Neurodevelopmental Outcome in Full Term Infants with Neonatal Asphyxia: Relation to Complement 9

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ABSTRACT

Introduction: *Hypoxic-ischemic (HI) cerebral injury is a recognized cause of permanent long-term neurologic disability in children. Much of the cerebral injury following HI insult occurs during the reperfusion phase. Post-ischemic reperfusion injury is augmented by complement because the products of complement activation mediate inflammation and cytotoxicity. C9 is an acute phase protein that rises in serum in response to inflammation Deposition of C9, the major cytolytic complement component initiates mechanisms of injury that cause irreversible post-ischemic tissue damage, such as necrosis and apoptosis. Studies in animal models have provided that complement activation participates in the development of post-ischemic cerebral injury, however no studies about the role of complement in human neonatal HIE were reported.* ***Objectives:*** *To test the hypothesis that C9 rises in both serum and CSF of full-term newborn infants with HIE and this rise may correlate with poor neurodevelopmental outcome at the age of 6 and 12 moths.* ***Methods:*** *A controlled, prospective study of 24 full-term newborn infants with HIE and 13 matched control neonates (with suspected but unproven sepsis). HIE was classified according to the criteria of Sarnat and Sarnat (1976). Blood and CSF samples were obtained within the first 24 hours of life and stored until assay. Eleven HIE infants died in the neonatal period and two at about the age of 7 months. Neurological examination and Denver Developmental Screening Test II (DDST II) were performed at 6 and 12 months in the survivors. Survivors were reclassified into two groups of normal and abnormal outcome.* ***Results:*** *The serum and CSF levels of C9 were significantly higher in HIE patients than in control neonates and correlated to the severity of HIE. Elevated serum and CSF C9 related to poor outcome at both studied ages. Median C9 CSF/serum ratios were high in HIE patients compared to controls.* ***Conclusion:*** *Serum and CSF levels of C9 were elevated in HIE patients, and their elevated levels in both body fluids correlated to the severity of HIE and poor neurodevelopmental outcome at the age of 6 and 12 months. High CSF/serum ratios of C9 in HIE suggest that C9 is produced in the brain of these infants. Future studies to delineate the sites and times at which complement inhibition can effectively minimize amplification of tissue damage and enhance recovery after neonatal HIE would be warranted.* **(Int. J. Ch. Neuropsychiatry, 2004, 1(1): 51-64)**

INTRODUCTION

Perinatal asphyxia is an insult to the fetus or newborn due to a lack of oxygen (hypoxia) and/or a lack of perfusion (ischemia) to various organs. Both factors probably contribute to asphyxial injury. The incidence of perinatal asphyxia is about 1.0 to 1.5% in most centers and is usually related to gestational age and birth weight[1]. Perinatal asphyxia is the leading cause of hypoxic ischemic encephalopathy (HIE) which is

classified during the first week of life as mild (stage I), moderate (stage II), or severe (stage III) according to the criteria described by Sarnat and Sarnat (1976)[2].

At the cellular level, neuronal injury in HIE is an evolving process. Reperfusion injury is a second determinant of the extent of brain damage. By 6-24 hours after the initial injury, a new phase of neuronal destruction sets in, characterized by apoptosis (i.e., programmed cell death)[3]. Experimental models suggest that a cytokine network participate in ischemic brain damage. Acute brain injury, evoked by cerebral hypoxic-ischemia, results in transient marked increases in expression of IL-1β and TNF-α mRNA in brain regions susceptible to irreversible injury[4].

Post-ischemic reperfusion injury is augmented by complement because the products of complement activation mediate inflammation and cytotoxicity[5]. Complement activation augments ischemic injury to a variety of tissues, including the adult mouse brain[6], and complement inhibition has been proposed as a strategy to ameliorate ischemic tissue injury[7].

It is hypothesized that analysis of the CSF and serum samples obtained from HIE newborns will show that after birth asphyxia concentration of complement9 (C9) rises in both serum and CSF and its rise may be correlated with adverse clinical outcome.

Aim of Work

The aim of this study was to asses the levels of complement9 in the serum and cerebrospinal fluid in full term newborns with HIE and its relation to the neurodevelopmental outcome at the ages of 6 and 12 months.

SUBJECTS AND METHODS

Subjects

Study design:

Prospective study including twenty four full term newborn infants born in Mansoura University Hospital and referred to the neonatology unit of Mansoura University Children's Hospital with the diagnosis of HIE during the period of December 2001-November 2003.

A control group of 13 matched full-term newborns with no history of perinatal asphyxia, were used from those admitted for exclusion of sepsis. Patients whose blood and/or CSF cultures become positive were excluded from the study.

The following criteria were used for the diagnosis of HIE, with one or more of the following: (1)5 minutes Apgar score ≤ 3, metabolic acidosis (serum bicarbonate <12 mmol /L in first hour), and/or delayed first breath beyond 5 minutes after birth; (2) mechanical ventilation at birth; (3) evidence of encephalopathy and (4) evidence of multisystem involvement (i.e. encephalopathy and at least one other system) [(8)].

Exclusion criteria included congenital anomalies, prematurity (Gestational age < 37 weeks), and intracranial hemorrhage.

Methods:

All studied newborns were subjected to thorough history, full clinical examination with stress on neurologic evidence of HIE including reflexes, tone, posture, movement and cranial nerve function, estimation of serum and CSF levels of complement component C9, and other investigations as required. Infants who survived neonatal

period were prospectively followed up by routine clinical examination at 2 months intervals until they reached 1 year of age. Their detailed neurological examinations and developmental assessment using Denver II test were performed at 6 and 12 months of age.

(A) History taking

1. **Maternal history:** Gravidity and parity, maternal age, complications of the present pregnancy (e.g. antepartum hemorrhage, pre-eclamptic toxemia, premature rupture of membranes), maternal illness, and maternal intake of drugs.
2. **Labor and delivery history:** Mode of delivery, amniotic fluid (normal, offensive or meconium stained), analgesia or anesthesia, documented history suggestive of perinatal asphyxia (e.g. Apgar score at 5 minutes ≤ 3), and the need of resuscitation by oxygen ambu bag, endotracheal intubations and drugs.

(B) Clinical evaluation

1. **General examination:** Vital signs, weight, gestational age by Dbowitz criteria [9], and major congenital anomalies.
2. **Neurological assessment:**
   * Consciousness (hyper-alertness, lethargy, stupor or coma).
   * Convulsions: onset, frequency, response to anticonvulsant drugs and number of the drugs used.
   * Neonatal reflexes (suckling, grasp, Moro).
   * Apnea.
   * Tone (normal tone, hypotonia and flaccidity, hypertonia).
3. **Cardiovascular, respiratory and other systems assessment.**

   Patients group were classified according to Sarnat and Sarnat's[2] criteria into 3 groups:
   - *Group I (Mild HIE):* This group included 5 full term newborn infants (4 males and 1 female).
   - *Group II (Moderate HIE):* This group included 7 full term newborn infants (4 males and 3 females).
   - *Group III (Severe HIE):* This group included 12 full term newborn infants (10 males and 2 females).

(C) Sample Collection:

CSF samples were collected by lumbar puncture within 1st 24 hours of postnatal life of infants with HIE. Grossly blood contaminated CSF samples were discarded. Blood samples were collected at the same time into test tubes without additives. Control CSF and blood samples were obtained from matched neonates with suspected, but unproved sepsis.

(D) Complement Assessment:

Quantitation of C9 in CSF and serum samples was done by Radial immunodiffusion kits (Human C9 NL RID Kit). These kits were supplied from The Binding Site (San Diego, CA). It's principally derived from the work of Manchini et al.[10,11], and Fahey and McKelvey[12]. The concentration of C9 was calculated from the regression equation derived from the concurrent assays of control sera, provided by the vendor, which contained known concentration of C9. The minimal concentration of C9 detected by these kits was 42.3 mg/L.

(E) Neurodevelopmental assessment:

Detailed neurological examinations and DDST II were performed at 6 and 12 months of age.

1. *Neurological Examinations[13]:*
   - Mental function
   - Cranial nerves examination
   - Motor system examination:
     a. Examination of the muscle tone
     b. Examination of the reflexes including pathological reflexes.
2. *DDST II Assessment [(14)]:*

A. Scoring: Items that can be passed by report of caregiver are denoted with a letter R. Each item that intersects or is just adjacent to the age line should be scored. Items should be scored as pass, fail, no opportunity, or refused to cooperate. Assess each item as follows:

One) **Advanced:** Child passes item that falls completely to the right of age line.

Two) **Normal:** Child passes, fails, or refuses item on which the age line falls between the 25th and 75th percentile.

Three) **Caution:** Child fails or refuses item on which the age line falls between the 75th and 90th percentile.

Four) **Delayed:** Child fails or refuses item that falls completely to the left of age line.

B. Assessment: A child fails a Denver screen if he or she has two or more delays noted. Re-evaluate the child in 3 months if there is one delay and/or two or more cautions. A child passes the screen with no delays and a maximum of one caution. Additionally, some children may be termed untestable if there are a significant number of refusal or no opportunity test items. Indications for referral are a failed test or a classification of untestable on two consecutive screenings.

(F) Statistical analysis:

Statistical analysis was done by using SPSS (statistical package for social science) program version 10, 1999. The data was parametric by using kolmogrov-smirnor test. The qualitative data were presented in the form of number and percentage. Chi-Square with Yates correlation was used. The quantitative data were presented in the form of median, range, mean and standard deviation.

Kruskal-Wallis H test was used to compare between three groups. Mann-Whintney U test was used to compare two groups. Spearman correlation coefficient was used to study relation between each two items in the same groups, and between more than two groups using one-way ANOVA test according to the distribution of variables. Significance was considered when p value less than 0.05.

ROC (Reciever operating curve) was done to determine a cut off point of selected items that help in the prediction of abnormal neurodevelopmental outcome. Sensitivity, specificity, and accuracy were calculated for a selected cut off point.

RESULTS

No significant differences were found between patients with different grades of HIE and control group as regards; birth weight, gestational age and gender (Tables 1, 2)

Serum and CSF levels of C9 were significantly elevated in patients with HIE compared to control neonates [median values: 106 (42.3 - 500) vs 42.3 (0-72.2) & 369 (106-648) vs 42.3 (0-53.8); p=0.001 & p<0.001 respectively] (Tables 3 and Figure 1).

Serum and CSF levels were significantly positively correlated with the severity of HIE (r=0.62, 0.61; p<0.001 for both respectively) (Table 4 and Figure 2).

Elevated levels of C9 in both body fluids were significantly associated with the poor neurodevelopmental outcomes at the ages of 6 and 12 months. Among patients with the poor outcome, two patients died after the age of 6 months (one with grade II and the other with grade III) (Table 5, Figure 3 and Picture 1).

Ratio of the median values of C9 in CSF and serum was high (3.48) indicating that C9 is produced in the brains of patients with HIE.

Predictability of serum C9 for the poor neurodevelopmental outcomes at 6 and 12 months was accurate in 61.3% (sensitivity: 62.5%and specificity 40 % respectively). However, the accuracy of predictability of CSF C9 for poor neurodevelopmental outcomes at 6 and 12 months was 82.5% and 80% respectively (Figures 4, 5).

Table 1. Birth weight and gestational age of controls and patients with different grades of HIE.*

		Control (n = 13)	Group I (n = 5)	Group II (n = 7)	Group III (n =12)	F	P
Birth Weight (kg)	Mean	3.22	3.33	3.5	3.25		
	SD	40	0.33	0.45	0.49		
	Range	(2.7-3.9)	(3-3.9)	(3-4.2)	(2.5-4)	0.67	0.57
Gestational Age (weeks)	Mean	37.53	39.6	39.71	39.75		
	SD	0.96	1.14	1.79	1.28		
	Range	(36-39)	(38-41)	(38-42)	(37-42)	2.12	0.08

* Oneway ANOVA Test was used.

Table 2. Gender of controls and patients with different grades of HIE.*

Sex	Control N (%)	Grade I N (%)	Grade II N (%)	Grade III N (%)	X^2	P
Male	7 (53.8%)	4 (80%)	4 (57.1%)	10 (83.3%)	2.1	0.35
Female	6 (46.2%)	1 (20%)	3 (42.9%)	2 (16.7%)		

* Chi-Square test was used.

Table 3. Serum and CSF levels of C9 in patients with HIE compared with control group.

C9	*Control N=13*	*Patients N=24*	*Z**	*P*
Serum Median (range)	42.3 (0-72.2)	106 (42.3-500)	4.82	0.001
CSF Median (range)	42.3 (0-53.8)	369 (106-648)	5.01	< 0.001

* Mann Whitney U test

Table 4. Correlation between serum and CSF levels of C9 and grades of HIE.

C9	*Grades of HIE*	
	r*	**P**
Serum	0.62	0.001
CSF	0.61	< 0.001

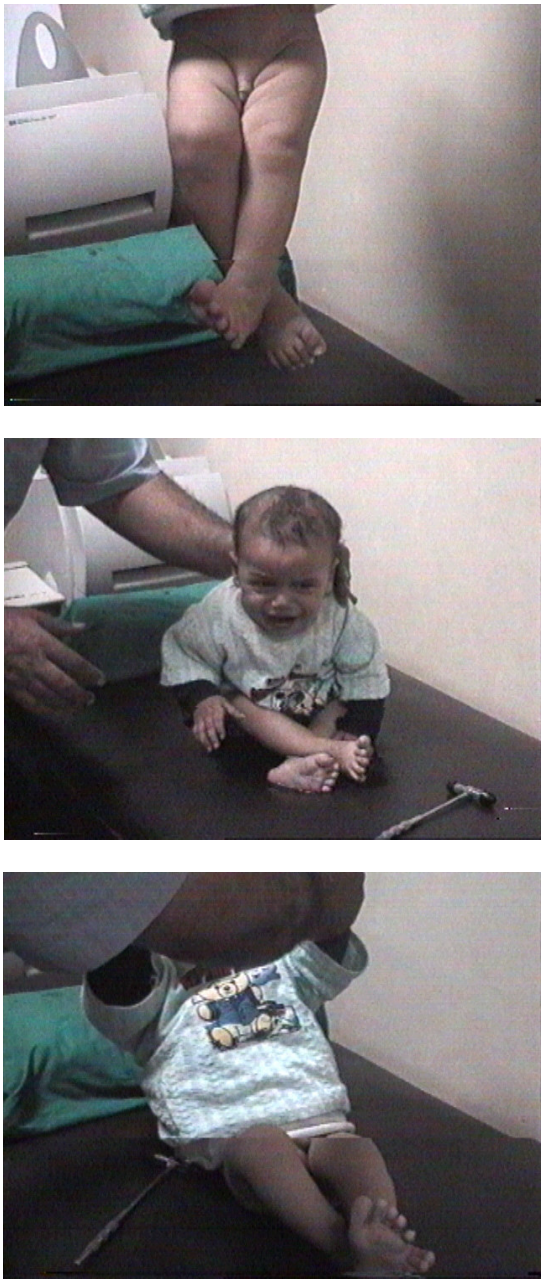
* Spearman correlation coefficient test

Table 5. Association of serum and CSF levels of C9 with the outcome of patients at the ages of 6 and 12 months.

C9	Out come at 6 Months				Out come at 9 months			
	Abnormal* N=9	**Normal N=4**	**Z**	**P**	**Abnormal * N=**	**Normal N=4**	**Z****	**P**
Serum (mg/L)	106 (42.3-500)	60.3 (42.3-438)	2.51	0.01	175 (42.3-500)	60.3 (42.3-438)	2.64	0.008
CSF (mg/L)	380 (236-648)	227.5 (106-295)	2.49	0.01	438 (226-648)	227.5 (106-295)	0.95	0.01

* Abnormal: abnormal neurological examination and / or abnormal DDST II.

** Mann Whitney U test. $p \leq 0.05$ is significant



Picture (1): Male infant 7 months –old with HIE grade III presented with early spasticity.

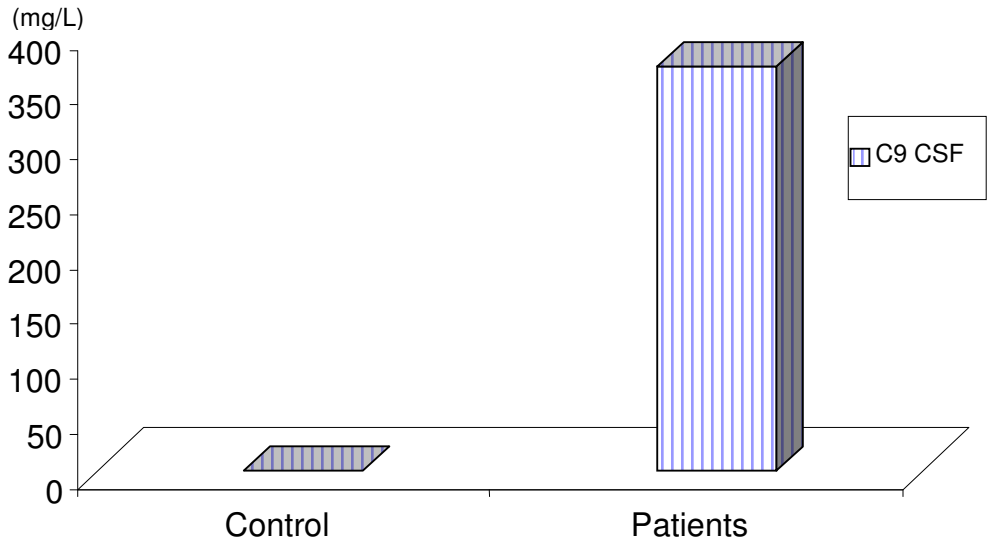


Fig. (1): Comparison between CSF and serum levels of C9 in control and patients groups.

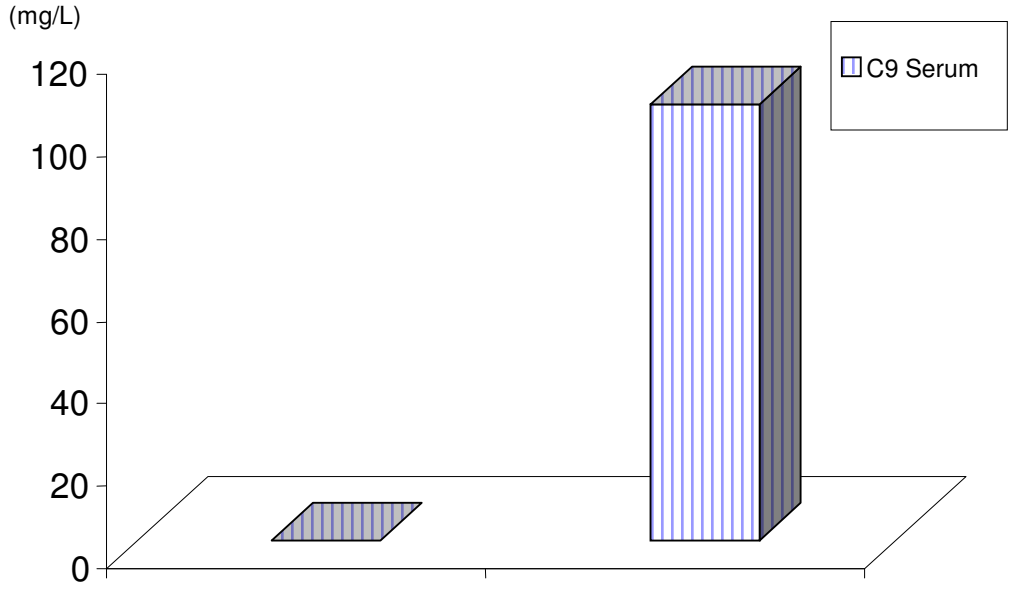


Fig. (2): Correlation between serum and CSF levels of C9 and grade of HIE.

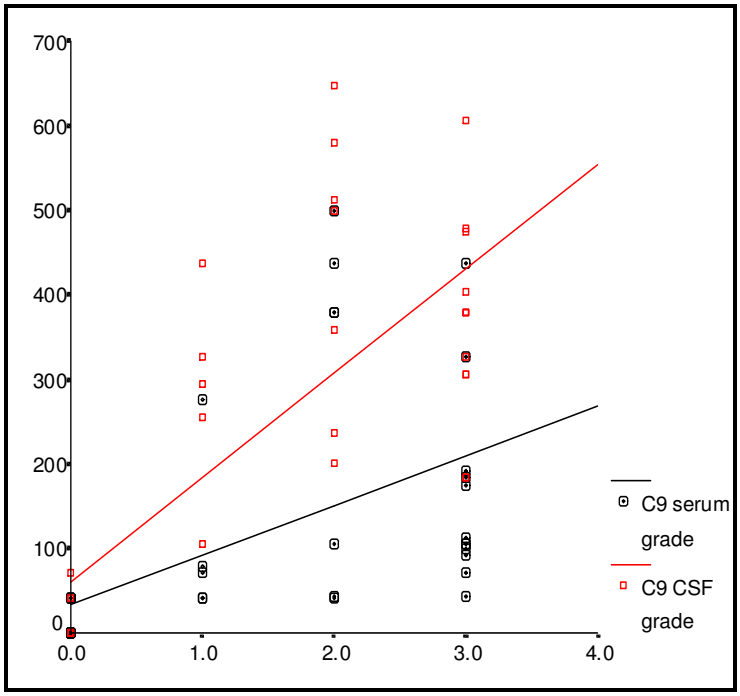
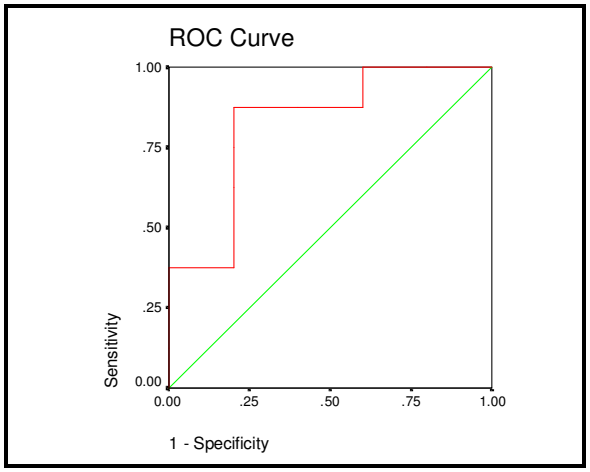
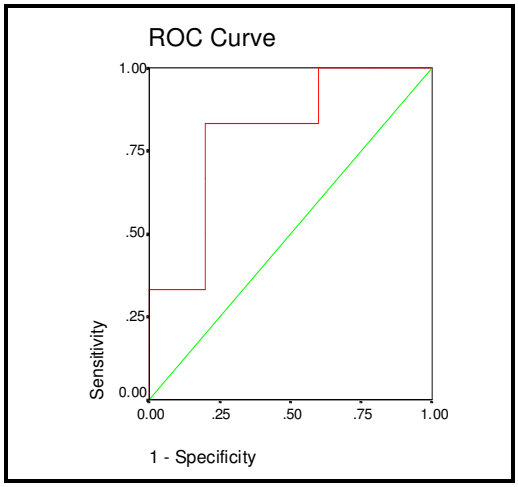


Fig. (3): Relation between C9 levels in both serum and CSF and outcome at 12 months age.



- Cut off: 310.5
- Sensitivity: 87.5%
- Specificity: 80%
- Accuracy: 82.5%

Fig. (4): ROC curve of CSF C9 predictive value at 6 months age.



- Cut off: 310.5
- Sensitivity: 83.3%
- Specificity: 80%
- Accuracy: 80%

Fig. (5): ROC curve of CSF C9 at 12 months age.

DISCUSSION

Clinical data suggest that inflammatory mediators play a role in the pathogenesis of hypoxic-ischemic brain injury. There is abundant evidence suggesting that complement plays a direct role in neuronal cell death in the setting of CNS inflammation[15]. Furthermore, biosynthesis of complement components in CNS is now well established[16].

To our knowledge C9 concentration in the CSF and serum of human neonates with hypoxic-ischemic encephalopathy have not been studied before. In our study serum and CSF C9 concentrations of HIE patients is significantly higher than the control group and correlate significantly to the severity of HIE. We reported mild elevation of both serum and CFS levels of C9 in one control which may be due to the difficulty to rule out sepsis completely. In this respect, Lassiter et al.[17], reported that the serum of neonatal rats, like neonatal humans contained a diminished concentration of complement component C9 compared with adult serum and that deficiency restricts the cytolytic capacity of their sera.

Cowell et al. (2003) and others[18,19] determined that HI induces local complement cascade activation as early as 8 hr post-HI in neonatal rats; by immunoflorescence and confocal microscopy, C9 was localized to injured neurons 16 and 24 hr pos-HI. Confocal microscopy analysis demonstrated complement deposition on apoptotic cells and release of intracellular material from necrotic cells stimulate complement activation in vitro[20,21]. Therefore, it is likely that in the acutely injured neonatal brain, neuronal apoptosis may serve as a local stimulus for complement activation.

Although neuronal morphological changes indicative of injury (e.g., nuclear shrinkage) generally preceded complement deposition, local complement activation could exacerbate ischemic injury by multiple mechanisms, including recruitment of inflammatory cells, disruption of the blood–brain barrier, and amplification of local tissue damage via formation of membrane attack complex and release of intracellular contents[21,22].

In an attempt to determine if complement removal could provide protection for the brain during ischemic-reperfusion (I/R) injury, Huang at al.[23], found that treatment with a fusion protein consisting of soluble complement receptor 1 and Sialyl Lewis^x reduced neutrophil accumulation and cerebral infarct volumes in a mouse model of transient forebrain ischemia.

There is growing recognition that inflammatory mediators can exert beneficial as well as detrimental effects during the recovery period after acute brain injury[24]. Although a preponderance of data suggests that complement activation exacerbates neurodegeneration, C5a may also exert neuroprotective effects[25].

Our results demonstrated a significant relation between the elevation of C9 concentration in the serum and the elevation noticed in its CSF level in HIE infants. An elevation in C9 CSF/serum ratio in HIE infants was also found. This elevated ratio might suggest C9 production in the CNS in response to ischemic injury. To explain our results, Zhang et al., (1996) found that; under normal conditions, complement activity within the CNS is limited. The BBB, a function of micro-vascular endothelial cells, restricts CNS entry of plasma proteins including complement components[26]. Subsequent activation of complement (C) at the surface of injured endothelial cells and leakage of plasma proteins through a compromised BBB expose CNS cells to the potential toxicity of the complement system[27]. Severe ischemic brain damage per se did not elicit terminal complement complex (TCC) formation, suggesting that a considerable mixing of serum and CSF may be needed to cause subarachnoid fluid-phase C activation. The presence and colonization of the blood-born C products C3d and C9 indicate that BBB damage had occurred in areas of C activation. Furthermore, all of the activation component, regulatory proteins, and receptors of the C system are produced by healthy astrocytes, microglia, and neurons[16,28], as do post-ischemic cells.

In our study the survival rate of grade I, HIE, was 100%, 85.7% for grade II, and 8.3% for grade III. Mortality was mainly during the neonatal period (except two cases; one grade II and one grade III). This points to the fact that death in the neonatal period is one of the defining features of severe encephalopathy. Babies who survive the initial injury are expected to live and surely need close follow up especially grade III babies.

Subsequently we found that elevated serum and CSF concentrations of C9 have a significant relation to poor neuro-developmental outcome at the age of 6 months (measured by abnormal DDST II and/or abnormal neurological examinations) with accuracy of 61.3%, 82.5% respectively and at 12-months-old with accuracy of 53.3%, 80% respectively. No previous studies concerning the role of C9 in human neonatal HIE were present and hence no data agree or antagonize our predictive values of

serum and CSF C9 for poor neurodevelopmental outcome. C9, the major cytolytic C component, through its injurious effect on brain cells would be one of the determining factors for poor neurodevelopmental outcome.

Contrary to our results a prospective cohort study of newborn encephalopathy in Kathmandu, Nepal[29], has described an excess mortality (17%) for mild encephalopathy, 71% risk of severe impairment or death in moderate encephalopathy, whereas severe encephalopathy had a 97% risk of death or severe impairment.

Current clinical markers, including the Apgar score and cord blood gases, are very poor predictors of outcome, though a combination of these markers has been suggested to be more useful to identify high-risk infants[30].

Several studies had measured biochemical factors in serum or CSF, but the tests were performed several days after birth, when the infants may already have HIE[31-33]. Urinary lactate/creatinine ratio has been reported to predict HIE within 6 hours with H nuclear magnetic resonance spectroscopy[6], but a useful indicator for HIE requires a readily available laboratory technique. Besides this, infants with asphyxia often have oliguria, and urine sampling may not be possible.

In a more recent report, elevated serum concentrations of protein S-100 and brain-specific creatine kinase reliably indicated moderate and severe HIE as early as 2 hours after birth[34]. However, the data were obtained from only seven infants with moderate to severe HIE, and neurodevelopmental follow-up studies were not performed.

From our results, we conclude that C9 is an early indicator for HIE and useful marker for later outcome. Identification of outcome by DDST II and/or neurological examination at 6 months of age showed no difference when compared to 12 months age. Depending on this finding, it's reasonable to evaluate the babies at 6 months age, which help earlier intervention. We hope that this study will open the way for future synthesis of anticomplement for early intervention with neonatal asphyxia.

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