CHANGES IN ERYTHROCYTIC DEFORMABILITY AND PLASMA VISCOSITY IN NEONATAL ICTERICIA

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ABSTRACT

We studied 45 full-term newborns divided into 3 groups. Group 1: 17 newborns with bilirubin <10 mg/dl; Group 2: 18 newborns with hemolytic ictericia (bilirubin 11–20 mg/dl) and Group 3: 10 newborns with moderate hemolytic ictericia needing exchange transfusion. The following were studied: erythrocytic deformability, plasma viscosity, plasmatic osmolarity, seric bilirubin, bilirubin/albumin ratio, free fatty acids and corpuscular volume of the erythrocytes. In full-term newborns, the following are risk factors for increased erythrocytic rigidity: neonatal hemolytic illness ($p = 0.004$, odds ratio: 7.02), increases in total bilirubin ($p = 0.02$, odds ratio: 3.3) and increases in the bilirubin/albumin ratio ($p = 0.025$, odds ratio: 4.2). Hemolytic illness is the most important risk factor for high plasma viscosity is also neonatal hemolytic illness ($p = 0.01$, odds ratio: 2.30). The role of total bilirubin is also important ($p = 0.09$, odds ratio: 2.10), while that of the bilirubin/albumin ratio ($p = 0.07$ NS) is less so. The greater the hemolysis, the greater the erythrocytic rigidity and plasma viscosity ($p < 0.01$). In full-term newborns with moderate ictericia, hemolytic illness and increases in the bilirubin/albumin ratio are accompanied by rheological alterations that could affect cerebral microcirculation and cause a neurological deficit not exclusively related to the levels of bilirubin in plasma.

Keywords: Neonatal hyperbilirubinemia; erythrocytic deformability; plasma viscosity

Neonatal hyperbilirubinemia is considered a neurological risk factor leading to alterations in neuron conduction, in evoked visual potentials, in the behavior of the newborn infant, extrapyrmidal manifestations, etc. All these problems can be avoided or corrected by suitable early treatment.[1–5]

The mechanism by which bilirubin produces neuronal damage is not fully understood, although certain situations such as premature birth, acidosis, hypercapnia, etc. favor its appearance. It is still not known at what concentrations and under which circumstances a serious risk of brain damage arises[6] because there is no relationship between blood levels of bilirubin and postmortem findings of bilirubin inction.[7] No relationship has been found between maximum values of bilirubin and cognitive deficits

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or the later intellectual coefficient of the newborn infant.[8]

Plasma bilirubin has an affinity for cell membranes, affecting their permeability and function (inhibition of ATPase of membrane, reduction of membrane potentials, interaction with membrane phospholipids, etc.).[9-12]

The aim of this study was to determine whether alterations in erythrocytic deformability and plasma viscosity take place in full-term newborns with hyperbilirubinemia and to identify the factors that favor an increase in erythrocytic rigidity and plasma viscosity.

MATERIALS AND METHODS

We studied 45 full-term newborns (gestational age between 37 and 42 weeks) less than 4 days old, classified into 3 groups. Group 1 consisted of 17 healthy newborns (Coombs negative and without blood group incompatibility) with total bilirubin <10 mg/dL and not requiring treatment. Group 2 consisted of 18 full-term newborns with moderate hyperbilirubinemia (total bilirubin 11–20 mg/dL) treated by phototherapy (Coomb negative and without blood group incompatibility). Group 3 comprised 10 full-term newborns with hemolytic illness due to blood group incompatibility (Coombs Direct Test positive) requiring transfusion exchange (5 Anti-A, 1 anti-B, 2 Rh (D), 1 Anti-C, and 1 Anti-e) (total bilirubin between 11–21 mg/dL).

We excluded from the study those newborns with obstetrical records of maternal illness (diabetes, hypertension, metabolic illness, prolonged membrane breakage etc.), with any kind of perinatal or neonatal pathology (perinatal asphyxia, infection, cardiopathy, polycythemia, respiratory distress, congenital malformations, etc.), and also those that were expanded, tamponed, or had received parenteral nutrition.

Analytical Methods

Blood samples of 3 mL were obtained by peripheral venipuncture and a Coulter Electronic Counter was used to determine erythrocyte, hemoglobin (Hb), and hematocrit (Hct) counts, mean corpuscular volume (MCV), mean hemoglobin concentration (MHC), mean corpuscular concentration of hemoglobin (MCCH), platelets, and leucocytes. With the globular packet and following the recommendations of the International Committee for Standardization in Hematology,[13] a suspension of erythrocytes at 8% [Phosphate buffer suspension (PBS), pH = 7.4, osmolality = 295 ± 5 m Osm/kg] was obtained. Erythrocytic filtration was carried out using the method of Schmid-Schönlein et al[12] modified with a negative pressure of 10 cm of water and Isopore (Millipore Corporation, Bedford, MA) polycarbonate filters with mean pore size 5 μm and diameter 25 mm. Deformability was calculated using the rigidity index (RI) expressed as[13]:

$$RI = ((ts - t)/(t \times Hct^2)) \times 100,$$

where ts is the filtration time for 1 mL of the erythrocyte suspension, t the filtration time for 1 mL of buffer (PBS) and $Hct = 8\%$.

Plasma viscosity was quantified using the Harkness capillary viscosimeter[14] following International Committee for Standardization in Hematology guidelines. Plasma osmolality was determined by the cryoscopic method (Roebling Microosmometer), albumin by electrophoresis (Beckman P/N 655900, Beckman, Galway, Ireland), free fatty acids by the enzymatic colorimetric method and plasma bilirubin by the colorimetric method [dichlolaniline DIAZOADA (DPD) using a Hitachi: 917 Autoanalyzer (Boehringer Mannheim, Germany)].

Statistical Methods

The groups were compared using a one-way variance analysis (ANOVA). We carried out a study of correlation and regression (Pearson's "r") of the rigidity index and plasma viscosity with all the variables analyzed. Logistic regression analysis was used to determine the risk factors for high erythrocytic rigidity and plasma viscosity in full-term newborns with hyperbilirubinemia.

RESULTS

All newborns from the control group and the group with nonhemolytic bilirubinemia resulted asymptomatic. Among the full-term newborns with hemolytic icterica, three patients exhibited clinical

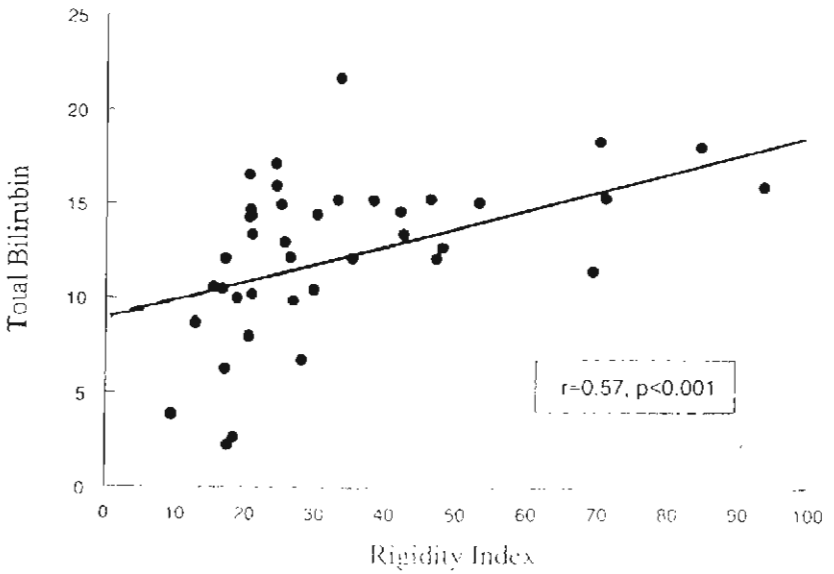

Figure 1. Rigidity Index and total bilirubin (full-term newborns).

hypotonia, in one case with rejection of food. These symptoms vanished after exchange transfusion.

During the early neonatal period, increases in plasma bilirubin ($r = 0.57$, $p < 0.001$) (Fig. 1), bilirubin/albumin ratio ($r = 0.58$, $p < 0.001$) (Fig. 2), and free fatty acids ($r = 0.41$, $p < 0.01$) were accompanied by increases in erythrocytic rigidity. In these newborns the following factors were related to increased erythrocytic rigidity: neonatal hemolytic illness ($p = 0.004$, odds ratio 7.02), total bilirubin, $p = 0.02$, odds ratio 4.3), and the bilirubin/albumin ratio ($p = 0.02$, odds ratio 4.25) (Table 1). However, when the influence of total bilirubin and of the bilirubin/albumin ratio was controlled in the logistic regression analysis, the most important risk factor for increased erythrocytic rigidity was neonatal hemolytic illness ($p = 0.04$, odds ratio 7.9), erythrocytic deformability being related to the MCV ($p < 0.06$, odds ratio 4.3).

Although in full-term newborns the rise in bilirubin was accompanied by increases in plasma viscosity ($r = 0.34$, $p < 0.05$), the most important risk

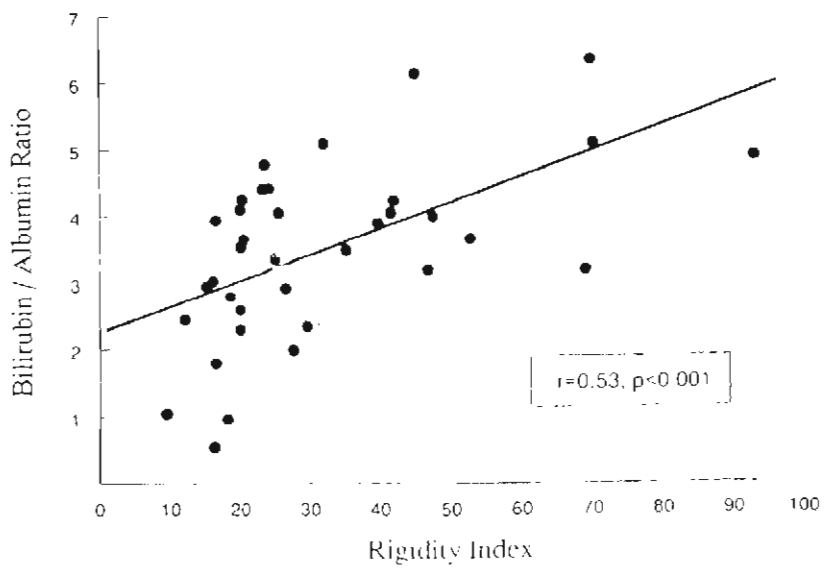

Figure 2. Rigidity Index and bilirubin/albumin ratio (full-term newborns).

Table 1. Risk Factors of Rheological Alterations in Neonatal Ictericia

	High Erythrocyte Rigidity		*High Plasma Viscosity*	
	P	*Odds Ratio*	*P*	*Odds Ratio*
Haemolytic disease	0.004	7.02	0.01	2.3
Bilirubin/Albumin index	0.025	4.25	0.12	-
Serum Bilirubin	0.02	4.3	0.09	2.00

factor for high plasma viscosity was also neonatal hemolytic illness ($p = 0.01$, odds ratio 2.30). The role of total bilirubin was also important ($p = 0.09$, odds ratio 2.10) and less so, that of the bilirubin/albumin ratio ($p = 0.12$, NS) (Table 1).

The increases in plasma viscosity negatively affected erythrocytic filterability ($r = -0.59$, $p < 0.001$) which is a risk factor for high erythrocytic rigidity ($p < 0.05$, odds ratio 6.4).

When only newborns with moderate hyperbilirubinemia (bilirubin 10–21 mg/dL) were analyzed, neonatal hemolytic illness was a risk factor for both high plasma viscosity ($p = 0.48$, odds ratio 3.38) and for decreased erythrocytic deformability ($p = 0.07$, odds ratio 2.52), in spite of their having serum bilirubin level almost identical to those of newborns with either hemolytic or nonhemolytic ictericia. In these newborns, the higher the hemolysis the greater the erythrocytic rigidity (IR/erythrocytes number: $r = 0.48$, $p < 0.01$), IR/Hb: $r = -0.49$, $p < 0.01$, IR/MCV: $r = 0.49$, $p < 0.01$) and with plasma viscosity (VP/erythrocytes number: $r = -0.43$, $p < 0.01$, Hb: $r = -0.40$, $p < 0.01$).

DISCUSSION

Bilirubin has an affinity for the membrane of erythrocytes.[5] Noncombined bilirubin is a polar molecule with low solubility in aqueous solutions, which, at plasma level, binds preferentially to albumin as a dianion at a high affinity site (fixation constant 6.4×10^7 m^{-1}) when molar bilirubin:albumin < 1 when this is $< 1/6$ it binds to two sites of low affinity (fixation constant 4.4×10^6 m^{-1}). Bilirubin fixed to albumin protects the cells from the toxic effects of free bilirubin. There is an equilibrium between the free bilirubin dianion (B^{--}) and that bound to the albumin, such that, when bilirubin:albumin $> 3:1$ as occurs in neonatal hyperbilirubinemia (Table 2), free bilirubin increases in the plasma as does its toxics effects at cellular level.[17–19]

The increases in plasma bilirubin and the bilirubin/albumin ratio lead to an increase in the acid salt of bilirubin (HB$^-$) and of acid bilirubin (BH^0) in the plasma. Being molecules of low solubil-

Table 2. Differences Between Study Groups (X ± SD)

				ANOVA	*Newman–Keuls*		
	Group 1	*Group 2*	*Group 3*	*P*	*Group 1–2*	*Group 1–3*	*Group 2–3*
GA	40 ± 1.2	39.6 ± 1.1	39.1 ± 1	NS	NS	NS	NS
Weight	3.36 ± 0.5	3.42 ± 0.4	3.54 ± 0.6	NS	NS	NS	NS
RI	20 ± 6	30 ± 12	52 ± 14	‡	NS	†	†
PV	0.93 ± 0.06	0.92 ± 0.07	1.06 ± 0.1	‡	NS	†	†
TB	7.8 ± 3.1	14.2 ± 1.6	15.8 ± 3.1	†	†	†	NS
TB/Alb	2.24 ± 0.9	4.2 ± 0.4	3.8 ± 1.3	†	†	†	NS
TP	5.5 ± 0.3	5.3 ± 0.3	4.9 ± 0.5	†	NS	†	†
Albumin	3.5 ± 0.3	3.3 ± 0.3	3.3 ± 0.3	NS	NS	NS	NS
Globulin	1.99 ± 0.27	1.9 ± 7	1.6 ± 0.29	NS	NS	NS	NS
Osmol	28 ± 6	286 ± 0.3	290 ± 1.2	NS	NS	NS	NS
FAA	0.7 ± 0.2	0.7 ± 0.3	1 ± 0.4	*	NS	*	*
Hb	16.6 ± 2.2	17.2 ± 2.8	13.7 ± 2.1	‡	NS	†	‡
Hct	47.3 ± 5.7	50 ± 8.2	40.3 ± 6.4	‡	NS	*	‡
MCV	106 ± 5	106 ± 4	109 ± 10	NS	NS	NS	NS
MCHC	3402 ± 1.5	34 ± 1.7	34.2 ± 1.1	NS	NS	NS	NS

* $p < 0.05$; † $p < 0.01$, ‡ $p < 0.001$.

NS, nonsignificant; GA, gestational age; RI, rigidity index; PV, plasma viscosity; TB, total bilirubin; Alb, albumin; Osmol, osmolality; FFAA, fatty acids; Hb, hemoglobin; Hct, haematocrit; MCV, medium corpuscle volume; and MCCH, mean corpuscular concentration of hemoglobin.

ity with capacity for aggregation and precipitation,[18,20] they produce alterations in plasma viscosity and erythrocytic deformability.[21–23]

Although, at physiological pH, free bilirubin is found generally as bilirubin dianion ($B^{=}$), it is found also in lesser quantities as bilirubin monoanion or acid bilirubin (BH^{2}) represented by the equilibration: $(B^{=})\ (BH^{-})\ (BH^{2})\ (BH^{2})_{n}$ precipitate.[20]

In neonatal ictericia the fraction of bilirubin not bound to the albumin increases, favoring the production of electrostatic complexes between the cationic lipidic groups of the membrane and the bilirubin monoanion (BH^{-}). The monovalent anion accepts a hydrogen ion on penetrating the cell membrane and frees it on entering the cytoplasm. By this means, bilirubin can pass through the cell membrane.[20] When the cell membrane is saturated with bilirubin, aggregation and precipitation of acid bilirubin take place, induced by the cell membrane.[24]

Alteration produced in cellular permeability and the functionalism of the red globules when the free bilirubin binds to the cell membranes[9,11,18] contribute to the lower erythrocytic deformability in newborns with hyperbilirubinemia. This takes place: (a) when the free fatty acids displace the bilirubin from the albumin ($r = 0.48$, $p < 0.05$),[25] (b) when plasma bilirubin increases (Fig. 2), and (c) when the bilirubin/albumin ratio increases (Fig. 2).[15,19] These situations are of special interest in newborns exhibiting a reduction in the capacity of the albumin to bind bilirubin.[26]

These observations suggest that the alterations in erythrocytic deformability are secondary to the influence of bilirubin on the erythrocyte membrane rather than to the existence of an intrinsic primary defect of the red globule.[27]

In neonatal hemolytic illness, erythrocytic deformability is also affected by phagocytosis by macrophages of part of the erythrocytic membrane resulting in a decrease in the surface: volume ratio of the red globule,[28] which could lead to confusion, as a result of the increase in the number of spherocytes recorded by the automatic erythrocyte counters in cases of immune derived hemolysis. The damage produced in the erythrocytic membrane makes MCV a limiting factor of erythrocytic filterability[29–31] and of the severity of the hemolysis related to the lower deformability of the red globules.[22] The differences in erythrocytic filterability of the newborns with ictericia cannot be attributed to the size of the pores used for filtration[15,29] because the VCM of the erythrocytes of the different groups studied were identical and there is no relationship between VCM and erythrocytic rigidity in newborns with nonhemolytic ictericia.

It is probable that small aggressions to the erythrocyte membrane associated with the cellular toxicity of bilirubin have important effects of erythrocytic deformability. This would explain why full-term newborns with hemolytic ictericia, in spite of having the same seric levels of bilirubin as the newborns with nonhemolytic ictericia, show important alterations of erythrocytic deformability and plasma viscosity as well as clinical symptoms in 30% of the cases (Table 2).

At present, it is not known at what concentrations and under what circumstances an increase in bilirubin can create a serious risk of brain damage.[9] The decrease in erythrocytic deformability and/or increases in plasma viscosity that we observed in newborns with ictericia are rheological factors related to the decrease in capillary blood flow and local changes in microcirculation, acidosis, and hypoxia.[32–35]

These are situations that at the cerebral level, can produce an aperture in the hematoencephalic barrier allowing free bilirubin bound to the albumin to penetrate the encephalic parenchyme[32] and/or to cause microthromboses of agglutinated erythrocytes in the small vessels of the encephalon like those observed in the kernicterus.[33]

In necropsic studies of newborns with ictericia, some authors attribute a bilirubin-stained necrosis to a direct toxic effect on the tissues,[34] while others claim that the necrosis occurs first, perhaps with an ischemic or hypoxic basis, and that the bilirubin subsequently stains zones that are already damaged or necrotic.[35,36] Bilirubin encephalopathy is a dynamic process in which cells may be damaged to a varying extent: the first histologic manifestations of cerebral damage are caused by ischemic changes, dilation and an increase in capillary permeability (observed in 30–50% of newborns[35]), a circumstance that might lead to a localized accumulation of bilirubin and increased cell damage (especially in cellular regions affected by kernicterus, which are particularly vulnerable to anoxic neonatal damage).

Newborns dying from ictericia and/or kernicterus present multiple micro-infarcts of the encephalon which might be caused by blockage of small vessel by microthromboses of the erythrocytes[37] and necrotic lesions arising from ischemia

and hypoxia in different organs. They are usually the cause of death in newborns; moderate to severe necrotic lesions have been observed in the spleen (38%), intestines (55.5%), pancreas (7.5%), kidneys (24–26.5%), adrenal (29.5%), heart-lung (60%), etc.[35,36,38]

The observation of changes in erythrocytic deformability due to plasmatic factors[39,40] in victims of cerebrovascular accidents suggests that fluctuations in perfusion and cerebral blood flow, less important than rheological alterations, could explain neurological deficits unrelated to bilirubin levels in plasma[7,8,41,42] in neonatal ictericia. This is the case particularly when hypoxia, acidosis, or hyperosmolarity are present. In these situations, besides the increase in neurotoxicity of free bilirubin,[43,44] changes in microcirculation take place due to an increase in blood viscosity[21-29] making tissue perfusion depend upon the levels of cutting in the capillary.[23]

CONCLUSION

In full-term newborns with moderate ictericia, changes in erythrocytic deformability and plasma viscosity take place, being especially related to the existence of hemolysis and increases in seric bilirubin and the bilirubin/albumin ratio. These rheological alterations can lead to disruptions of cerebral microcirculation and the hematoencephalic barrier that can cause neurological deficits and anatomopathological injuries in addition to those caused by the neurotoxicity of the bilirubin.

In the future, advances in the study of cerebral tissue perfusion and metabolism in newborns may improve our knowledge of the influence of bilirubin and rheological alteration on the modification in cerebral blood flow and deficits of the newborn with hyperbilirubinemia.

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