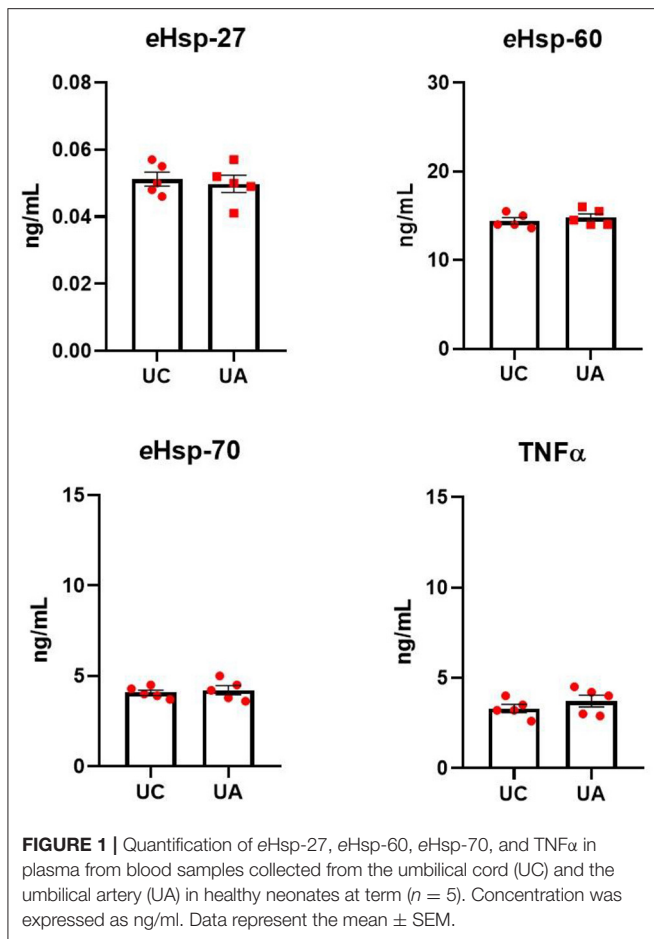
Microbiological Analysis

Table 2 shows the bacteria detected in blood culture, maternal diagnosis, and evidence of sepsis. Blood culture was positive in 27.3% of samples taken from infants with early-onset neonatal sepsis (three of 11 cases). The bacteria identified in these samples were *E. coli* (1 case, S2), *E. epidermidis* (1 case, S6), and *S. dysgalactiae* (1 case, S8). Blood cultures of samples taken from healthy neonates at term were negative (Table 2).

Extracellular Heat-Shock Proteins and Inflammatory Cytokine in Plasma

To assess whether blood from different sampling sites does not affect the quantification of eHsp and TNF α , blood samples from both the umbilical cord and the umbilical artery from five different healthy neonates at term were collected. All samples were assessed for eHsp and TNF α . No differences were found (Figure 1) between sampling sites for eHsp-27 (0.051 ± 0.004 vs. 0.04 ± 0.005 , $p = 0.6851$), eHsp-60 (14.4 ± 0.79 vs. 15.1 ± 1.02 , $p = 0.1958$), eHsp-70 (4.08 ± 0.31 vs. 4.22 ± 0.55 , $p = 0.6396$), and TNF α (3.3 ± 0.50 vs. 3.7 ± 0.72 , $p = 0.3207$).

Figure 2 shows the levels of eHsp-27, eHsp-60, eHsp-70, and TNF α in the plasma of healthy neonates at term and infants with early-onset neonatal sepsis. The levels of eHsp-27 decreased 2.2-fold in infants with early-onset neonatal sepsis compared with healthy neonates at term (0.045 ± 0.02 vs. 0.019 ± 0.006 pg/ml, $p = 0.008$; Figure 2). In contrast, the levels of eHsp-60, eHsp-70, and TNF α increased significantly in all infants with early-onset

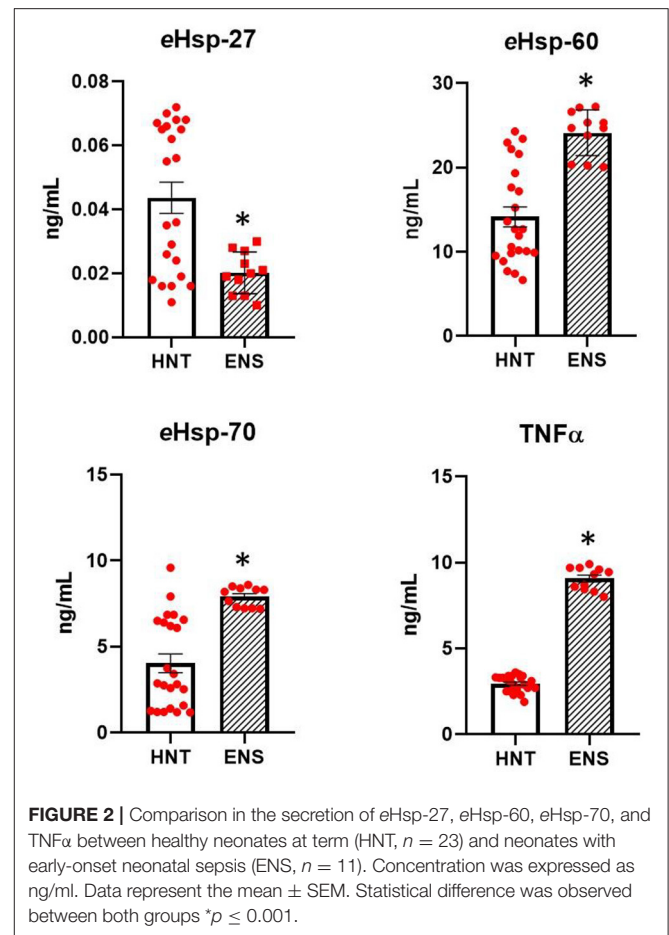


neonatal sepsis compared with healthy neonates at term 1.6-fold (14.15 ± 5.7 vs. 24.7 ± 3.0 pg/ml, $p \leq 0.001$), 2.0-fold (4.03 ± 2.6 vs. 7.9 ± 0.62 pg/ml, $p \leq 0.001$), and 3.0-fold (2.94 ± 0.46 vs. 8.96 ± 0.72 pg/ml, $p \leq 0.001$), respectively (Figure 2).

Table 3 shows the relevance of assessing eHsp levels as part of routine clinical laboratory tests for early-onset neonatal sepsis. The sensitivity and specificity of eHsp compared with that in CRP test of 11 samples from infants with early-onset neonatal sepsis were 73.3 and 63.2%, respectively. In addition, positive predictive value (PPV) and negative predictive value (NPV) values were 47.8 and 36.4%, respectively. Finally, the sensitivity and specificity of eHsp compared with that of blood culture were 73.3 and 60.0%, whereas PPV and NPV values were 47.8 and 33.3%, respectively.

DISCUSSION

eHsp have traditionally been considered as intracellular molecules involved in cellular protections (48, 49). However, in recent years, they have been reported as molecules related to different components of the immune response (5, 50). It has been shown that eHsp-60 and eHsp-70 proteins are associated with the inflammatory response (21, 38, 51) and are increased in the plasma of children with septic shock (52, 53). Notwithstanding,



the role of eHsp in the plasma of infants with clinical evidence of early-onset neonatal sepsis is poorly unknown.

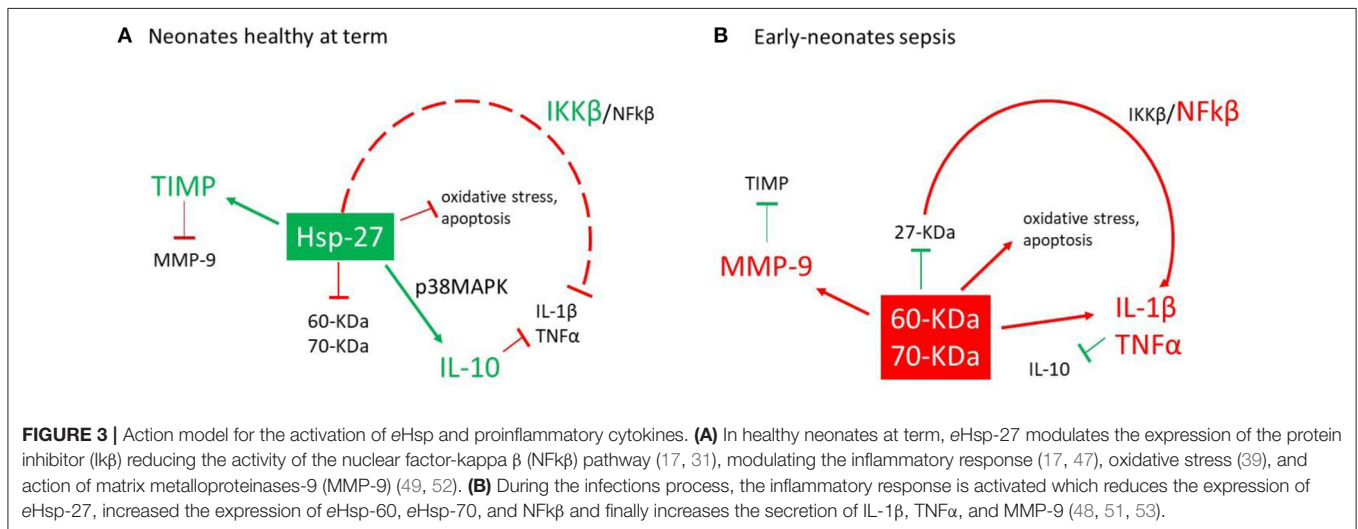
The main findings of this study are as follows: (1) there is an upregulation of eHsp-60 and eHsp-70 in plasma of patients with early-onset neonatal sepsis, in parallel with an increment of TNFα level that has been previously reported as an early-onset neonatal sepsis biomarker; (2) the downregulation of eHsp-27 in plasma of patients with early-onset neonatal sepsis indicates an inverse relationship with the levels of eHsp-60 and eHsp-70; (3) the high levels of eHsp-60 and eHsp-70 in plasma were consistently detected in neonates with visible signs and symptoms of sepsis even in cases with an undetectable level of CRP and bacteria in blood cultures (Table 3); (4) eHsp-60 and eHsp-70 tests showed higher sensitivity and specificity compared with CRP and blood culture tests.

Studies by Wheeler et al. (54) and He et al. (55) in children with severe sepsis have shown a significant increase in the levels of eHsp-70, TNFα, IL-1β, IL-6, IL-8, IL-13, IL-27, macrophage inflammatory protein-1α, and matrix metalloproteinase-8 (MMP-8) in blood and plasma (54, 56). Studying the systemic inflammatory response syndrome in children, Fitrolaki et al. (51) demonstrated increased levels Hsp-72, Hsp-90, IL-8, IL-6, and TNFα in patients diagnosed with sepsis and considered these as biomarkers associated with fatal

TABLE 3 | Comparison between clinical laboratory and biochemical test in early-onset neonatal sepsis.

Neonatal blood sample	Clinical laboratory test		Biochemical test		
	CRP (ng/ml)	BC	eHsp-60 (ng/ml)	eHsp-70 (ng/ml)	TNF α (ng/ml)
1	ND	-	20.2	7.3	8.3
2	57	+	20.3	7.2	7.8
3	ND	-	25.3	7.7	8.0
4	ND	-	27.1	8.2	8.45
5	6	-	26.6	7.2	9.7
6	ND	-	24.7	8.4	9.6
7	8	+	24.7	8.6	9.4
8	56	+	25.3	8.5	9.3
9	ND	-	27.2	7.2	9.7
10	ND	-	23.8	6.9	8.6
11	ND	-	17.9	8.3	9.9

CRP, C-reactive protein; BC, blood culture; eHsp, extracellular heat-shock protein; TNF α , tumor necrosis factor-alpha; ND, not detected.



outcome in these patients (51, 57). In this investigation, we showed that infants with early-onset neonatal sepsis presented increased levels of eHsp-60 and eHsp-70 that are correlated with an increment in TNF α (Table 3), supporting previous evidence reported by our group and replicating previous reports (46, 51).

A positive blood culture is considered the gold standard for the diagnosis and identification of many clinical infections (27, 58). However, it has a low sensitivity and specificity when used to diagnose neonatal sepsis (59). Recently, it has been shown that neonatal sepsis produced by Group B *Streptococcus*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Neisseria meningitidis* is associated with increased levels of eHsp-70, eHsp-90, and TNF α in blood and plasma (51, 55). Our findings provide new evidence and support previous results showing that infants with early-onset neonatal sepsis with positive blood culture for *E. coli*, *S. epidermidis*, and *S. dysgalactiae* also display marked increase levels of eHsp-60, eHsp-70, and TNF α in plasma.

Using experimental models of infection Campisi et al. (60) showed that *E. coli* induces a dose-dependent expression of eHsp-72, which is mediated by Toll-like receptor (TLR) by recognizing different structural components of bacteria (60–62). The secretion of eHsp-72 has been associated with increased levels of other biomarkers, such as nitric oxide, TNF α , IL-1 β , and IL-6 (60).

Figure 3 shows a proposed model for the differential actions of the anti-inflammatory (eHsp-27) and proinflammatory (eHsp-60 and eHsp-70) response (5, 9). In healthy patients, eHsp-27 is the mainly expressed eHsp, and it is related to protein inhibitory β , a negative regulator of the classical nuclear transcription factor-kappa β (NF κ B) pathway (5, 50), which reduce the production of molecules associated with oxidative stress (63), apoptosis (64, 65), IL-1 β , TNF α (12, 21), and collagenolytic action of MMP-9 (66, 67) (Figure 3A). During infection, the inflammatory response is activated, reducing the expression of eHsp-27, increasing the activity of NF κ B and enhancing the levels of eHsp-60 and eHsp-70. This chain of events upregulates IL-1 β , TNF α , and MMP-9

(68–70). In sepsis, this activation is mediated by TLR-4 (61, 62) (**Figure 3B**). Interestingly, our data demonstrate a significant imbalance between a decrease of anti-inflammatory eHsp-27 and an increase of pro-inflammatory eHsp-60 and eHsp-70 in infants with early-onset neonatal sepsis.

Clinical guidelines in cases of possible early-onset neonatal sepsis require both CRP assessment and positive blood culture. CRP is produced by the liver and is increased in response to early-onset neonatal sepsis (71). However, several studies have often show inconsistency in the assessment of CRP, possibly due to the gestational age and volume required for testing (30). Nevertheless, the sensibility of these tests increases when additional markers are assessed (72).

The most remarkable finding of our study is that the levels of eHsp-60 and eHsp-70 were consistently increased in all infants with early-onset neonatal sepsis (**Table 3**), whereas blood culture and CRP tests when combined were able to detect 27.27% of the positive cases. The sensitivity, specificity, PPV, and NPV for eHsp with regard to the CRP test was 73.3, 63.2, 47.8, and 36.4%, respectively.

CONCLUSION

Our study highlights that eHsp-60 and eHsp-70 measured in the plasma of infants could be used as a reliable biomarker of early-onset neonatal sepsis, because the levels of these proteins are consistently elevated and show high sensitivity, specificity, PPV, and NPV. These results provide a strong indication that the assessment of these proteins, together with conventional tests such as CRP and blood culture, can provide a highly sensitive and accurate diagnostic tool to confirm diagnosis of early-onset neonatal sepsis.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética en Investigación. Instituto Nacional de Perinatología. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC-E, GZ-G, and RM-C obtained blood samples from neonates with and without evidence of early-neonatal sepsis. AC-E and GZ-G provided clinical data on neonates. AC-E, GZ-G, JP-L, and HF-H performed the quantification of eHsp. JP-L performed the quantification of TNF α . PS-T, PG-M, and HF-H conceived and designed the study. AC-E, GZ-G, ND, PS-T, OD-R, and HF-H analyzed the data and interpreted the results. PS-T, PG-M, OD-R, and HF-H wrote the manuscript. All other authors gave approval for the final version of manuscript.

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