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Effect of acidosis on bilirubin-induced toxicity to human erythrocytes

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Abstract

Unconjugated bilirubin binds to erythrocytes, eliciting crenation, lipid elution and hemolysis. The present work attempts to establish the role of acidosis on bilirubin-induced toxicity to human erythrocytes. To this end, pH values ranging from 7.0–8.0 were used to induce a different representation of acid and anionic bilirubin species, respectively. Erythrocytes from healthy donors were incubated with bilirubin and albumin (3:1, molar ratio), during 4 h. Erythrocyte-bound bilirubin was evaluated by albumin or chloroform extraction in an attempt to assess either mono- and dianion bilirubin adsorbed on the cell surface or colloidal aggregates, respectively. Cytotoxicity indicators, such as the morphological index, and the extent of phospholipids and hemoglobin release were also determined. The results showed that as pH drops from 8.0–7.0, less bilirubin is removed by albumin and more become recovered by chloroform. The data corroborate the predominance of anionic and non-aggregated bilirubin species at pH 8.0 with dimers and precipitates occurring at 7.0. In accordance, crenation and cell lysis were four times increased at acidic pH. In contrast, elution of phospholipids was 1.5 times less evident at the same pH, thus suggesting that formation of bilirubin complexes with membrane phospholipids may have contributed to prevent their release. In conclusion, both anionic and acid bilirubin species interact with human erythrocytes leading to cytotoxic alterations that may determine definitive lesions. Nevertheless, increased vulnerability to crenation and hemolysis are more likely to occur in acidic conditions pointing to the bilirubin precipitates as the main candidates of bilirubin-induced toxicity to erythrocytes. (Mol Cell Biochem **247**: 155–162, 2003)

Key words: bilirubin species, erythrocyte membrane, hemolysis, morphology, phospholipid exfoliation, acidosis

Introduction

In blood plasma, unconjugated bilirubin (UCB) circulates almost entirely bound to albumin. Only one molecule of UCB is firmly bound to the high affinity binding site of the albumin molecule [1–3]. Therefore, with a normal albumin concentration of 435 μmol/l (3 g/100 ml) as much as 435 μmol/l (25 mg/100 ml) of UCB will be transported. However, manifestations of UCB toxicity, and even cases of kernicterus, were already described for UCB concentrations far below those levels [4, 5], specially in premature neonates [6, 7]. To this fact shall contribute the decreased albumin concentration, as well as its lower affinity and/or capacity for UCB binding, together with the immaturity of the detoxifying mechanisms and an eventual immaturity of the blood-brain barrier, conditions that are particularly relevant in low weight premature infants [8–10]. In addition, UCB cytotoxicity was shown to be enhanced by acidosis [11–13], a frequent condition during neonatal life.

The influence of pH on UCB binding to albumin remains to be conclusively understood. In fact, there are studies indicating that the high affinity binding constant does not vary in the range of pH from 7.0–9.0 [3, 14], while others point to its decrease with the acidification of the medium [2, 15, 16]. Regarding UCB binding to circulating cells there are several lines of evidence indicating its increase with acidosis [14, 17–20]. In addition, pH is a determinant of the distribution of the three different UCB molecular species: the protonated

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or acid, the monoanion and the dianion UCB. In fact, a pH decrease from 8.0–7.0 promotes the sharp rise of the acid UCB from 47–93% and the correspondent decrease of the ionised species from 57–7%. The protonated UCB is the predominant form at physiological pH, representing around 83% of the total UCB, and the monoanion is the major ionised species [15].

We have recently demonstrated that exposure of either intact neural cells or isolated mitochondria to UCB results in impairment of several crucial aspects of cell function, that can culminate in cell death [21–25]. Binding of UCB to erythrocytes was also demonstrated [26–28], wherein two different entities of membrane-bound UCB appear to be implicated: one entity corresponds to the UCB fraction responsible for the reversible toxic manifestations, which is removed by albumin washing of the erythrocytes, while the other respects to UCB probably aggregated within the membrane, implicated in irreversible toxicity, only extractable by chloroform [29]. As observed for other cell types, UCB toxicity towards erythrocytes increases with the pigment concentration and the UCB to albumin molar ratio, as well as with temperature and the decrease in pH [28–31]. Among the induced-toxicity, there are morphological alterations, which were observed either in erythrocytes from adults or from newborn infants, and both *in vivo* and *in vitro* [32–34]. It was also reported the release of membrane components, as well as the exposure of phosphatidylserine in the outer membrane leaflet, which shall promote the phagocyte engulfment of precocious senescent erythrocytes [35].

Our previous studies were extended, in the present work, in an attempt to establish the role of the distinct UCB molecular species and acidosis on the UCB-induced toxicity to human erythrocytes. To this end, erythrocytes were exposed to UCB at pH values ranging from 7.0–8.0, to enhance the representation of the acid or the anionic UCB species, respectively. Erythrocyte-bound UCB was evaluated by albumin or chloroform extraction in order to assess either the anion UCB adsorbed on the cell surface or colloidal aggregates, respectively. Cytotoxicity indicators, such as the morphological index, and the extent of phospholipids and hemoglobin release were also determined.

Materials and methods

Chemicals

Bilirubin IXα, human serum albumin (Fraction V, essentially fatty acid free), dithiothreitol and phosphorus standard solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A 20% solution of human albumin was acquired from Octapharma Pharmazeutika (Viena, Austria). Osmium tetroxide and propylene oxide were obtained from BDH Chemicals (Poole, UK); 2-hydrazinobenzothiazole was from Eastman Kodak Co. (Rochester, NY, USA); Titrisol sodium hydroxide, Titrisol hydrochloric acid, glutaraldehyde, and all the other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Cell preparation

Human blood was collected from seven healthy donors into 50 U/ml sodium heparin, and used within 30–60 min. Erythrocytes were separated from plasma and buffy coats by centrifugation at 600 *g* for 10 min at 4°C, and washed 3 times with cold 145 mmol/l NaCl, 5 mmol/l sodium phosphates, pH 7.4 (isotonic saline buffer). The washed erythrocytes were then resuspended, at a hematocrit of 50%, in 7.5 mmol/l phosphates, 145 mmol/l NaCl, 5 mmol/l glucose, 1 mmol/l $MgSO_4$ (incubation buffer), at pH 7.0, 7.4 or 8.0.

Interaction of bilirubin with erythrocytes

UCB was purified according to McDonagh and Assisi [36]. A 6.84 mmol/l UCB stock solution was prepared, just before use, in 100 mmol/l NaOH. Albumin stock solutions, at 0.912 mmol/l, were prepared in incubation buffer at pH 7.0, 7.4 or 8.0. Erythrocyte suspensions (at pH 7.0, 7.4 or 8.0) were added to a UCB-albumin solution, in order to obtain a 10% hematocrit, and UCB and albumin concentrations of 340 and 114 μmol/l, respectively. A 100 mmol/l HCl solution was used to restore the pH value. Cells were incubated during 4 h at 37°C, under light protection and gentle shaking. Controls, prepared in the absence of UCB, were run in parallel. A UCB to albumin molar ratio of 3 was used to produce a toxicity similar to that occurring during episodes of serious hyperbilirubinemia by severe illness in neonatal life or Crigler-Najjar type I syndrome. In these conditions cells can be exposed to UCB to albumin molar ratios as high as 1–2 during several days [37–39], an exposure that greatly surpasses the 4 h incubation period used in the present study.

Following the incubation, 600 μl of the cell suspensions were taken for determination of albumin-extractable erythrocyte-bound UCB (A-EUCB). The cell suspensions were then centrifuged at 600 g for 10 min, at 4°C, and supernatants were collected for evaluation of hemolysis and phospholipid elution. Aliquots of the pelleted erythrocytes were used for morphological analysis. The remaining cells were further incubated with a 4% albumin solution (in incubation buffer at the corresponding pH value), for 30 min, at 37°C, under gentle shaking. After centrifugation (600 g, 10 min, at 4°C), chloroform-extractable erythrocyte-bound UCB (C-EUCB) was determined in isolated erythrocyte membranes.

In order to minimise bilirubin photodegradation, all the manipulations and experiments were conducted under light protection (tin foil wrapping of the vials and dim light).

Morphological analysis

Analysis of erythrocyte morphology was performed by scanning electron microscopy, as previously described [32, 33], using a JEOL-JSM-T220 scanning microscope operating at 15 KV.

According to morphological appearance, cells were classified as discocytes, echinocytes 1, 2, 3 and spheroechinocytes [40], as shown in Fig. 1. In order to express quantitatively the extent of shape changes, the morphological index was calculated as follows:

Morphological index = Σ (morphological score) × [(number of distorted cells)/(total cell number)] [41].

Scores were considered as: 0 (discocytes), +1, +2 and +3 (echinocytes 1, 2 and 3, respectively), and +4 (spheroechinocytes). A total of 200–300 cells were counted in 4–6 fields, randomly selected.

Evaluation of erythrocyte-bound unconjugated bilirubin

Erythrocyte-bound UCB was evaluated by albumin extraction (A-EUCB) and by chloroform extraction after albumin washing of erythrocytes (C-EUCB).

A-EUCB was determined in the 10% hematocrit cell suspensions, as previously described [29]. Briefly, after three times washing with 150 mmol/l NaCl, containing 0.05% dithiothreitol, erythrocytes were incubated with an albumin solution (3.5% in the washing solution) for 10 min at room temperature. UCB was determined in the albumin phase, recovered by centrifugation (600 g, 10 min), by a colorimetric micromethod using 2-hydrazino-benzothiazol [42, 43]. Absorbance due to UCB was obtained by deducting interference of test reactives and hemoglobin eventually released during the extraction with albumin.

For quantification of C-EUCB, erythrocyte membranes (ghosts) were isolated from albumin-washed erythrocytes by the method of Dodge *et al*. [44] and protein content was determined by the method of Lowry *et al*. [45]. UCB was extracted from membranes (100 µl) with chloroform (1 ml) by vigorous mixing followed by 5 min sonication. The chloroform phase was recovered and UCB concentration was determined by direct measurement of the absorption at 454 nm (ε = 60,700), according to McDonagh [46].

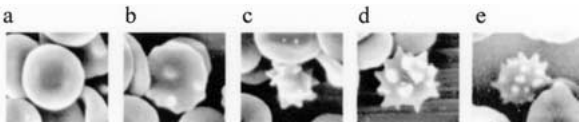

Fig. 1. Classification of human erythrocytes according to their morphology, as discocytes (a), echinocytes 1 (b), echinocytes 2 (c), echinocytes 3 (d) and spheroechinocytes (e). Details of microscopic preparation and analysis are given in 'Materials and methods'.

Evaluation of hemolysis and lipid elution

The extent of hemolysis was evaluated by direct measurement of the supernatant absorption at 542 nm, as already described [34]. Results were expressed as percentage of total hemolysis achieved by complete lysis of erythrocytes with water.

To quantify the amount of phospholipids released from erythrocytes, lipids were extracted from 3 ml aliquots of the supernatants, as usual in our laboratory [29, 47]. Lipid phosphorus was determined in 10 µl aliquots of the reconstituted extracts by using the Fiske and Subbarow reagent [48], as previously described [33], based on a calibration curve prepared using an inorganic phosphorus standard solution (20 µg/ml).

Statistical analysis

All data are expressed as means ± S.E.M. from 7 independent experiments. Comparisons were made using the unpaired two-tailed Student's *t*-test performed on the basis of equal or unequal variances, accordingly with the variance ratio test (F-test). Differences were considered statistically significant when p values were lower than 0.05.

Results

Effect of pH on bilirubin-induced morphological alterations

Evidence of UCB binding to erythrocytes is easily achieved by morphological analysis. In order to relate the extent of shape changes with the predominant UCB molecular species, the morphological index was determined following exposure of erythrocytes to UCB at pH values varying from 7.0–8.0. While controls showed a majority of normal biconcave disc appearance cells, samples treated with UCB presented a wide variety of echinocytic forms, ranging from echinocytes 1 to spheroechinocytes, besides some intact discocytes. Accordingly, the morphological index obtained following UCB interaction was higher than that obtained for controls, specially at physiologic and acidic pH ($p < 0.001$) (Fig. 2). Moreover, the quantification of the shape changes revealed that the extent of crenation progressively increases with the acidification of the medium ($r = -1.000$, $p < 0.001$).

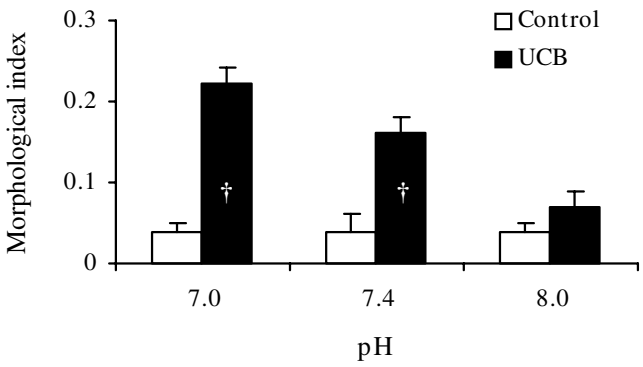

Fig. 2. Effect of pH on the morphological index of human erythrocytes. Erythrocytes were incubated in the absence of bilirubin (control), or in the presence of unconjugated bilirubin (UCB), at a bilirubin to human serum albumin molar ratio of 3, for 4 h, at 37°C, at different pH values. Details of the microscopic preparation and of the morphological index determination are given in 'Materials and methods'. Each bar represents the mean ± S.E.M. of 7 independent experiments. $^{\dagger}p < 0.001$ from control; $*p < 0.05$, $**p < 0.001$ from pH 7.0; $^{\S\S}p < 0.01$ from pH 7.4.

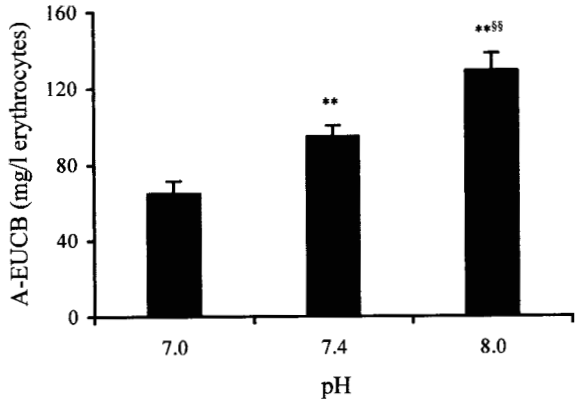

Fig. 3. Effect of pH on the concentrations of albumin extractable erythrocyte-bound unconjugated bilirubin (A-EUCB). Human erythrocytes were incubated in the absence or presence of unconjugated bilirubin, at a bilirubin to human serum albumin molar ratio of 3, for 4 h, at 37°C, at different pH values. Details of the A-EUCB determination are given in 'Materials and methods'. No A-EUCB was detected in control assays. Each bar represents the mean ± S.E.M. of 7 independent experiments. $**p < 0.001$ from pH 7.0; $^{\S\S}p < 0.001$ from pH 7.4.

Effect of pH on erythrocyte-bound bilirubin

Having verified that a decrease in pH markedly aggravates the UCB-induced changes in erythrocyte morphology, we further assessed the influence of pH on the UCB binding to erythrocytes. This interaction increases proportionally with the elevation of UCB in the medium and is irrelevant in physiological conditions. Erythrocyte-bound UCB was determined by albumin (A-EUCB) and by chloroform (C-EUCB) extraction, following incubation of cells with UCB at the three different pH values.

As expected, no A-EUCB was detected in control assays, while in UCB-treated samples significant amounts of UCB were recovered from the membrane by albumin washing (Fig. 3). Although the concentrations of A-EUCB varied in a pH-dependent manner, the profile was opposite to that observed regarding the morphological alterations. In fact, the lowest levels of A-EUCB were found in erythrocytes incubated at acidic pH, while a double concentration was detected in cells incubated with UCB in an alkaline medium ($p < 0.001$).

Another fraction of UCB, inaccessible to albumin, was recovered from erythrocyte membranes by chloroform extraction. This C-EUCB was undetectable in controls but was undoubtedly present in UCB-treated samples, varying its concentration with the pH of the medium (Fig. 4). In fact, C-EUCB levels nearly doubled as the pH decreased from 8.0 to 7.4 and 7.0 ($p < 0.05$), similarly to the observed shape changes and contrasting with the data obtained for A-EUCB.

Effect of pH on bilirubin-induced lipid elution and hemolysis

Having confirmed that pH influences the binding of UCB species to erythrocytes, probably by favouring different physical states that distinctly interact with the erythrocyte membrane, we next determined the amount of lipids and hemoglobin released to the incubation medium, as markers

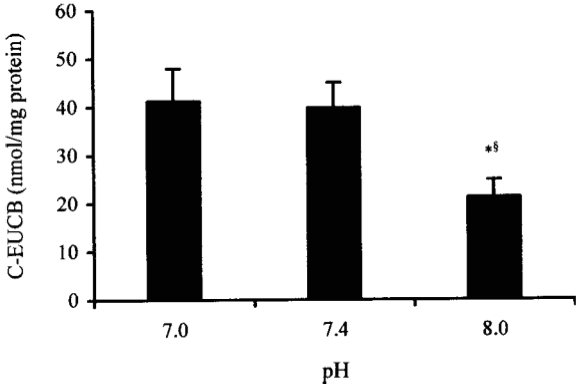

Fig. 4. Effect of pH on the concentrations of chloroform extractable erythrocyte-bound unconjugated bilirubin (C-EUCB). Human erythrocytes were incubated in the absence or presence of unconjugated bilirubin, at a bilirubin to human serum albumin molar ratio of 3, for 4 h, at 37°C, at different pH values. Details of the C-EUCB determination are given in 'Materials and methods'. No C-EUCB was detected in control assays. Each bar represents the mean ± S.E.M. of 7 independent experiments. $*p < 0.05$ from pH 7.0; $^{\S}p < 0.05$ from pH 7.4.

of definitive cell lesion. As shown in Fig. 5, UCB promoted the elution of phospholipids to the incubation medium at all the pH values. The loss of phospholipids was also pH-dependent, being the maximum release achieved at pH 8.0, followed by the one attained at pH 7.4 and finally by that at pH 7.0 ($p < 0.001$, $p < 0.01$ and $p < 0.05$ from control, respectively). In fact, the concentration of phospholipids in the supernatants obtained following incubation with UCB at pH 8.0 was 1.5 times more evident than that induced by incubation at lower pH values ($p < 0.05$), and paralleled the profile obtained for A-EUCB. In contrast, the extent of hemolysis significantly increased as the pH of the incubation medium decreased from 8.0 to 7.4 and 7.0 (Fig. 6), similarly to the data obtained for the morphological alterations and C-EUCB. It is still worthwhile to mention that all the UCB species were able to markedly increase the lysis of erythrocytes ($p < 0.01$) since hemolysis of controls was lower than 0.5% of total lysis.

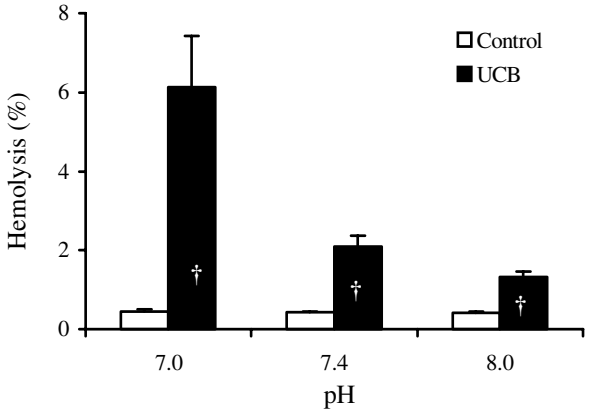

Fig. 6. Effect of pH on the bilirubin-induced lysis of human erythrocytes. Erythrocytes were incubated in the absence (control) or presence of unconjugated bilirubin (UCB), at a bilirubin to human serum albumin molar ratio of 3, for 4 h, at 37°C, at different pH values. Details of the hemolysis determination are given in 'Materials and methods'. Results are expressed as percentage of total lysis of erythrocytes with water. Each bar represents the mean ± S.E.M. of 7 independent experiments. $^{\dagger}p < 0.01$ from control; $^{*}p < 0.05$, $^{**}p < 0.01$ from pH 7.0; $^{\S}p < 0.05$ from pH 7.4.

Discussion

We have previously shown that UCB elicits toxicity to human erythrocytes, which may culminate in irreversible cell lesion, manifested by the release of membrane lipids and hemolysis [33–35]. While monomers of UCB are probably extractable from the membrane by albumin, another fraction of the pigment appears to be inaccessible to albumin, and more implicated in the irreversible toxic manifestations [29]. In the present work, those studies were extended to evaluate the interaction of UCB at different physical states with the erythrocyte membranes, by using pH values ranging from 7.0–8.0. The results obtained demonstrate that acidosis aggravates the cytotoxic effects of UCB, probably by favouring aggregation of acid UCB within the erythrocyte membrane.

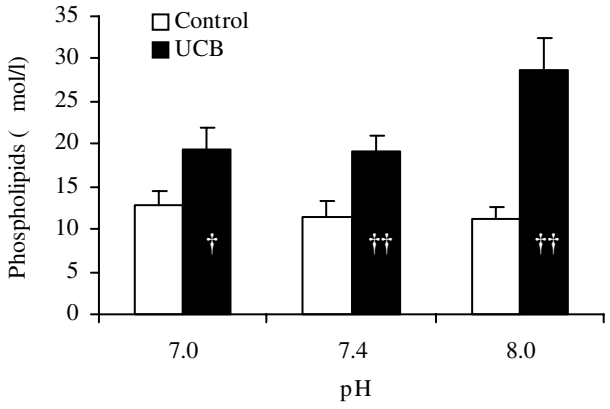

Fig. 5. Effect of pH on the bilirubin-induced elution of phospholipids from human erythrocytes. Erythrocytes were incubated in the absence (control) or in the presence of unconjugated bilirubin (UCB), at a bilirubin to human serum albumin molar ratio of 3, for 4 h, at 37°C, at different pH values. Details of the determination of the phospholipids released to the incubation medium are given in 'Materials and methods'. Results are expressed as lipid phosphorus. Each bar represents the mean ± S.E.M. of 7 independent experiments. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ from control; $^{*}p < 0.05$ from pH 7.0; $^{\S}p < 0.05$ from pH 7.4.

Morphological alterations of human erythrocytes are among the UCB-induced effects, observed both *in vivo* and *in vitro* [29, 32–34]. The increase in the echinocytosis extent with the acidification of the medium (Fig. 2) was expected since the binding and toxicity of UCB to erythrocytes was shown to rise with a reduction of pH [18, 30, 31, 49, 50]. Therefore, the acidic pH would have promoted the interaction of UCB with the erythrocyte membrane, thus leading to a sharpening of the morphological alterations.

The highest concentration of A-EUCB and the lowest levels of C-EUCB observed at pH 8.0, together with the opposite profile at pH 7.0, suggest that at alkaline pH occurs a superficial membrane deposition of UCB as monomers, which are apparently eluted by albumin washing (Fig. 3). In contrast, an acidic pH would favour the formation of UCB aggregates which are only recovered by chloroform (Fig. 4). This concept is highly relevant because it explains why a correlation of hyperbilirubinemia and erythrocyte-bound UCB, evaluated by albumin extraction, was never found in jaundiced neonates.

Based on previously proposed models for the interaction of UCB with erythrocyte membranes, liposomes and synaptosomes [51–53], the dominant anionic UCB species at pH 8.0 would interact with the polar head groups of lipids at the membrane interface. In contrast, UCB aggregation and precipitation would be favoured at pH 7.0, where acid species prevail. So, and in agreement with the studies of UCB interaction with the neuroblastoma cell line N-115 [19], the

UCB molecules involved in the formation of electrostatic interactions with the polar groups of erythrocyte membrane lipids would correspond to the fraction removed from the membrane by albumin. On the other hand, aggregated UCB in the membrane, progressively originating acid UCB microcrystals [53], shall correspond to the UCB remnant in the membrane after albumin washing, responsible for the irreversible damage that culminates in cell lysis (Fig. 6). This assumption is supported by the profiles obtained for C-EUCB and hemolysis (Figs 4 and 6), both increasing when the pH drops from 8.0–7.0 ($p < 0.05$ and $p < 0.01$, respectively). Similar findings were achieved when erythrocytes obtained from the umbilical cord blood were used, although the effects were remarkably enhanced, which is in accordance with an increased vulnerability of fetal erythrocytes to UCB toxicity [54].

The aggravating toxicity of UCB to erythrocytes by acidosis is in line with the UCB-induced alterations of the membrane dynamic properties of erythrocytes under the same pH range [55]. In fact, a sharp increase in the lipid fluidity and polarity was observed upon a pH decrease, also pointing to the uncharged or acid UCB as the species preferentially interacting with the membrane leaflet and eliciting toxicity.

The loss of lipids is a characteristic feature resulting from the interaction of UCB with erythrocyte membranes, already referred [29, 33, 56]. The lower levels of phospholipids released to the aqueous medium at pH 7.0 and 7.4, as compared to pH 8.0 (Fig. 5), may result from an increased complexation of UCB with the membrane phospholipids, thereby preventing their elution. This concept agrees with the observations of Eriksen *et al*. [51] and Vázquez *et al*. [53] indicating that the protonation of UCB and its aggregation with phospholipids is favoured by low pH values. The absence of parallelism between the release of phospholipids and the hemolysis profiles indicate that the mechanisms of UCB-induced hemolysis and lipids release are independent. On the other side, the simultaneous increase of phospholipids exfoliation and the A-EUCB concentrations at pH 8.0, suggests that binding of UCB monomers may be somehow facilitated by membrane lipid depletion.

In accordance with the model proposed for the hemolytic process induced by surfactants [57], it is conceivable that adsorption of UCB monomers at the cell surface is followed by the formation of oligomers and aggregates within the membrane, favoured by acidosis. The interaction of UCB with membranes showed to induce alterations of membrane properties, such as permeability and phospholipid packing. These events were observed following exposure of both human erythrocytes and rat mitochondria to the pigment [25, 55], which indicates a generic perturbation mechanism. Moreover, alterations on the composition and organisation of the lipid bilayer with exposure of phosphatidylserine in the outer leaflet also occur [35], which shall facilitate the rupture of erythrocytes and hemoglobin release. The increase in the extent of morphological alterations and hemolysis, as well as on the C-EUCB levels, showed to increase as the pH decreased from 8.0 to 7.4 and 7.0, thus corroborating the protonated or acid UCB as the species accelerating drastic and irreversible cell damage.

Taken together, the results obtained in this study demonstrate that UCB binding to erythrocyte membranes involves both acid and anionic species. Moreover, they show that the different molecular species interact with the membrane by distinct pathways, leading to cytotoxic alterations that may culminate in cell lysis. In fact, although anionic species revealed ability to promote cell alterations, and since UCB is recovered by albumin, the data indicate that those changes probably result from a superficial deposition of UCB monomers. In contrast, acid UCB tends to form aggregates inducing the most marked toxic signs, namely the highest extension of morphological changes and hemolysis. The increased vulnerability to cytotoxicity in acidic conditions, that frequently occur in neonatal hyperbilirubinemia, points to the bilirubin precipitates as the main candidates of bilirubin-induced toxicity to human erythrocytes.

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