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Invited critical review

Bilirubin toxicity to human erythrocytes: A review

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Abstract

Neonatal jaundice, a physiologic condition reflecting the interplay between developmentally modulated changes in bilirubin production and metabolism, affects virtually all newborn infants. Usually, it is an entirely benign process that is resolved at the end of the first week of life without treatment or sequelae. However, in a small percentage of neonates, unconjugated hyperbilirubinemia can pose a neurotoxic risk especially in the presence of aggravating conditions such as a diminished albumin binding capacity and/or affinity, acidosis, displacing drugs and prematurity. Although neuronal cells are considered the main target for unconjugated bilirubin (UCB) toxicity, circulating cells are also affected during neonatal hyperbilirubinemia. Moreover, the UCB ability to cause hemolysis shall further aggravate neonatal jaundice through a vicious circle. In this review, we summarize the most relevant data obtained by our group regarding UCB toxicity and the role of some risk factors for kernicterus. In order to improve the risk assessment of neurotoxicity it is essential to understand the underlying mechanisms of UCB pathophysiology.

Keywords: Bilirubin; Cytotoxicity; Human erythrocytes; Newborn

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1. Bilirubin and neonatal jaundice

Bilirubin is the principal degradation end product of heme moiety from hemoglobin and other hemoproteins in mammals.

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Due to its structure, unconjugated bilirubin (UCB) is poorly soluble in aqueous medium (<70 nM). Therefore, over 99.9% UCB is transported in the blood tightly bound to albumin and biotransformation is required for excretion [1–3]. Due to the augmented hemolysis and to the immaturity of the mechanisms for bilirubin disposition in the neonatal period, virtually all newborn infants have moderately elevated serum UCB levels within the first days of life, a condition known as physiologic jaundice of the neonate [4,5]. However, in some newborn infants, plasma UCB levels can increase dramatically. In these cases, untreated hyperbilirubinemia can cause toxicity, leading to acute UCB encephalopathy or the classical kernicterus, depending on whether the clinical manifestations of neurological damage are reversible or progress to chronic and permanent clinical sequelae, that can culminate with the infant's death [6–9]. The early hospital discharge of term neonates and the increased survival of prematurely born infants [2,9,10], together with the implementation of kinder and gentler approaches to the management of neonatal jaundice [11], led to the recently reported cases of kernicterus [10,12–16]. The reemergence of kernicterus brought back the interest in understanding the mechanisms that underlie UCB neurotoxicity. On the other hand, the evidence of minor neurologic dysfunctions throughout the first year of life in children that have presented a moderate neonatal jaundice indicates that the serum UCB level is not the only hazardous factor to consider in the management of neonatal hyperbilirubinemia. Moreover, it led to a growing concern to elucidate the role of other risk factors, such as the albumin binding capacity and/or affinity, acidosis, the presence of displacing drugs and prematurity [6,8], in order to identify the newborn infants at risk of neurological damage by UCB.

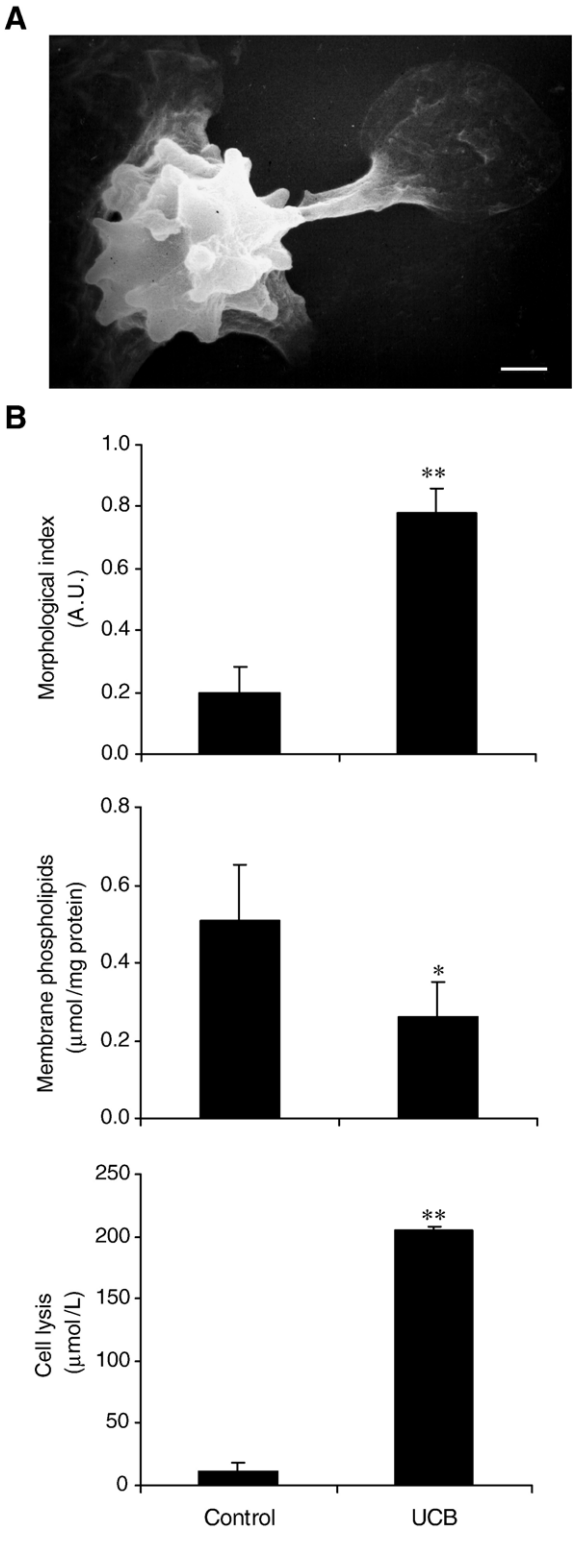

Fig. 1. Unconjugated bilirubin (UCB) induces morphological alterations, disruption of membrane structure and hemolysis of human erythrocytes from adult donors. Human erythrocytes obtained from adult donors were incubated in the absence (control) or in the presence of 171 μM purified UCB, at pH 7.4, for 3 h, at 37 °C. Morphological analysis, performed by scanning electron microscopy, revealed the presence of echinocytes, hemoglobin-depleted cell-like vesicles and fusion events in UCB-treated erythrocytes (A). The extent of shape changes was quantitatively expressed as the morphological index, and membrane phospholipids and hemolysis were determined as lipid phosphorus and hemoglobin release, respectively (B). *$P<0.05$ and **$P<0.01$ from control. Bar: 1 μM. Derived from data of Brito et al. [40] and Brites et al. [42].

1.1. The human erythrocyte as a model to study unconjugated bilirubin membrane binding and toxicity

Although the primary concern with respect to hyperbilirubinemia is the potential for neurotoxic effects, general cellular injury also occurs [17,18,22,24], as pointed out by the panoply of toxic events occurring in different study models [19–25], including erythrocytes [26,27]. The alterations in membrane potential, transport and enzymatic systems [28–33] point to the disruption of the cell membrane structure as a common denominator of the mechanisms underlying UCB cytotoxicity. It was even pointed out that the amount of UCB bound to the membrane, rather than its concentration in the medium, determines the magnitude of the toxic effect [34].

The human red blood cell (RBC) can be used as a model to study UCB cellular binding and toxicity due to its accessibility, the absence of intracellular organelles and the similarities of some membrane components with those of other cell types [35]. Moreover, UCB binding to RBC has been considered a sensitive indicator of cellular affinity and toxicity [36–38] and was proposed as a criterion to assess the risk of bilirubin encephalopathy [37,39].

1.2. Unconjugated bilirubin induces profound disturbances of human erythrocytes integrity

As shown in Fig. 1, exposure of human RBC from adult donors to high concentrations of free UCB (171 μM) leads to the appearance of echinocytic forms and fusion events, which may derive from an evagination of the membrane as a result of the interaction of UCB with the outer leaflet of the bilayer. The shape changes, expressed quantitatively as the morphological index [40,41], are accompanied by the loss of membrane phospholipids and most likely result in a subsequent and irreversible stage of UCB toxicity that culminates in cell lysis and appearance of hemoglobin-depleted cell-like vesicles. In fact, crenation is more evident in the 5–30 min period after UCB addition, where hemolysis is virtually absent, but the extent of shape changes becomes less pronounced as hemolysis starts to occur later on [42]. Therefore, membrane deposition of UCB leads to echinocytosis, phospholipid elution and cell destruction, reflecting different stages of UCB interaction with the RBC [40]. Interestingly, younger cells preferentially show the early stage of UCB toxicity, corresponding to cell crenation, while the final and irreversible step, demonstrated by hemolysis, is more evident in aged RBC [42].

UCB also perturbs the normal distribution of membrane phospholipids. In fact, we have observed that the pigment not only induces marked alterations in the membrane content of several classes of phospholipids, but also leads to the translocation of the inner leaflet aminophospholipids to the outer leaflet of the membrane [43]. These alterations point to an accelerated ageing of RBC exposed to UCB, which was referred to be accompanied by the loss of components as a result of membrane fragmentation and the release of vesicles [44]. The RBC that became senescent by UCB interaction shall be recognized and removed from circulation. This assumption is convincingly documented by the externalization of phosphatidylserine induced by UCB [43], which seems to constitute a signal for the phagocytic engulfment by macrophages [45].

1.3. The extent of cell damage increases with the serum unconjugated bilirubin concentration and with acidosis

The extent of the previously mentioned effects on human erythrocytes, namely the shape changes and hemolysis, as well as the release of phospholipids and their redistribution, increase with the UCB to human serum albumin (HSA) molar ratio (Fig. 2), as would be anticipated. In fact, in blood plasma UCB circulates almost entirely bound to HSA, so that with a normal albumin concentration of 435 μM (3 g/100 ml) as much as 435 μM (25 mg/100 ml) of UCB will be transported. However, when the UCB to HSA molar ratio exceeds the unity, the albumin capability for UCB binding is surpassed and the amount of unbound UCB sharply rises [34]. In these conditions, the free fraction of UCB, which is mainly in the non-ionised (protonated diacid) form at physiological pH, can passively diffuse across membranes and impair cell viability [3]. Our findings assume a particular relevance since a decreased

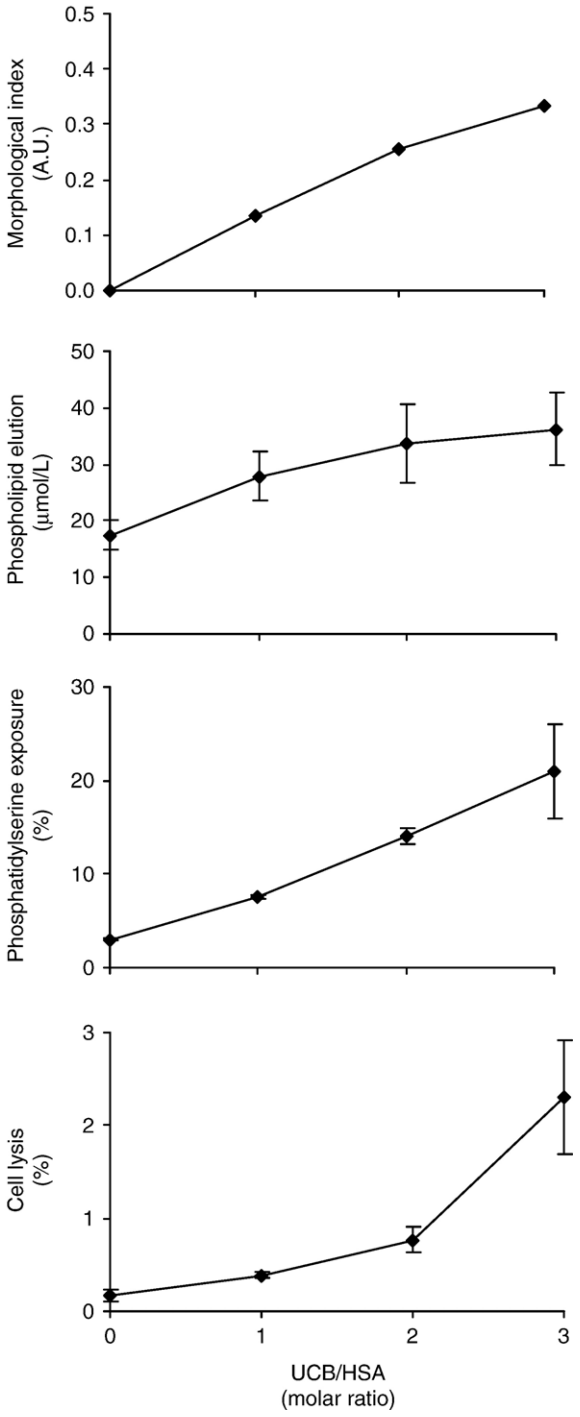

Fig. 2. Morphological alterations, disruption of membrane structure and hemolysis of human erythrocytes from adult donors increase with the unconjugated bilirubin (UCB) to albumin molar ratio. Human erythrocytes obtained from adult donors were incubated in the absence (control) or in the presence of 114–340 μM purified UCB, at different UCB to human serum albumin (HSA) molar ratio values, at pH 7.4, for 4 h, at 37 °C. Morphological analysis was performed by scanning electron microscopy and the extent of shape changes was quantitatively expressed as the morphological index; phospholipid elution, phosphatidylserine exposure and hemolysis were determined as lipid phosphorus, annexin V binding and hemoglobin release, respectively. Statistically significant differences from control were observed for all the UCB/HSA molar ratios. Derived from data of Brito et al. [57] and Brito [62].

albumin concentration and a lower affinity and/or capacity for UCB binding is frequently found in neonates, especially in the low weight premature infants [8,46]. Furthermore, the already statistically significant effects observed at an UCB to HSA molar ratio of one provide an explanation for the manifestations of toxicity, namely the cases of kernicterus, occurring in severely jaundiced newborns [2,8,47], where this ratio value can be attained.

Another frequent condition usually associated with an aggravation of UCB cytotoxicity during neonatal life is acidosis [8,34,48]. To this fact shall contribute the protonation of the propionic acid residues of the UCB molecule in an acidic medium and the aggregation of the so-called acid UCB within the membrane. In contrast, the ionisation of the side chain(s) at alkaline pH values renders the molecule more soluble and less toxic [49]. In our studies, acidosis revealed to enhance the extent of morphological alterations and hemolysis (Fig. 3), probably as a reflex of an increased binding of UCB to erythrocytes, which is in agreement with previous reports [50,51]. On the other hand, an acidic medium lessens the loss of phospholipids, as well as their redistribution, probably by promoting the formation of UCB complexes with membrane phospholipids. The aggregation of UCB dimers at the membrane level, progressively originating acid UCB microcrystals [52], shall be responsible for the irreversible damage that culminates in cell lysis. Thus, these data provide a basis for the role of acidosis as an aggravating condition of hyperbilirubinemia, pointing to acid UCB as the cytotoxic species, and rendering UCB precipitates as the entities mainly responsible for the demolition of the membrane architecture [53].

1.4. The erythrocyte membrane binding and morphological changes increase by sulfisoxazole displacement of unconjugated bilirubin from albumin

In order to better clarify the role of UCB-displacing drugs on the pigment binding and toxicity to erythrocytes, experiments were performed in the absence or presence of sulfisoxazole, a UCB displacer from the albumin molecule [54]. In our experimental conditions (UCB/HSA molar ratio of 0.5), the unbound UCB level of ~26 nM (Fig. 4A) assured that aggregation of UCB was avoided, while the erythrocyte-bound UCB fraction extractable by albumin washing (albumin extractable-UCB) content of ~5 μmol/l RBC was not enough to induce morphological changes (Fig. 4B). Following sulfisoxazole addition (sulfisoxazole/HSA molar ratio of 4.0), the small increase of unbound UCB (1.3-fold) compared with the highly significant rise (5.5-fold) in the erythrocyte-bound UCB indicates that a transfer of UCB from the albumin molecule to the RBC was achieved by the sulphonamide [42]. As a consequence of UCB binding to erythrocytes, morphological changes ranging from echinocytes 1 to spheroechinocytes were observed, in contrast with the normal morphology in the assays performed with no addition of the displacing drug (Fig. 4B). These data confirm that administration of a drug that binds competitively to the albumin molecule induces a rise in free UCB levels and a sharp increase in cellular uptake, consequently

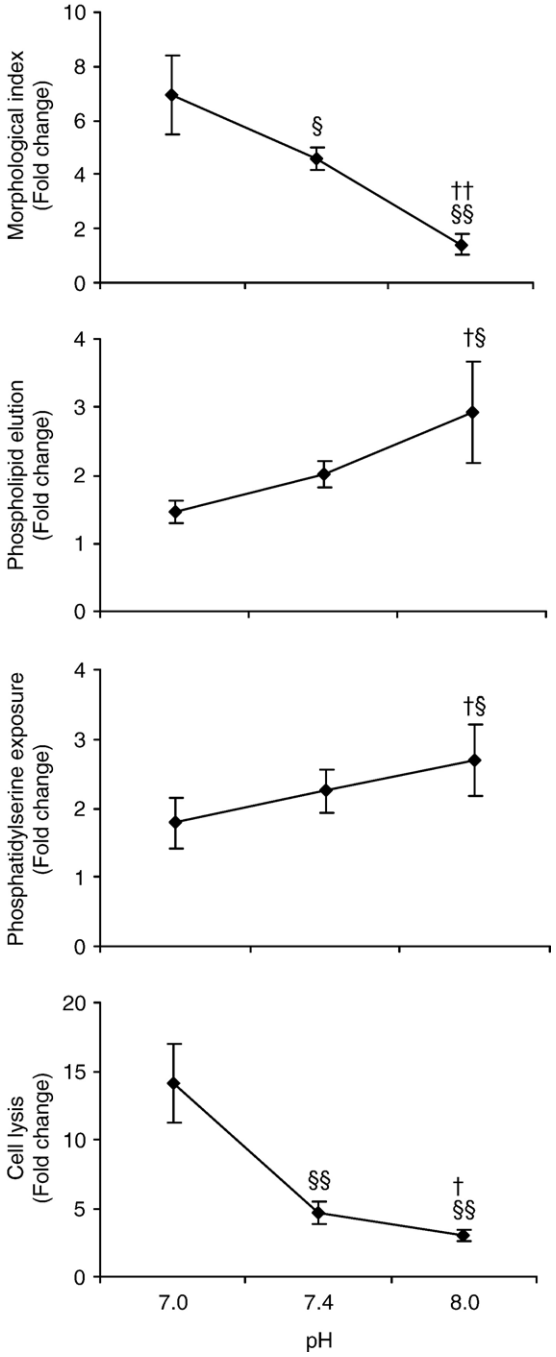

Fig. 3. Acidosis differently affects unconjugated bilirubin (UCB)-induced morphological alterations, disruption of membrane structure and hemolysis of human erythrocytes. Human erythrocytes obtained from adult donors were incubated in the absence (control) or in the presence of 340 μM purified UCB, at a UCB to human serum albumin molar ratio of 3, for 4 h, at 37 °C, at different pH values. Morphological analysis was performed by scanning electron microscopy and the extent of shape changes was quantitatively expressed as the morphological index; phospholipid elution, phosphatidylserine exposure and hemolysis were determined as lipid phosphorus, annexin V binding and hemoglobin release, respectively. Statistically significant differences vs. respective control were obtained for all the parameters except for morphological index at pH 8.0 and phosphatidylserine exposure at pH 7.0; fold changes were calculated based on corresponding controls. $^{\S}P<0.05$ and $^{\S\S}P<0.01$ from pH 7.0; $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ from pH 7.4. Derived from data of Brito and Brites [53].

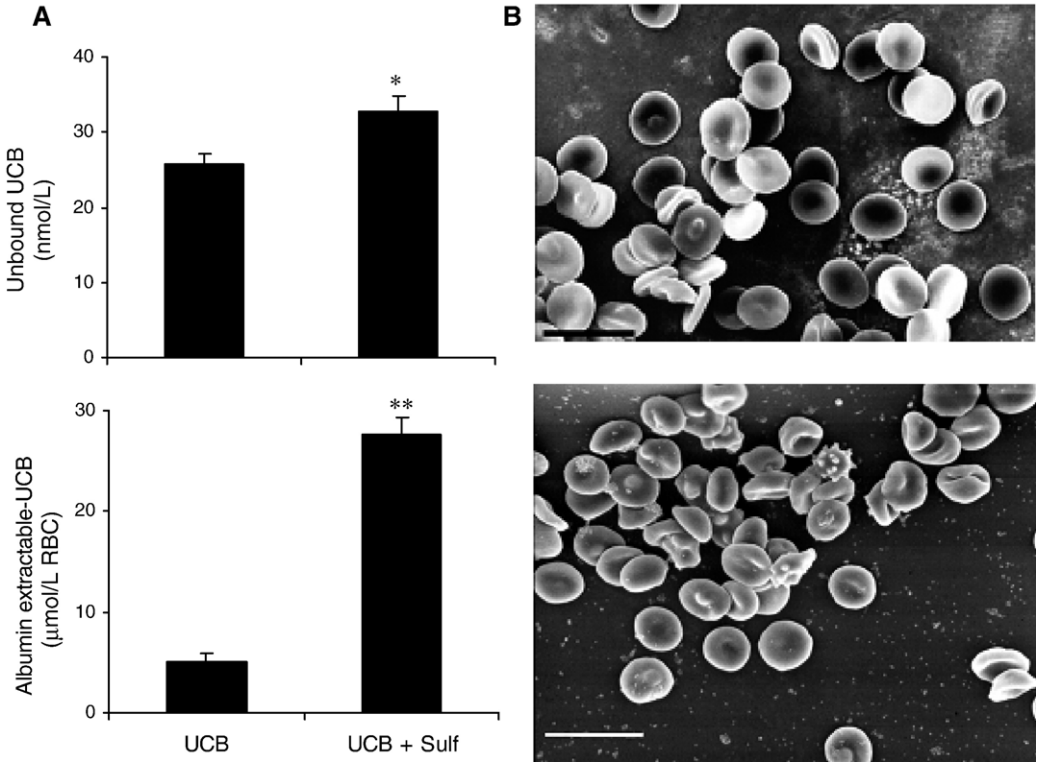

Fig. 4. Sulfisoxazole displaces unconjugated bilirubin (UCB) from albumin, and increases cellular uptake and morphological changes. Human erythrocytes obtained from adult donors were incubated with 171 μM purified UCB and 342 μM human serum albumin (HSA), in the absence or in the presence of 1.37 mM sulfisoxazole (Sulf), at pH 7.4, at 37 °C, for 60 min (A) or 210 min (B). Unbound UCB was determined by the peroxidase method and erythrocyte-bound UCB was evaluated by albumin extraction (albumin extractable-UCB) (A); morphological analysis was performed by scanning electron microscopy (B). *$P<0.05$ and **$P<0.01$ from UCB alone. Bar: 10 μm. Derived from data of Brites et al. [42].

leading to cytotoxic manifestations, despite the serum albumin surplus. These findings, by providing a basis for the occurrence of kernicterus at low UCB concentrations in infants that were quite inadvertently given sulfisoxazole in the 1950s [55] and for the clinical significance of the UCB displacing effects of antibiotics, anticonvulsants, diuretics and other important drug classes [56], highlight the potential risk for drug–UCB interactions in neonatal drug therapy.

1.5. Albumin only partially recovers erythrocyte-bound unconjugated bilirubin

In the clinical management of jaundice, a crucial aspect is the reversibility of the toxic manifestations of UCB by therapeutics, such as albumin administration. Nevertheless, the wash of erythrocytes with albumin after incubation with UCB, although removing some of the erythrocyte-bound UCB and decreasing the extent of shape changes, is not able to completely restore the normal cell morphology (Fig. 5). These observations suggest that only the superficial and non-aggregated UCB molecules are taken off by albumin and that no other than the initial stages of membrane toxicity can be entirely reverted by albumin. By contrast, the UCB aggregates involved in the irreversible mechanisms of UCB-induced toxicity can only be recovered by solubilization with chloroform. So, the determination of erythrocyte-bound UCB by chloroform extraction (chloroform extractable-UCB) [57] seems to be a more reliable technique to evaluate the extent of UCB bound to erythrocyte membranes than that based on albumin extraction (albumin extractable-UCB) [58]. This is in line with a previous study reporting the inability of albumin as a UCB eluting medium from erythrocyte membranes [59]. Therefore, appreciation of chloroform extractable-UCB levels may add valuable information to improve the assessment of the risk of UCB cytotoxicity during hyperbilirubinemia [57].

1.6. Binding of unconjugated bilirubin to human erythrocytes during neonatal jaundice elicits toxicity

In line with the in vitro studies, the examination of blood samples obtained from moderately jaundiced neonates (185 μM UCB, UCB/HSA molar ratio of 0.4), revealed the presence of echinocytosis, together with a decreased content of membrane phospholipids [40]. As expected, the magnitude of the effects observed in the physiologic and reversible neonatal hyperbilirubinemia (Fig. 6) is lower than that found in the in vitro study (Fig. 1), which intent to mimic the irreversible pathological condition of kernicterus. To this fact accounts the absence of albumin in the in vitro study, which leads to much higher levels of free UCB and favours its interaction with the membranes, despite the same range of concentrations of the pigment (<200 μM).

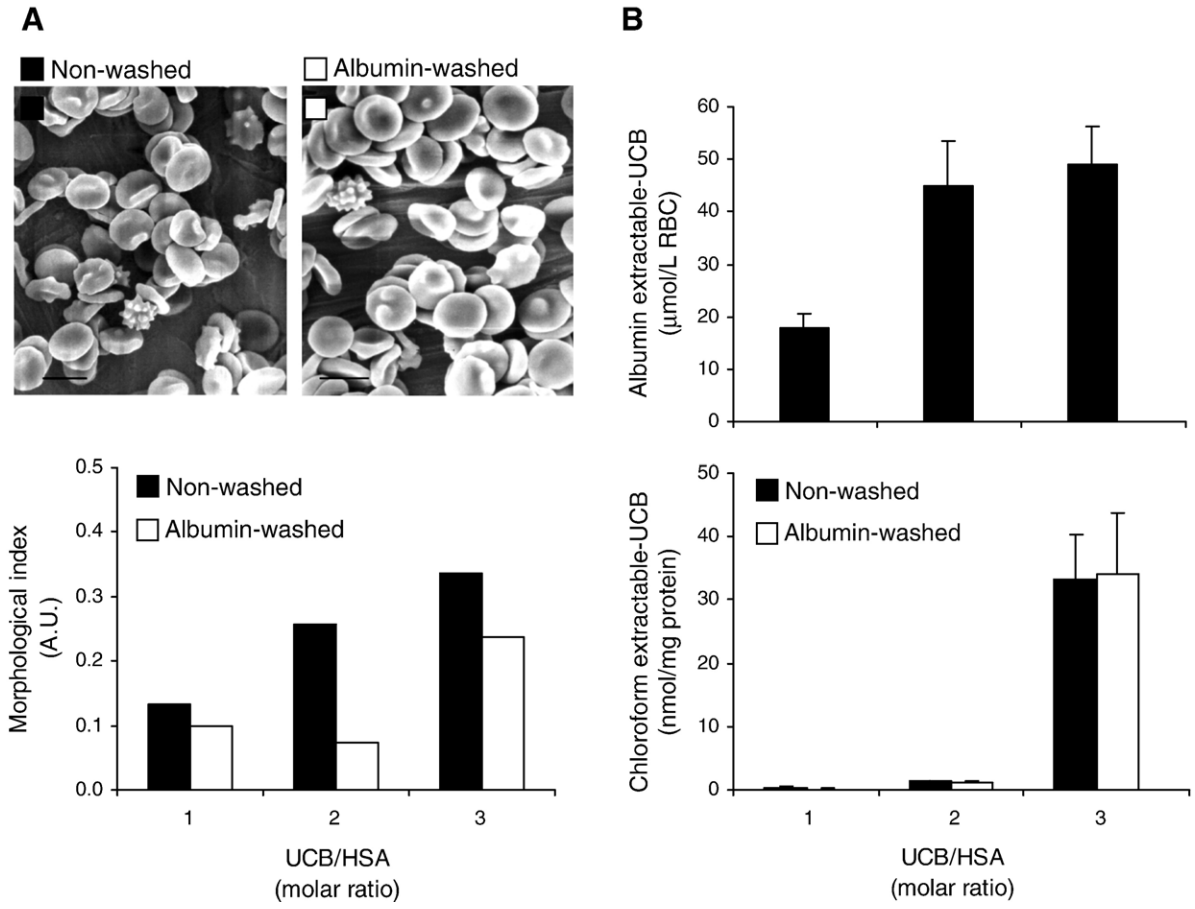

Fig. 5. Albumin is unable to completely revert the morphological alterations induced by unconjugated bilirubin (UCB) and to extract all the erythrocyte-bound UCB, which is only recovered by chloroform extraction. Human red blood cells (RBC) obtained from adult donors were incubated in the absence (control) or in the presence of 114–340 μM purified UCB at different UCB to human serum albumin (HSA) molar ratio values, at pH 7.4, for 4 h, at 37 °C. Morphological analysis was performed by scanning electron microscopy and the extent of shape changes was quantitatively expressed as the morphological index (A); erythrocyte-bound UCB was evaluated by both albumin extraction (albumin extractable-UCB) and chloroform extraction (chloroform extractable-UCB) in non-washed and albumin-washed RBC (B). Albumin extractable-UCB and chloroform extractable-UCB were not detected in control assays and no statistically significant differences were obtained between the chloroform extractable-UCB levels of non-washed and albumin-washed erythrocytes. Bar: 10 μm. Derived from data of Brito et al. [57].

Interestingly, jaundiced neonates exhibit significantly higher levels of membrane-bound hemoglobin, as compared with healthy babies or adults (Fig. 6). This finding reinforces the already mentioned accelerated ageing of erythrocytes exposed to UCB [43], since hemoglobin binding to membranes was shown to increase during in vivo erythrocyte senescence [60]. Furthermore, this observation raises the hypothesis that high levels of UCB may induce oxidative injury, considering that membrane-bound hemoglobin increases with the level of oxidative stress [61].

1.7. Neonatal erythrocytes are more prone to unconjugated bilirubin-induced toxicity than cells from adult donors

UCB binding and its pernicious effects are exacerbated in neonatal RBC [62]. In effect, cells obtained from the umbilical cord blood suffered more drastic changes than those from adult donors, following exposure to the pigment (UCB to HSA molar ratio of 3), as shown by the higher distortion of morphology and elution of membrane phospholipids, together with the more marked disruption of the normal bilayer asymmetry, indicated by the increased externalization of phosphatidylserine (Fig. 7). The proneness of neonatal cells to suffer such profound disturbances of membrane structure shall lead to a further acceleration of the UCB-induced erythrocyte senescence. As a result, the extent of hemolysis is nearly five times greater than that of adults. Underlying the vulnerability of neonatal cells to the injurious effects of UCB shall be a different binding and accommodation status of the pigment within the membrane, as indicated by the opposite profiles of albumin extractable-UCB and chloroform extractable-UCB in neonates as compared to adults. Actually, erythrocytes from adults appear to bind mainly UCB monomers at a superficial level of the membrane and, thus, UCB is easily removed by albumin washing. Cells from the umbilical cord blood, although presenting less albumin extractable-UCB, contain a bigger fraction of the pigment inaccessible to albumin, probably as aggregates of acid UCB with the membrane phospholipids, which is only recovered by chloroform. This

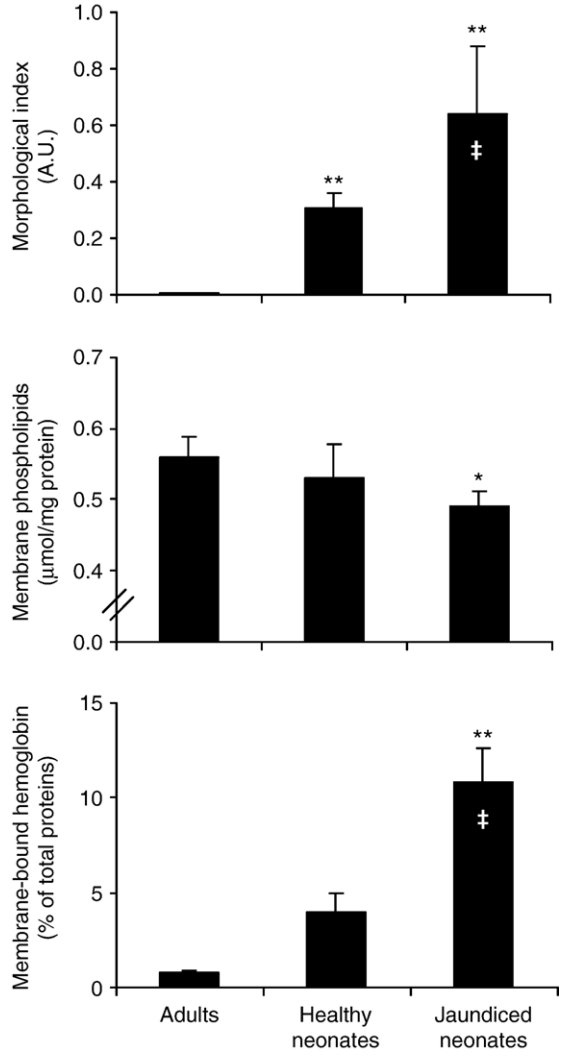

Fig. 6. Human erythrocytes from jaundiced neonates present alterations of morphology and membrane lipid composition, and avidly bind hemoglobin. Human erythrocytes were obtained from venous blood of moderately jaundiced neonates (~185 μM unconjugated bilirubin; bilirubin/albumin molar ratio of ~0.4 and adult donors, as well as from the umbilical cord blood of healthy newborn infants. Morphological analysis was performed by scanning electron microscopy and the extent of shape changes was quantitatively expressed as the morphological index; membrane phospholipids were determined as lipid phosphorus, and membrane-bound hemoglobin was evaluated based on the peroxidase activity of hemoproteins. *$P<0.05$ and **$P<0.01$ from adults; ‡$P<0.01$ from healthy neonates. Derived from data of Brito et al. [40] and Brito [62].

chloroform extractable-UCB presumably corresponds to the fraction implicated in the enhanced irreversible toxicity in neonatal cells. Our observations are in line with the higher capacity for UCB incorporation referred by Vázquez et al. [52] in synaptosomal plasma membrane vesicles obtained from neonatal rats.

1.8. Unconjugated bilirubin enhances the formation of intracellular vesicles in neonatal erythrocytes

In agreement with the hemolysis and the membrane effects of UCB, the induction of the endovesiculation process is also enhanced in neonatal erythrocytes. It is well described that neonatal erythrocytes are more prone to spontaneous endovesiculation than adult ones, and that this process can be induced more easily in the former cells by a variety of stimuli [63–67]. The presence of endovesiculation can be analyzed through a stereological method that quantifies the numerical density of the intracellular vesicular profiles by volume unit (Nv,vi, μm^{-3}) [68,69]. Probably due to its ability to interact with cell membranes, UCB is also able to induce the endovesiculation in neonatal erythrocytes (Fig. 8A), as demonstrated by the presence of intracellular vesicles observed when cells from the umbilical cord blood were exposed to the pigment (UCB to HSA molar ratio of 3). Furthermore, this in vitro effect of UCB seems to be also observed in vivo [70]. In fact, the number of intracellular vesicular profiles in RBC collected by venous puncture of moderately jaundiced babies (UCB/albumin molar ratio of ~0.5) was significantly higher than that observed in healthy neonates (Fig. 8B). Once again, these observations suggest that UCB induces the senescence of neonatal erythrocytes since throughout the life span of these cells there is a reduction of the cell superficial area that is not accompanied by a parallel loss of phospholipids which can, therefore, account for the internalization of the membrane as endocytic vesicles [71].

Collectively, the data obtained using neonatal cells indicate that erythrocytes from newborn infants, by binding UCB more avidly and showing signs of a marked viability decline, represent sensitive targets of UCB toxicity, thus playing a pivotal role during hyperbilirubinemia. The enhanced senescence rate shall favour erythrophagocytosis, therefore contributing to raise hemolysis that further aggravates neonatal jaundice through a vicious circle. Interestingly, the description of UCB-induced hemolysis is not without precedent since other studies have also reported erythrocyte lysis [72] and increased membrane rigidity [73] and fragility [74] due to hyperbilirubinemia.

Therefore, these findings provide a basis for the increased susceptibility of newborn infants to UCB injury and for the enhanced effects induced by UCB in less differentiated astroglial cells in accordance with our recent publication [75].

1.9. Unconjugated bilirubin impairs membrane dynamic properties of human erythrocyte right-side-out vesicles, which are further aggravated by acidosis

Previous data have pointed to the cell membrane as a primary target of UCB cytotoxicity. To clarify whether UCB has a special avidity for different regions of the membrane, namely the lipid–water interface, the carbonyl or the methylene chain regions or, eventually, the middle of the bilayer, we performed electron paramagnetic resonance (EPR) spectroscopy analysis of RBC right-side-out vesicles, using several spin labelled phospholipids, in order to selectively establish the UCB-induced perturbations of membrane dynamic properties at different depths of the leaflet [76]. Interestingly, UCB leads to a dual effect in the membrane lipid package and polarity, decreasing the phospholipids mobility and the polarity environment at C-5 region, while inducing their increase at C-7 and less markedly in deeper regions of the leaflet (Fig. 9A). The alteration of membrane dynamics caused by UCB

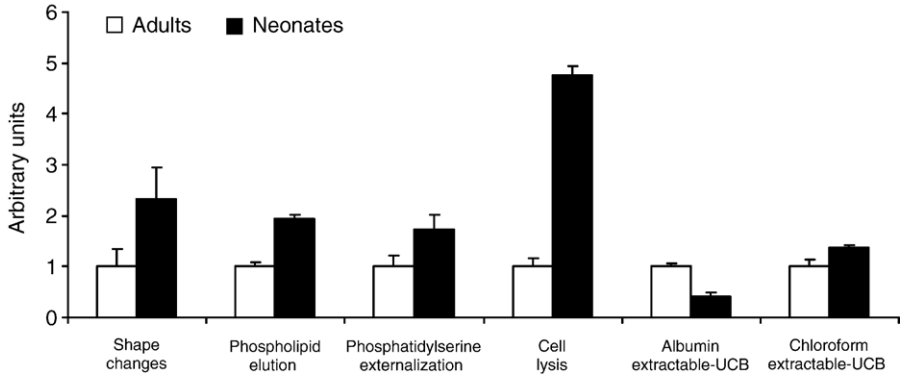

Fig. 7. Unconjugated bilirubin (UCB)-induced toxicity is exacerbated in human neonatal erythrocytes as compared to adults. Human erythrocytes were obtained from either adult donors or umbilical cord blood of healthy neonates and incubated in the absence (control) or in the presence of 340 μM purified UCB, at a UCB to human serum albumin molar ratio of 3, at pH 7.4, for 4 h, at 37 °C. Morphological analysis was performed by scanning electron microscopy and the extent of shape changes was quantitatively expressed as the % of echinocytes 3 plus spheroechinocytes; phospholipid elution, phosphatidylserine externalization and cell lysis were determined as lipid phosphorus, annexin V binding and hemoglobin release, respectively. Erythrocyte-bound UCB was determined by albumin extraction (albumin extractable-UCB), and the amount of the pigment not removed by albumin was recovered using chloroform as a solvent (chloroform extractable-UCB). Statistically significant differences were obtained for all the parameters vs. respective control, in both groups, and between adults and neonates. Concentrations of the different parameters were expressed as arbitrary units (AU) where results obtained for adults were normalized to unity to allow the comparison with the neonatal values. Derived from data of Brito [62].

appears to have common features regardless the type of membrane model, since a similar pattern of membrane perturbation was observed in isolated rat brain mitochondria [77]. These results indicate that accommodation of UCB occurs at C-5, therefore decreasing the already low chain motion of phospholipids. The intercalation of foreign molecules shall render the inner regions of the leaflet more fluid and more permeable to water diffusion, as a second-hand effect, which is maximal at C-7. The decrease in the extent of membrane perturbation from C-7 to C-12 may be understood as a wave progressively attenuating as the distance from the primary interaction target increases. Thus, from our data we may conclude that at least a portion of the UCB molecule interacts to some extent with the hydrocarbon core at the superficial regions of the membrane, which is in agreement with previous studies [78–80], and sustains the appearance of echinocytic forms following exposure to UCB. Moreover, the solubility characteristics of the molecule would not favour its intercalation between the phospholipids acyl chain [81], while the

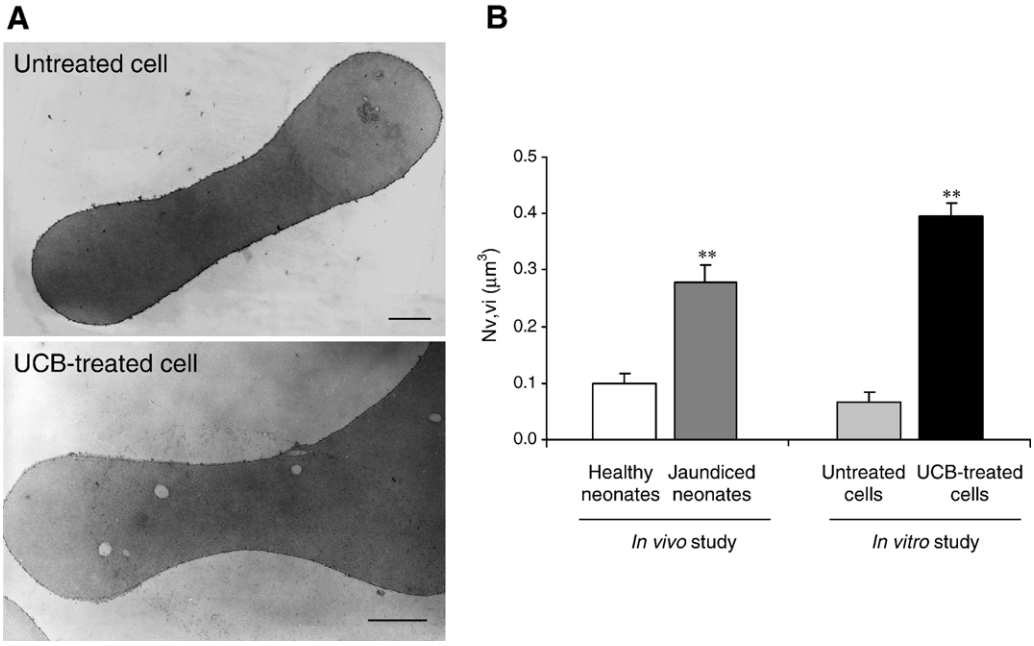

Fig. 8. Unconjugated bilirubin (UCB) induces in vivo and in vitro formation of intracellular vesicles in human neonatal erythrocytes. Human erythrocytes were obtained either from the umbilical cord blood of healthy neonates or venous puncture of 2–3-day-old jaundiced neonates (~200 μM UCB; UCB/albumin molar ratio of ~0.5). For in vitro studies, umbilical cord blood cells were either untreated or treated with 340 μM purified UCB at a UCB to human serum albumin molar ratio of 3, at pH 7.4, for 4 h, at 37 °C. Ultrathin sections of all cells were observed under transmission electron microscopy (A) and the number of endocytic vesicles quantified stereologically by the numerical density of the intracellular vesicular profiles by volume unit (Nv,vi) (B). $^{**}P<0.01$ from healthy neonates or untreated cells. Bar: 1 μm. Derived from data of Silva [70].

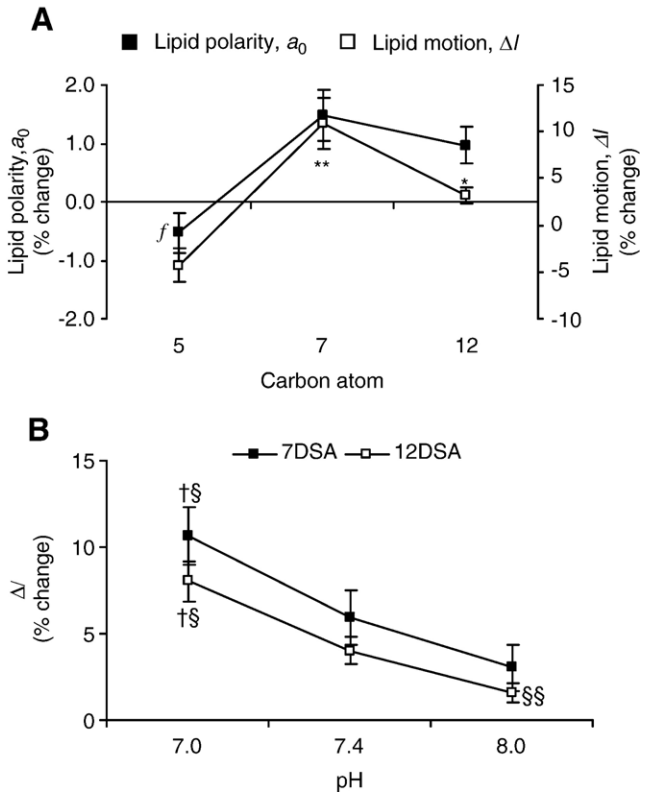

Fig. 9. Unconjugated bilirubin (UCB) impairs membrane dynamic properties of human erythrocyte membranes, which are further aggravated by acidosis. Right-side-out vesicles were obtained from human erythrocytes of adult donors and were spin labelled with stearic acid molecules bearing a reporter group at different positions down the lipid chain, prior to incubation with either no addition (control) or 8.6 μM purified UCB, for 15 min, at 37 °C. Alterations of the polarity environment and phospholipid motion were determined by electron paramagnetic resonance spectroscopy analysis, through measurement of the isotropic hyperfine splitting constant (a_0) and/or the outer half-width at half-height of the low field extremum (ΔI), following incubation at pH 7.4 (A) or at different pH values (B). Changes in percentage were calculated based on corresponding controls. *$P<0.05$ and **$P<0.01$ from respective control for ΔI; $^{f}P<0.05$ from respective control for a_0; $^{\S}P<0.05$ and $^{\S\S}P<0.01$ from pH 7.4; $^{\dagger}P<0.01$ from pH 8.0. Derived from data of Brito et al. [76].

lack of effect at C-16 [76] is also consistent with the accommodation of UCB in lipid bilayers at regions different from the methyl terminal end of the acyl chain [78–80].

Our studies also showed that the membrane properties are further impaired by acidosis (Fig. 9B), reinforcing the concept that uncharged diacid UCB is the molecular species involved in the UCB-induced disruption of cell integrity [76].

2. Conclusions

In this review, we show that in vitro exposure of human RBC to UCB induces profound disturbances of cell integrity and morphology and that cellular uptake of UCB is enhanced by the presence of the bilirubin-displacing drug, sulfisoxazole. These perturbations increase with the UCB to albumin molar ratio and with acidosis, pointing to diacid UCB as the cytotoxic species and implicating the UCB precipitates in the irreversible mechanisms of cytotoxicity. This late finding was corroborated by the inability of albumin to remove UCB aggregates from erythrocyte membranes, which were only recovered by chloroform. In line with the in vitro studies, blood samples from moderately jaundiced neonates present similar morphological and structural alterations. Interestingly, they also reveal higher levels of membrane-bound hemoglobin, an indicator of oxidative stress. The ability of UCB to interact with the erythrocyte membrane is also demonstrated by the induction of endovesiculation in neonatal cells either in vivo or in vitro. Fetal cells, by binding UCB more avidly and showing early signs of decreased viability, demonstrate to be sensitive targets for UCB toxicity. Altogether, such profound alterations can accelerate the normal life cycle of neonatal erythrocytes, resulting in increased senescence and precocious elimination of the cells from the circulation. Consequently, the enhanced extent of hemolysis shall further aggravate neonatal jaundice through a vicious circle. Mechanistically, the interaction of UCB with erythrocytes results in an impairment of membrane dynamic properties, namely by altering the phospholipid packing and the polarity of membrane microenvironment, perturbations that are more severe in an acidic medium. Collectively, some of the events occurring during hyperbilirubinemia are clarified, providing insights into the mechanisms of UCB cytotoxicity, essential to improve the risk assessment of UCB encephalopathy.

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