

Inflammatory Cytokines in Neonatal Hypoxic Ischemic Encephalopathy and their Correlation with Brain Marker S100 Protein: A Case Control Study in Saudi Arabia

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Abstract

Background: Neonatal HIE is still one of the major causes of morbidity and mortality in the perinatal period. The effective management and early intervention for these infants depends on early identification of the condition. This study aimed to identifying biomarkers of inflammation in HIE in Saudi Arabia and their association with serum level of S100 protein.

Patients and methods: 100 cases of full-term infants were classified into two groups. The first was comprised of normal healthy infants, while the second was composed of infants with hypoxic-ischemic encephalopathy. Blood gases, electrolytes, liver and renal functions were evaluated, along with some inflammatory cytokines IL1, IL6 and TNF α . Correlation between cytokine levels and S100 protein level was adjusted with time.

Results: The hypoxic infants recorded decreased pH level and disturbed blood electrolytes in comparison with healthy control. Also, significant increase in serum levels of IL1, IL6 and TNF α was detected in hypoxic group. Positive correlation between serum cytokine levels and S100 protein was evident.

Conclusion: The data revealed some of the vital inflammatory cytokines that were most affected due to a decreased blood pH in newborn babies. Such information is useful for predicting treatment to overcome the severe side-effects arising in cases of hypoxic-ischemic encephalopathy. The study predicted early diagnosis and treatment as important for the avoidance of serious complications.

Keywords: Inflammatory cytokines; S100 protein; Hypoxic ischemic encephalopathy; Acidosis

Introduction

Perinatal Hypoxic-Ischemic Encephalopathy (HIE) is a major cause of neonatal death and long-term disability. Approximately 15% to 25% of affected newborns die in the postnatal period and 25% develop severe and permanent neuropsychological sequelae [1], including cerebral palsy, seizures, visual impairment, mental retardation, learning impairment and epilepsy [2].

It is characterized by clinical and laboratory evidence of acute or sub-acute brain injury due to asphyxia leading to hypoxia and acidosis. HIE injury induces release of cytokines and chemokines, which amplify inflammatory cascades and recruit neutrophils and monocytes to sites of injury. Serum inflammatory proteins are readily measurable and may be useful biomarkers of phases of injury. Combinations of cytokines are beginning to be used as early, discriminating predictors of severe traumatic brain injury and multiorgan system failure in adults and children [3,4].

In the case of Saudi Arabia, Itoo et al. [5] concluded that HIE continues to be an important cause of morbidity and mortality in the western part of the country. Moreover, Al-Shehri and Eid [5,6] recorded a total of 57 full-term infants with clinical evidence of HIE at birth over a period of three years in Abha: a high altitude region of Saudi Arabia.

Cytokines are important inflammatory mediators, and cerebral ischemic injury can trigger a cascade of cytokine induction that acts to orchestrate an in situ inflammatory reaction [7] and maintains brain tissue homeostasis [8]. In general, the roles of cytokines are pleiotropic, and whether the overall effects are pro- or anti-inflammatory in the context of ischemic insults remains controversial even in adult models, for which there are more data than for HIE. The most studied cytokines related to the inflammatory responses to stroke are IL-1, IL-6, IL-10, tumor necrosis factor- α (TNF- α), and transforming growth factor- β (TGF- β) [9].

Despite advances in perinatal care, the outcome of newborns with HIE is poor and the issue still remains challenging in neonatology. The use of an easily approachable and practical biomarker not only could identify neonates with severe brain damage and subsequent adverse

outcome, but could also target the group of infants that would benefit from a neuroprotective intervention [10].

Therefore, this study aimed to evaluate the mechanisms underlying cerebral ischemic injury and the following immune response through detection of the levels of the inflammatory cytokines TNF, IL-1 and IL-6 in cases of HIE. Also, to correlate their levels with the serum level of neurotrophic factor S100 protein.

Patients and Methods

Patients

A case-control study was performed which included all full-term neonates born at Al-Hada Armed Forces hospital (a tertiary care hospital belonging to Ministry of Defence) with a diagnosis of perinatal asphyxia during the first 48 hours of life to investigate early onset neonatal sepsis, during the period between January 2011 to June 2012.

A diagnosis of perinatal asphyxia was made in the presence of at least three of the following criteria, with criteria 3 and 4 being obligatory [11]: 1.signs of fetal suffering revealed by intrapartum monitoring (IPM) such as; persistent decelerations, sustained fetal bradycardia or "silent IPM"; 2. Apgar score less than or equal to 4 during the first minute and less than or equal to 6 at the fifth; 3.umbilical pH value less than 7.20; 4. Umbilical cord arterial lactate greater than 3.0 mmol/L; 5.need for positive pressure ventilation for at least two minutes in order to start respiration. The diagnosis of hypoxic-ischemic encephalopathy was established by the presence of perinatal asphyxia associated with neurological manifestations resulting from hypoxemia and ischemia [12]. A total number of 8 males and 37 females diagnosed as HIE were recruited in the study.

The control group was made up of non-asphyxiated neonates with Apgar scores >9 at the first and fifth minutes of life including 15 males and 40 females full-term infants born or referred to the hospital.

Consent was obtained from the parents before study enrollment, using a consent form approved by the by the Regional Research and Ethics Committee at Taif University, Saudi Arabia.

All neonates were born after more than 36 weeks' gestation. Exclusion criteria included maternal chorioamnionitis, sepsis at birth and either birth weight or head circumference <10th percentile for gestational age. One milliliter of blood for serum cytokines was collected via umbilical catheter in the early morning of age 24 hours and 72 hours after birth, in conjunction with routine venous blood sampling for biochemical and metabolic screening. All blood samples were collected in Lithium-Heparin tubes (Sarstedt, Nürnberg, Germany) and were stored at room temperature for no longer than 6 hours before processing.

Arterial blood gases, PH and serum electrolytes (Na⁺, K⁺, Cl⁻, Ca⁺², Ph⁺ and Mg⁺²) were estimated via delivering to Biosentia Lab, Konrad Adenauer Str. 17; Ingelheim; Germany. Liver and kidney functions (e.g. Serum Glutamic Oxaloacetic Transaminase (SGOT); Alanine Aminotransferase (SGPT); total protein, albumin, alkaline phosphatase, urea and creatinine) were analysed with Cobas 6000 (Roche Diagnostics, Basel, Switzerland).

Serum blood samples for Evaluation of a neurotrophic factor, S100 binding protein (S100 P). In addition, the inflammatory cytokines e.g.

IL-1, IL-6 and TNF- α , were analyzed in blood plasma using a commercial enzyme-linked immunoassay according to the manufacturers's instructions (Coulter/Immunotech, Krefeld, Germany). All samples were analyzed in duplicate. The optical density was determined photometrically at 405 nm using the ELISA reader Spectra Classic (SLT Lab. instruments GmbH, Crailsheim, Germany) and plotted against a standard curve. The intra-assay coefficient of variation was <4% for all tests. Cytokine levels were within the assay's detection limit in all stimulated samples.

Statistical analysis

The statistical tests were performed with the SPSS (version 16) software. For analysis, all values are given as the means \pm SD. Significant differences among values were statistically calculated by two-way Multi-varieties Analysis of Variance (MANOVA), and then determined by Duncan's test. Differences with $p < 0.05$ were considered statistically significant. Relationships between the inflammatory cytokines and S100 protein were done by pearson correlation (r) analysis and the correlation coefficients (r) were tested. $P < 0.05$ was considered statistically significant.

Results

100 cases comprising 55 normal patients (15 males and 40 females) and 45 hypoxic infants (8 males and 37 females) were enrolled in the present work. Characteristics of the neonates that enrolled in the study and their mothers were presented in (Table 1).

Mothers	HIE	Control
Mode of delivery		
Caesarean (emergency)%	65%	40%
Caesarean (elective)%	25%	10%
Vaginal %	10%	50%
Age, Y (mean \pm SD)	28 \pm 9	24 \pm 3
Gravida (mean \pm SD)	3 \pm 1	2 \pm 1
Gestational hypertension %	45%	-----
Gestational Diabetes Mellitus %	25%	-----
Placental abruption %	30%	-----
Neonates		
Weight (Kg) mean \pm SD	2.76 \pm 0.54	3.41 \pm 0.36
Apgar score (mean \pm SD)		
At 1 min	2.95 \pm 1.13	8.17 \pm 1.1
At 5 min	5.47 \pm 1.07	9.28 \pm 0.65
Sex		
Male (N)	8	15
Female (N)	37	40

Table 1: Characteristics of the mothers and infants recruited in the study served as control or HIE neonates.

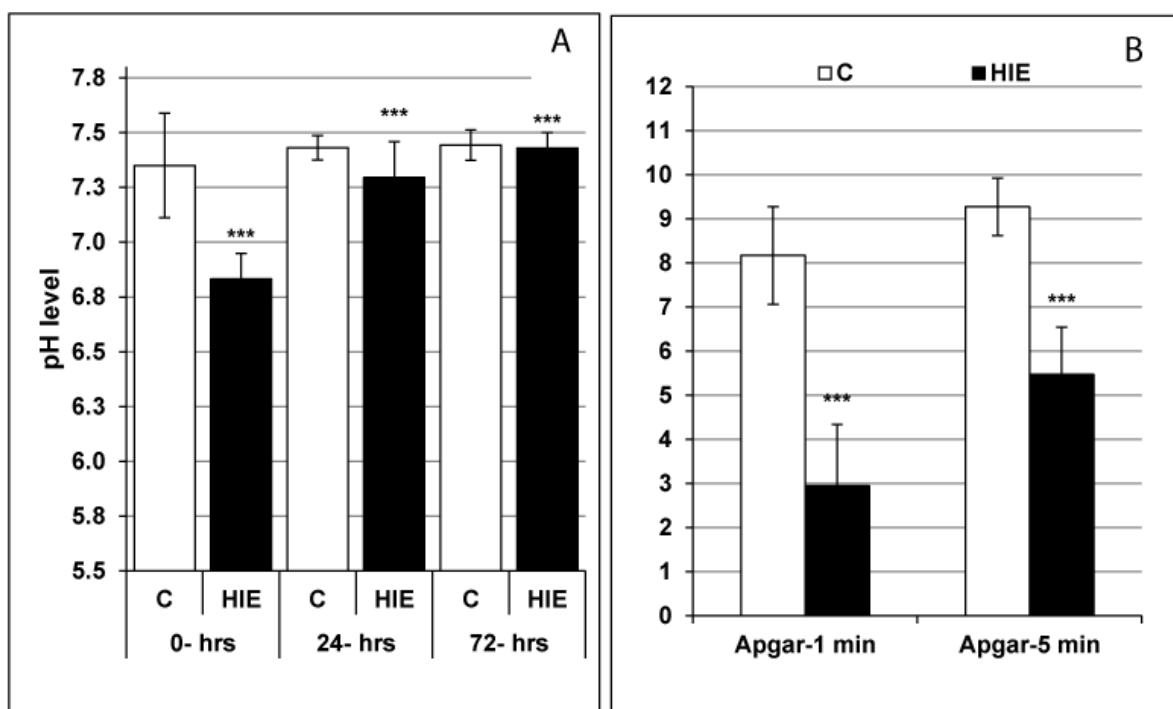


Figure 1: Changes in pH, Apgar at 1 min and 5 min; in healthy control newborns (C) and hypoxic patients (HIE) along 24 and 72 hours post labouring. Values within same parameter and at same time-interval not sharing common superscripts denote significant differences (***) $p < 0.001$.

	Control		HIE	
	At 24 hrs	At 72 hrs	At 24 hrs	At 72 hrs
PCO ₂ (mmHg)	32.14 ± 5.93	27.07 ± 5.65	33.89 ± 7.13 ^{NS}	39.84 ± 9.35 ^{***}
PO ₂ (mmHg)	46.10 ± 10.22	60.31 ± 14.54	54.32 ± 29.01 ^{NS}	44.74 ± 7.15 ^{***}
CO ₂ (mEq/L)	17.93 ± 1.94	18.31 ± 2.16	16.89 ± 4.15 ^{NS}	21.37 ± 3.90 ^{***}
HCO ₃ (mEq/L)	21.52 ± 2.50	19.45 ± 2.61	19.84 ± 5.48 ^{NS}	23.37 ± 3.11 ^{***}
Sodium (mEq/L)	139.90 ± 2.55	138.45 ± 2.57	141.58 ± 8.40 ^{NS}	140.16 ± 5.97 ^{NS}
Potassium (mEq/L)	4.98 ± 0.41	4.91 ± 0.61	3.82 ± 0.58 ^{***}	4.32 ± 1.19 ^{***}
Chloride (mEq/L)	108.93 ± 2.67	106.48 ± 2.87	104.32 ± 4.68 ^{***}	103.42 ± 6.83 ^{***}
Calcium (millimol/L)	2.16 ± 0.19	2.14 ± 0.27	2.05 ± 0.23 ^{NS}	2.05 ± 0.23 ^{NS}
Phosphorus (millimol/L)	2.07 ± 0.25	2.36 ± 0.23	1.68 ± 0.33 ^{***}	1.99 ± 0.54 ^{***}
Magnesium (millimol/L)	0.86 ± 0.19	0.84 ± 0.08	0.80 ± 0.15 ^{NS}	0.84 ± 0.11 ^{NS}

***Significant values $P < 0.001$, NS Non-significant values $P > 0.05$.

Table 2: Mean ± SD of arterial blood PH, blood gases and electrolytes in both control and HIE neonates that included in the study at 24 and 72 hours after labour.

The average body weight (B. wt.) was significantly decreased in hypoxic cases (2.76 ± 0.54 Kg with $p < 0.001$) when compared with control (3.41 ± 0.36 Kg). The healthy control newborns had blood pH

levels at birth of 7.35 ± 0.24 , which was higher than that of the hypoxic patients (6.83 ± 0.12) ($p < 0.001$). It was further observed that the pH level of the hypoxic group was elevated between 24 hrs and 72 hrs of

birth, but still remained significantly lower than that of the healthy group (Figure 1A). Also, Apgar scores of the hypoxic patients ($2.95 \pm 0.1.39$ and 5.47 ± 1.07 at 1 and 5 minutes, respectively) were less than those of healthy control newborns (8.17 ± 1.10 ; 9.28 ± 0.65 at 1 and 5 minutes, respectively) (Figure 1B).

Concerning blood electrolytes, the present data revealed that sodium levels were not significantly increased but the level of

potassium showed a very highly significant decrease after both 24 hrs and 72 hrs, which may be due to acidosis correction. Moreover, the concentration of phosphorus recorded a highly significant decrease and the level of magnesium showed a non-significant decrease). The HIE patients also had a highly significant increase in blood bicarbonate levels 72 hrs after birth (23.37 ± 3.11) which can be explained by proper ventilator management (Table 2).

	Control		HIE	
	At 24 hrs	At 72 hrs	At 24 hrs	At 72 hrs
Urea (millimol/L)	2.91 ± 0.70	2.29 ± 1.60	3.21 ± 0.79 NS	3.26 ± 1.82 NS
Creatinine (millimol/L)	59.66 ± 8.45	48.86 ± 7.92	69.79 ± 10.44 ***	47.63 ± 8.99 NS
Alkaline Phos. (IU/L)	188.48 ± 41.61	200.31 ± 32.45	161.37 ± 12.64 ***	163.37 ± 15.65 ***
SGPT (U/L)	17.90 ± 3.85	18.38 ± 2.96	28.11 ± 6.24 ***	45.05 ± 9.89 ***
SGOT (U/L)	60.17 ± 17.74	56.59 ± 9.21	77.26 ± 6.81 ***	58.89 ± 12.69 NS
Total protein (g/L)	55.69 ± 5.27	56.17 ± 6.66	44.53 ± 5.78 ***	47.79 ± 7.49 ***
Albumin (g/L)	35.03 ± 2.58	34.52 ± 3.42	31.42 ± 4.85 ***	30.00 ± 4.68 ***

***Significant values $P < 0.001$, NS non-significant values $P > 0.05$

Table 3: Biological parameters of non CNS organ involvement in all studied cases at 24 and 72 hours.

	Control			HIE		
	0- hrs	24- hrs	72- hrs	0-hrs	24- hrs	72- hrs
S100 protein	1.55 ± 0.42	1.971 ± 1.038	1.26 ± 0.56	$2.45 \pm 0.42^*$	$4.11 \pm 1.26^*$	$2.7 \pm 0.239^*$
IL-1	5.38 ± 1.35	6.345 ± 2.3	5.45 ± 1.57	$21.26 \pm 3.93^*$	$21.526 \pm 5.35^*$	$11.47 \pm 4.48^*$
IL-6	4.86 ± 1.6	7.28 ± 3.4	5.6 ± 2.69	$41.8 \pm 6.67a$	$24.1 \pm 3.78 a$	$40.68 \pm 6.72 a$
TNF α	10.9 ± 2.2	13.43 ± 3.59	19.51 ± 4.64	$26.63 \pm 2.75^*$	$24.73 \pm 4.97^*$	$27.96 \pm 5.27^*$

*Significant with control $p < 0.05$, a high significant with control $P < 0.001$, NS non-significant with control

Table 4: Mean \pm SD of inflammatory cytokines at birth, 24 and 72 hours after delivery in both control and HIE neonates.

	S100 protein	
	R [*]	P value
IL1	0.0018	0.03
IL6	0.0019	0.005
TNF- α	0.0014	0.01

*Correlation coefficients express change in levels of PH and S100 protein per unit increase in cytokine adjusted with time.

Table 5: Correlation coefficient of neonatal cytokine levels with serum levels of S100 protein.

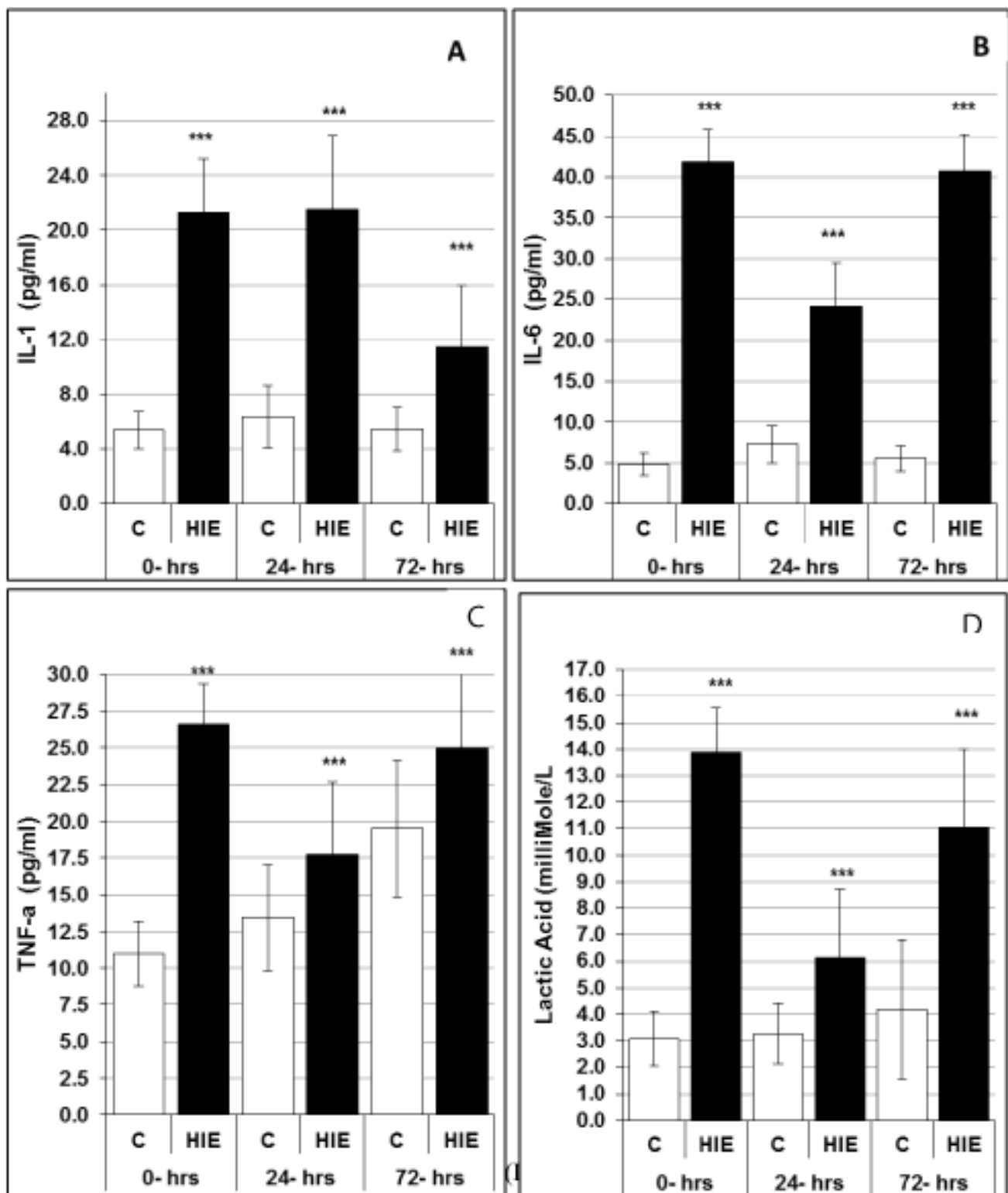


Figure 2: Changes in Inflammatory cytokines (IL-1; IL-6; TNF-a; pg/ml) and Lactic acid (mmol/L) in healthy control newborns (C) and hypoxic patients (HIE) along 0, 24 and 72 hours post labouring. Values within same parameter and at same time-interval not sharing common superscripts denote significant differences (***) $p < 0.001$.

Data of non CNS organ involvement including liver and kidney involvement revealed non-significant increase in urea and creatinine levels except that, at 24 hrs after birth, the creatinine level recorded an increase to 69.79 ± 10.44 millimol/L with a significance level of <0.001 . Moreover, the present data of liver functions showed a highly significant increase in the level of SGPT at 24 and 72 hrs after birth (28.11 ± 6.24 and 45.05 ± 9.89 U/L, respectively). Meanwhile, SGOT recorded a very highly significant ($P < 0.001$) increase only after 24 hrs (77.26 ± 6.81 U/L) and a non-significant increase after 72 hrs (58.89 ± 12.69 U/L) in comparison with those of healthy control infants. On the other hand, total protein and albumin levels recorded a very highly significant ($P < 1.001$) decrease 24 and 72 hrs after birth (Table 3).

Also, serum cytokines recorded significant increase in HIE group when compared with control group at different times after labour (Table 4). Significant increase in mean \pm SD of TNF- α (Figure 2C) and IL1 (Figure 2A) were also recorded at birth, 24 and 72 hours in comparison to the control neonates at the corresponding age. However, IL-6 showed high significant increased levels at birth, 24 and 72 hours (Figure 2B). All of the previous data that associated with low PH was also accompanied with high significant increase in lactic acid level in comparison to control group at birth and also after 24 and 72 hours after birth (Figure 2D).

Correlation coefficients analysis between the cytokine levels and S100 P revealed a significant positive correlation between the levels of S100BP and IL1, IL6 and TNF α (Table 5). Increase in the level of the inflammatory cytokines was associated with increase in the serum level of S100 protein.

Discussion

Despite advances in perinatal care, the outcome of newborns with HIE is poor and the issue still remains challenging in neonatology. Accumulating data have linked post-ischemic inflammation to the exacerbation of brain damage. The present study aimed to evaluate some biochemical and inflammatory markers in asphyxiated newborns in Saudi Arabia and the possible association with the level of neurotrophic factor S100 protein [10].

The collected data revealed that the pH of 18 new born hypoxic patients was significantly lower than those of healthy controls. As regard serum electrolytes, significant increase in potassium, phosphorus and chloride after 24 and 72 hours after birth were recorded in our study. Similar results were recorded by Douglas-Escobar and Weiss [13], who identified many potential biomarkers in neonates with HIE. Their biomarkers were obtained from CSF, serum, and urine.

As kidneys are very sensitive to oxygen deprivation, renal insufficiency may occur within 24 hours of a hypoxic ischaemic episode, which if prolonged, may even lead to irreversible cortical necrosis [14]. Early recognition of renal failure is important in babies with HIE to facilitate appropriate fluid and electrolyte management as a stable biochemical milieu is vital. Regarding hepatic enzymes, they are used as diagnostic tool to detect the severity of perinatal asphyxia and thus early treatment can be provided on the basis of liver function tests particularly whose birth details are not well recorded [15].

Evaluation of serum cytokines in HIE neonates in the present study revealed significant increase in serum levels of IL1, IL6 and TNF α in comparison to healthy control newborns at birth, 24 hours and 72

hours after birth. Also, serum level of S100 protein was significantly increased in HIE neonates when compared with control in all times.

Cerebral hypoxia-ischemia enhances rapid expression of brain inflammatory cytokines (IL-6, IL-1) [16] and leads to an inflammatory cell response to injury that includes neutrophils, lymphocytes, and microglia [17,18]. There are also elevated concentrations of the cytokines, tumour necrosis factor (TNF α), and the interleukins 1 β (IL-1 β) and IL-6 in the amniotic fluid and umbilical cord plasma of foetuses and prematurely born infants who sustain Periventricular Leukomalacia (PVL) [19,20]. Taken together, Yoon et al. [20] concluded that the amniotic fluid IL-6 is a sensitive test for the prospective diagnosis of acute histologic chorioamnionitis and the identification of neonates at risk of significant morbidity and mortality.

The main cause of perinatal asphyxia is the interruption in placental blood flow leading to brain cell ischemia-anoxia triggering anaerobic glycolysis. This in turn results in high consumption of ATP reserves, accumulation of lactic acid and failure of trans-cellular ion pumps with subsequent accumulation of Ca⁺², cytotoxic edema and release of neurotransmitters [22,23].

One of the good predictors of HIE outcomes such as death and long-term neurodevelopmental handicaps, is interleukin-6 (IL-6), which is an inflammatory cytokine produced by T-cells and macrophages [24]. The present study recorded higher levels of IL-6 in the hypoxic patients at birth and 72 hrs after birth. These findings agree with those of Chiesa et al. [24] who consider IL-6 as being a good predictors of HIE outcomes. Inflammatory cytokines may have a direct toxic effect via increased production of iNOS, cyclooxygenase and free radical release [25,26]. Hagberg et al. [16] detected high expression levels of IL-1 and TNF- α mRNA in the area of infarction within 1-4 h of HIE.

Umbilical cord IL-1b was also proposed as a potential biomarker of brain damage, as its levels were significantly higher in neonates with HIE and predictive of severe HIE and abnormal outcome at 6-12 months of age [27]. Regarding TNF- α , results among studies were very heterogeneous. A recent clinical study showed that increased levels of cytokines were correlated with seizures secondary to HIE; more specifically, most cytokines increased within the first 24 hours and subsequently decreased after 72 hours [28,29].

Ameboid microglia in the developing brain respond vigorously to hypoxia and accumulate in injured tissue [30], producing excess amounts of inflammatory cytokines (TNF- α , IL-1 β , etc) along with glu-tamate, Nitric Oxide (NO) and ROS, which collectively cause oligodendrocyte death, axonal degeneration and disruption of the immature blood brain barrier [30,31]. Both astrocytes and microglia are activated within minutes after injury by pro-inflammatory mediators, cytokines, and ROS that are secreted by injured neurons and glial cells [32]. The activation of astrocytes has both detrimental and beneficial roles in brain ischemia. Astrocyte support of neurons after a stroke can be achieved by several mechanisms, including the release of glutathione and Superoxide Dismutase (SOD) [33], enhanced extra-synaptic glutamate uptake, and the maintenance of ion gradients, such as that for potassium [34]. However, activated astrocytes can also produce pro-inflammatory cytokines, including IL-6, TNF- α , IL-1 α , and β and interferon γ [35,36]. Rapid increases in the levels of these cytokines exacerbate an ischemic injury by directly inducing the apoptosis of neuronal cells [37], increasing toxic NO levels and inhibiting neurogenesis. Apart from cytokines, reactive

astrocytes also secrete chemokines after ischemia, which results in the attraction of immune cells to the ischemic site and worsening of the brain injury [38].

The highly significant elevation of a neurotrophic factor, S100 protein (S100 P) in this study parallels the work of others, such as Gazzolo [39] and Qian et al. [40]. They noticed a significantly high concentration of S100 P after birth in HIE patients compared to controls in both urine and cord blood samples, and linked these findings to HIE. In Qian et al. [40] study, concentrations of S100 P greater than 2.02 µg/L had a sensitivity of 86.7% and a specificity of 88% for predicting the development of moderate or severe HIE. Moreover, urinary S100 P concentrations for 132 infants were higher in cases of perinatal asphyxia, and urine S100B above 1 mcg/L predicted neonatal death with a sensitivity and specificity of 100%. Also, the urinary S100 P concentrations were not affected by renal failure [41].

Disorders of the Central Nervous System (CNS) can be assessed with the help of biochemical markers. Especially in recent years, there has been an increased interest in the clinical use of brain markers such as S100 proteins. S100 protein is a calcium-binding peptide produced mainly by astrocytes that exerts paracrine and autocrine effects on neurons and glia [42]. An increased level of S100B is associated with pathological injury or clinical severity in a variety of disorders affecting the CNS. Elevation in serum or CSF S100B concentrations is associated with a variety of disorders affecting the CNS. Although in many instances its release may be an effect of the condition rather than the cause, it is nonetheless strongly implicated that S100B can be considered a strong candidate as a marker of CNS injury [43]. Serum and CSF concentrations of S100B could discriminate patients with good and bad outcome, but CSF measurements do not provide a higher accuracy than serum samples. Thus, when favouring S100B analysis, sampling serum values is sufficient for outcome prognosis and the detection of secondary complications [44].

CSF and serum concentrations of S100B were reported in adults to be diagnostic of cerebral damage (i.e. traumatic brain injury, stroke, subarachnoid hemorrhage) and reflective of the clinical course and severity of the disease [45]. In healthy children, S100B is measurable within the first 3 years of life and was moderately inversely correlated with age [46]. Serum and CSF S100 correlated with brain damage in children with traumatic brain injury and in preterm neonates with Intraventricular Hemorrhage (IVH) [47]. Women in preterm labour, with intact membranes and intramniotic infection have also been reported to have significantly higher S100B levels in amniotic fluid [39]. Increased serum S100 levels were reported to be higher in asphyxiated neonates compared to control groups and to be progressively decreasing from day 1 to day 4 or 7 [48,49] Gazzolo in 2004 indicated that urinary S100B in the asphyxiated group was significantly higher at all time-points monitored and that it was significantly higher in the group with moderate or severe HIE compared to that with mild HIE [39].

In conclusion, serum cytokines IL1, IL6 and TNFα were elevated significantly with HIE and this elevation were correlated with increased serum level of brain neurotrophic factor S100 protein. The present study hopes to contribute to the awareness, validation and clinical use of these biomarkers in an established neonatal brain injury. We also draw attention to serum levels of biomarkers which could be utilized to monitor the neonate's response to certain pharmacologic agents, which may be helpful in anticipating the potential prognosis or outcome after the brain injury.

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Authors' contributions

Authors confirmed that they are equally contributed in the present article processing.

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