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Iron Release in Erythrocytes and Plasma Non Protein-bound Iron in Hypoxic and Non Hypoxic Newborns

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Iron is released in a desferrioxamine (DFO)-chelatable form (DCI) when erythrocytes are challenged by an oxidative stress. In β-thalassemic erythrocytes, both DCI content and release (after aerobic incubation for 24 h) are increased and correlated with the fetal hemoglobin (HbF) levels. Since erythrocytes from newborns have an extremely high content of HbF and are exposed to conditions of oxidative stress, the release of iron in these erythrocytes was investigated. The erythrocyte DCI content was increased in preterm but not in term newborns as compared to adults, while the release was increased in both preterm and term erythrocytes. The level of plasma non protein-bound iron (NPBI), which was not detectable in adults, was much higher in preterm than in term newborns. When term plus preterm newborns were divided in two groups, normoxic and hypoxic, according to cord blood pH, it was found that both iron release and NBPI were markedly higher in the hypoxic newborns compared to normoxic ones. Similar results were also obtained when the preterm and term infants were considered separately on the basis of cord blood pH. Therefore, iron release and NPBI are higher when conditions of hypoxia occur. In fact, when the values for iron release and NPBI were separately plotted against cord blood pH values, significant negative correlations were seen in both cases. NPBI was correlated with iron release seen in all the newborns and a significant part of the released iron could be recovered into the incubation medium at the end of the incubation.

Keywords: Iron release; Erythrocytes; Oxidative stress; Newborns; Plasma non protein-bound iron; Hypoxia

INTRODUCTION

It is well known that redox cycling of iron promotes the Fenton reaction in which the potent oxidant hydroxyl radical is produced.[1–4] Also it is known that iron, to be redox-cycling-active, has to be released from its macromolecular complexes (mainly transport or store proteins).[5,6] Our previous studies have shown[7–9] that iron is released in a desferrioxamine (DFO)-chelatable form (DCI) (some times referred to as "free iron") when mouse erythrocytes are incubated with a number of oxidizing agents, such as phenylhydrazine, divicine, isouramil, acrolein, phenylhydroxylamine, dapsone hydroxylamine, etc. Iron is released from hemoglobin or heme[10] and the release is accompanied by methemoglobin (MetHb) formation.[8] When the cells are depleted of glutathione (GSH) the release is also accompanied by lipid peroxidation and hemolysis.[7,8,11] Intracellular chelation of the released iron prevents such alterations,[8] which suggests that free iron promotes oxidative membrane damage acting from the inside of the cell.

A similar release of iron also occurs during erythrocyte ageing, experimentally reproduced by a prolonged (48–60 h) aerobic incubation of erythrocytes in buffer.[12] The release is followed by oxidative alterations of membrane proteins as well

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as by the appearance of senescent cell antigen, a signal for termination of old erythrocytes.[12–14]

In hemolytic anemias by hereditary hemoglobin alterations an accelerated removal of erythrocytes occurs and an increased susceptibility to oxidative damage has been reported.[15–18] We have observed that erythrocytes from patients with major and intermedia β-thalassemia show an increased DCI content and an increased susceptibility to release iron after aerobic incubation (24 h), which suggests that these erythrocytes are more susceptible to oxidative stress.[19] Both DCI content and release significantly correlated with the level of fetal hemoglobin (HbF) in β-thalassemic erythrocytes (major and intermedia).[19] This strongly suggests that the presence of HbF is a condition favourable to iron release and consequently to oxidative stress. The markedly increased level of "free iron" may represent, in this case too, the trigger for events responsible for the early removal of erythrocytes from the blood stream.[19]

Erythrocytes from newborn infants show a decreased lifespan,[20] an extremely high content of HbF (nearly 100%), and an increased susceptibility to oxidant induced injury.[21,22] HbF has greater oxygen affinity than adult hemoglobin (HbA) and is likely more subjected to denaturation and oxidation.[23,24] It has been repeatedly suggested that newborns are exposed to conditions of oxidative stress resulting from the change from a low oxygen pressure in utero to a high oxygen pressure at birth.[25,26] Also it is known that in several newborns non protein-bound iron (NPBI) is detectable in plasma.[27–29] This iron was initially looked for and detected in patients with ≥100% transferrin saturation,[30,31] but subsequently, as techniques for its detection became more sophisticated, non-transferrin-bound iron (NTBI), subsequently referred to as NPBI, was also found in conditions where transferrin was not fully saturated.[27] This led to a revision of the original view of NPBI as a simple spillover phenomenon. Anyway the origin of this form (or forms) of iron which is also detectable in plasma of many patients with iron overload, such as primary and secondary hemocromatosis,[32,33] is not clear. In view of some similarities between newborn and thalassemic erythrocytes, mainly related to increased HbF level, decreased lifespan, appearance of plasma NPBI and increased susceptibility to oxidative stress, we studied in term and preterm newborns the erythrocyte DCI content and the release after oxidative stress (aerobic incubation) and the possible correlations between these parameters and plasma NPBI. Since newborns are exposed to hypoxic conditions, as mentioned above, and since the most important marker of hypoxia is the cord blood pH, we studied iron release in newborns with different pH values and the respective correlations with plasma NPBI. The results suggest that the release of iron from erythrocytes in these different conditions may contribute to the appearance of plasma NPBI.

MATERIALS AND METHODS

Subjects: One hundred and thirty newborn infants (64 term: 45 male, 19 female; 66 preterm: 29 male, 37 female) were examined at birth. Gestational age ranged from 37 to 41 weeks (38.8 ± 0. 2) for term and from 24 to 36 weeks (32.7 ± 0.4) for preterm infants. Birthweight ranged from 1.92 to 5.30 kg (3.24 ± 0.07) for term and from 0.59 to 2.82 kg (1.92 ± 0.07) for preterm infants. Thirty-seven babies (25 term, 12 preterm) were born by vaginal delivery; sixty-nine babies (34 term, 35 preterm) were born by elective caesarean section; twenty-four babies (5 term, 19 preterm) were born by emergency caesarean section. Malformed or suspected septic subjects were not enrolled in this study. HbF levels ranged between 82 and 100%. No subjects with thalassemias or other abnormal hemoglobins or with G-6-PD deficiency were present in this study.

The babies were then divided in two groups on the base of the pH value of cord blood.[34] The pH value of 7.21 was chosen as a cut off value taking into account that the normal values of venous blood which are reported in the literature are 7.32 ± 0.055 (mean ± standard deviation). The range of values is 7.18–7.41.[35]

Heparinized blood samples were obtained from the umbilical vein, immediately after cord clamping. Venous blood was also taken from a control group of 28 healthy adults.

Informed consent was obtained from the parents of the newborn infants and from the adult controls. The study was approved by the Human Ethics Committee of the Medical Faculty of the University of Siena.

Erythrocyte incubation: The erythrocytes were prepared by centrifugation from heparinized blood and washed three times with 0.123 M NaCl, 28 mM sodium phosphate/potassium phosphate buffer, pH 7.4, and resuspended in the same buffer as a 50% (v/v) suspension as previously reported.[7,8,12,19] Iron contamination was removed from buffer as previously described.[7] The incubation was carried out aerobically in a shaker apparatus for 24 h at 37°C in the presence of antibiotics (20 units penicillin and 20 μg streptomycin/ml of buffer). Anaerobiosis was obtained by bubbling separate erythrocyte suspensions with a nitrogen stream for the entire incubation period. At 0 time and at the end of incubation, samples were withdrawn for the determination of DCI, MetHb[36] and hemolysis.[7] DCI was determined as a DFO-iron complex (ferrioxamine) as

TABLE I Desferrioxamine-chelatable iron (DCI) and methemoglobin (MetHb) in erythrocytes from newborns and adults at 0 time (real content) and after 24 h of aerobic incubation

	Incubation time (hours)	DCI (nmol/ml)	MetHb (nmol/ml)	NPBI (nmol/ml)
Newborns (term + preterm)	0	2.4 ± 0.2*[a]	92 ± 4*[c]	2.53 ± 0.29
	24	12.9 ± 0.6***	645 ± 26***	–
Term newborns	0	1.9 ± 0.2	85 ± 5	1.54 ± 0.26
	24	12.5 ± 1.0**[a]	613 ± 31***	–
Preterm newborns	0	2.7 ± 0.3*[b]	96 ± 6**[b]	3.35 ± 0.50**[c]
	24	13.1 ± 0.8***	672 ± 41***	–
Adults	0	1.4 ± 0.2	68 ± 6	n.d.
	24	7.4 ± 0.7	341 ± 30	–

The plasma non protein-bound iron (NPBI) is also shown. The results reported derive from determinations carried out in 130 newborns and 28 adult controls. In some cases (about 8%) it was not possible to run all the determinations, due to the limited amount of blood available. The results (means ± SEM) are expressed as nmol/ml of incubation mixture or nmol/ml plasma. n.d.: not detectable *$p < 0.05$ [a]compared to adult 0 time value; [b]compared to term newborn 0 time value; [c]compared to adult 0 time value. **$p < 0.01$ [a]compared to adult 24 h time value; [b]compared to adult 0 time value; [c]compared to NPBI in term newborns. ***$p < 0.001$ compared to adult 24 h time value.

previously reported.[7] Briefly, at the end of the incubation, 25 μM DFO was added to the samples. The erythrocytes were then hemolysed by adding water (1 vol) and by freeze (−70°C)-thawing. The hemolysate was ultrafiltered in centrifugal filter devices (Centriplus® YM-30; Amicon, Millipore) and the excess of DFO was removed by silicic acid column chromatography. The DFO-iron complex was determined by HPLC.[7]

In order to evaluate whether DCI released in erythrocytes after aerobic incubation diffuses from the cells into the incubation medium, erythrocytes from adult subjects were incubated for 24 h as above. After the incubation the samples were centrifuged at 600*g* for 10 min and DCI was measured in both packed cells and incubation medium as above.

Plasma NPBI: After centrifugation of the heparinized blood samples, the plasma was used for NPBI determination. The latter was performed essentially according to Singh *et al*.[37] Briefly this assay relies on the use of a large excess of a low affinity ligand (nitrilotriacetic acid, NTA) which complexes the iron non specifically bound to plasma proteins, but does not remove that bound to transferrin or ferritin. The Fe-NTA complex was then ultrafiltered as reported[38] and quantified using an HPLC procedure in which on-column derivatization with a high affinity iron chelator (3-hydroxy-1-propyl-2-methyl-pyridin-4-one, CP22) is used. In this way, iron bound to NTA is converted to form the colored $(CP22)_3$-Fe complex which absorbs in the visible region at 450 nm.

Other determinations: pH and base excess (BE) were measured with a Radiometer ABL505 pH meter (Copenhagen, DK), immediately after blood sampling.

RESULTS

As shown in Table I, erythrocytes from newborns (term plus preterm) present an higher DCI content (0 time value) as compared to adult controls. Upon aerobic incubation for 24 h, the release of iron was much greater in newborn than in adult erythrocytes, suggesting that newborn red blood cells are more susceptible to oxidative stress. MetHb content (0 time) and formation (after 24 h incubation) were higher in newborn than in adult erythrocytes and a positive significant correlation ($r = 0.270$; $p < 0.01$) was found in newborn erythrocytes between iron release and Met-Hb formation. No relevant hemolysis was observed at 0 time and after incubation. No iron release or Met-Hb formation occurred after 24 h anaerobic (under nitrogen) incubation. Incubation in the presence of glucose (6 mM) delayed the release of iron[†] and this was probably due to the decrease of oxidative stress produced by the aerobic incubation. Such a decrease could be caused by a recharge of NADPH and thus of GSH.

Plasma NPBI was detectable in 69% of newborns, while it was not detectable, as noticed by many others,[39,40] in healthy adults (controls). As shown in Fig. 1), when the individual values for iron release seen in newborn erythrocytes were plotted against the corresponding values for plasma NPBI a positive correlation was observed.

When the newborns were divided in two groups (term and preterm) on the ground of gestational age,

[†]The DCI value for 24 h of aerobic incubation in glucose supplemented (g.s.) adult erythrocytes was 5.8 ± 0.3 versus 7.4 ± 0.7 nmol/ml (see Table I) in non supplemented (n.g.s.) cells.The delay in iron release was even more evident after 48 h of aerobic incubation (DCI 8.8 ± 1.1 in g.s. erythrocytes versus 21.7 ± 1.6 nmol/ml in n.g.s. cells). At this time, the erythrocytes GSH decrease (0 time values 884 ± 22 nmol/ml) in g.s. erythrocytes was 48.1% versus 89.1% in n.g.s. cells. The formation of MetHb was also delayed.

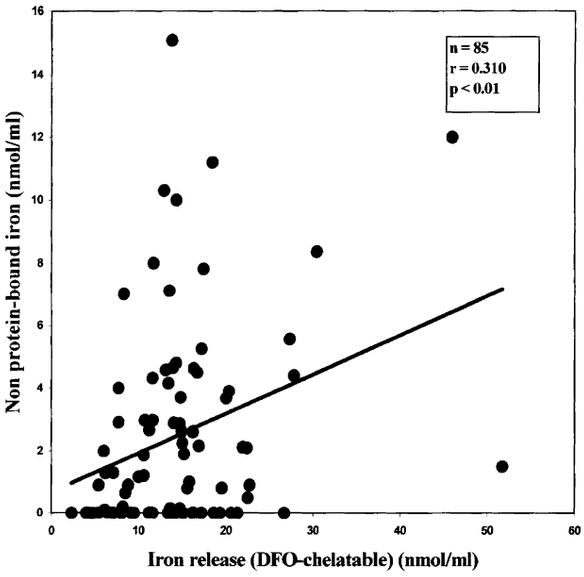

FIGURE 1 Correlation between iron release (24 h incubation) in erythrocytes from newborns (term plus preterm) and plasma non protein-bound iron (NPBI).

the erythrocytes from preterm infants showed (Table I) a higher DCI content as compared to term infants in whom the DCI level was not different from that of adults. The iron release after aerobic incubation was increased in both term and preterm erythrocytes in comparison to adult controls. Likewise the MetHb content and formation were higher in both groups of babies. Plasma NPBI was detectable in 58% of term infants and in 78% of preterm infants and the level was higher in preterm than in term infants.

The newborns (term plus preterm) were divided in two groups, normoxic and hypoxic, on the base of the pH cord blood value. As shown in Table II, it was found that both iron release (after aerobic incubation) and plasma NPBI were markedly higher in the newborns with pH $\leq$ 7.21 as compared to those with pH $>$ 7.21. Plasma NPBI was detectable in 64% of the non hypoxic infants and in 83% of the hypoxic infants. No difference was observed in MetHb (content and formation) between the two groups. Similar results were also obtained when the preterm and term newborns were considered separately still on the base of cord blood pH (Table III). Therefore, iron release and plasma NPBI are higher when conditions of hypoxia occurs. In fact, when the values for iron release and plasma NPBI were separately plotted against cord blood pH values, significant negative correlations were seen in both cases (Fig. 2 A and B, respectively). Likewise, significant negative correlations were seen (Fig. 3 A and B, respectively) when the same values for iron release and plasma NPBI were separately plotted against cord blood BE values.

Since a significative correlation between iron release in the erythrocytes and NPBI in plasma was observed and since the origin of NPBI is not yet clearly known, further experiments were carried out to investigate whether the iron released in erythrocytes after aerobic incubation could cross the erythrocyte membrane and diffuse outside. To this aim, DCI was measured in control erythrocytes (50% suspension) (Fig. 4 A) and in both packed cells and incubation medium (Fig. 4 B) after the sample was centrifuged at the end of aerobic incubation. It was observed that iron released in the erythrocytes diffused into the incubation medium in which it could be recovered at the 66% level of that detected in the cells. Similar determinations with the blood of newborns did not give reliable results due to the little amounts of blood available after the other measurements.

DISCUSSION

The present work shows that erythrocytes of newborns exhibit a higher content of DCI as compared to adults (Table I), which confirms the results of a previous paper from our group.[41] Also, erythrocytes of preterm show a higher content of DCI as compared to term infants. Upon aerobic incubation a higher release of iron occurs in erythrocytes of newborns in comparison to adults, indicating that newborn erythrocytes are more susceptible to oxidative stress than adult ones. The release of iron is much higher in newborns with hypoxia at birth (cord blood pH $\leq$ 7.21) as compared to non hypoxic newborns (Table II), suggesting that an "hypoxic

TABLE II Desferrioxamine-chelatable iron (DCI) and methemoglobin (MetHb) in erythrocytes from non hypoxic (pH $>$ 7.21) and hypoxic (pH $\leq$ 7.21) newborns, at 0 time (real content) and after 24 h of aerobic incubation

	Incubation time (hours)	DCI (nmol/ml)	MetHb (nmol/ml)	NPBI (nmol/ml)
pH $>$ 7.21	0	2.4 $\pm$ 0.2	97 $\pm$ 5	2.05 $\pm$ 0.32
(7.31 $\pm$ 0.01)	24	11.5 $\pm$ 0.6	623 $\pm$ 31	–
pH $\leq$ 7.21	0	1.8 $\pm$ 0.2	82 $\pm$ 8	3.63 $\pm$ 0.74*[b]
(7.13 $\pm$ 0.02)	24	16.4 $\pm$ 2.1*[a]	701 $\pm$ 62	–

The plasma non protein-bound iron (NPBI) is also shown. The results are the means $\pm$ SEM of 87 non hypoxic newborns and 29 hypoxic newborns. Results are expressed as nmol/ml of incubation mixture or nmol/ml plasma. *$p < 0.05$ [a]compared to non hypoxic newborn 24 h time value; [b]compared to non hypoxic newborns.

TABLE III Desferrioxamine-chelatable iron (DCI) and methemoglobin (MetHb) in erythrocytes from term and preterm newborns divided on the ground of cord blood pH value, at 0 time (real content) and after 24 h of aerobic incubation

	pH value	Incubation time (hours)	DCI (nmol/ml)	MetHb (nmol/ml)	NPBI (nmol/ml)
Term newborns	>7.21[36]	0	1.6 ± 0.2	94 ± 8	1.17 ± 0.28
		24	9.6 ± 0.8	599 ± 28	–
	≤7.2[19]	0	2.0 ± 0.3	77 ± 9	2.38 ± 0.54**
		24	17.2 ± 2.7*	623 ± 82	–
Preterm newborns	>7.21[51]	0	2.9 ± 0.4	99 ± 7	2.60 ± 0.46
		24	12.8 ± 0.8	646 ± 49	–
	≤7.21[10]	0	1.5 ± 0.2	90 ± 13	5.70 ± 1.63***
		24	15.1 ± 3.2	812 ± 90	–

The plasma non protein-bound iron (NPBI) is also shown. The results are the means ± SEM. The number of samples is in brackets. Results are expressed as nmol/ml of incubation mixture or nmol/ml plasma. *$p < 0.01$ compared to non hypoxic newborn 24 h time value. **$p < 0.05$ compared to non hypoxic newborns. ***$p < 0.02$ compared to non hypoxic newborn.

environment" is a factor in promoting iron release. Thus the aerobic incubation, when imposed to erythrocytes formerly stressed, such as those of hypoxic newborns, can reveal conditions favourable to iron release.

Iron release in erythrocytes seems to be related to the conditions of the infants at birth, as also shown by the significant correlations between iron release and cord blood pH (Fig. 2 A) and iron release and BE

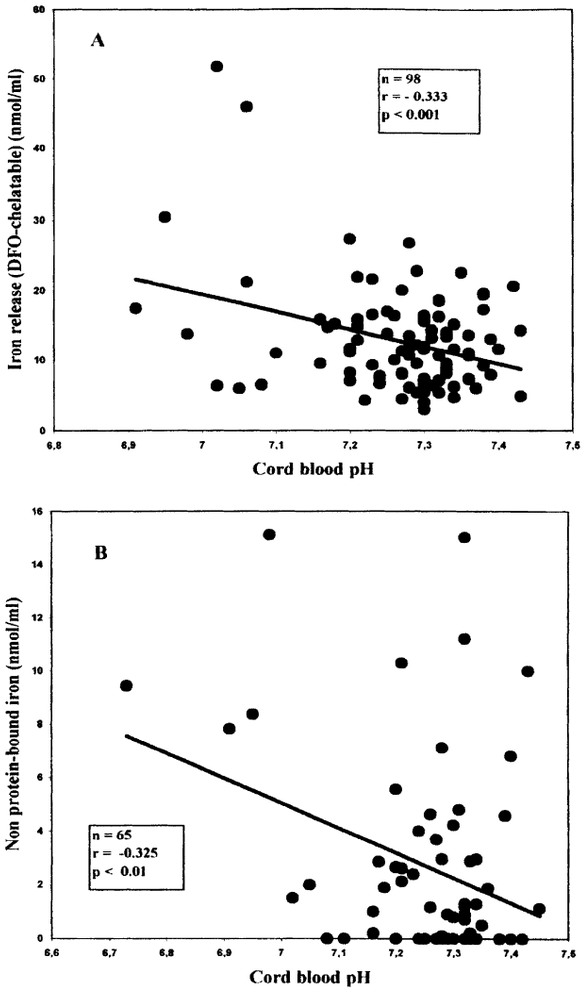

FIGURE 2 (A) Correlation between iron release (24 h incubation) in erythrocytes and cord blood pH in newborns (term plus preterm). (B) Correlation between plasma non protein-bound iron (NPBI) and cord blood pH in newborns (term plus preterm).

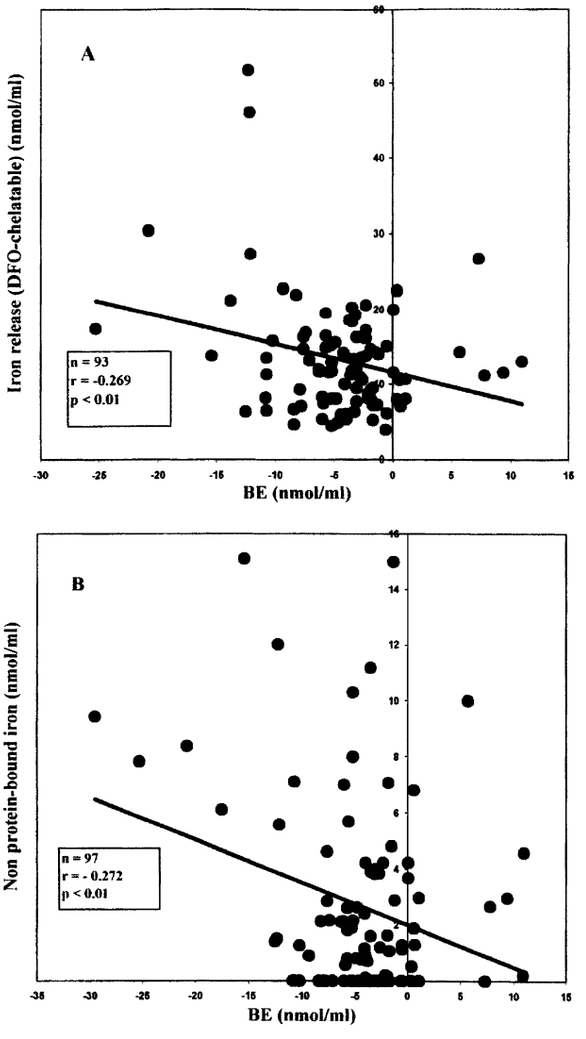

FIGURE 3 (A) Correlation between iron release (24 h incubation) in erythrocytes and cord blood base excess (BE) in newborns (term plus preterm). (B) Correlation between plasma non protein-bound iron (NPBI) and cord blood base excess (BE) in newborns (term plus preterm).

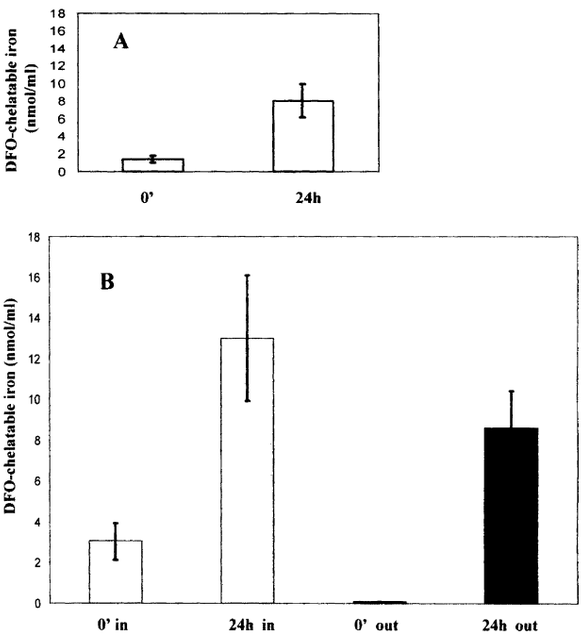

FIGURE 4 Diffusion of iron released from erythrocytes into the incubation medium. (A) DFO-chelatable iron in the incubation mixture (50% suspension of erythrocytes) at 0 time and after aerobic incubation (24 h). (B) DFO-chelatable iron in both packed cells (in) and incubation medium (out), at 0 time and after aerobic incubation (24 h). The results are the means ± SEM of 5 experiments.

values (Fig. 3 A). Furthermore, iron release is correlated with plasma NPBI (Fig. 1) which is higher in preterm than in term (Table I) and in hypoxic than in non hypoxic infants (Table II). Also NPBI is correlated with the cord blood pH (Fig. 2 B) and BE values (Fig. 3 B). So it appears that hypoxia increases iron release in erythrocytes and NBPI in plasma. It must be observed that the above correlations, although significant, are with a low *r* value, which indicates that they are somewhat weak. It should be considered, however, that they are all in the same direction (iron release and NPBI versus acidosis) and that in several instances *in vitro* versus *in vivo* results were considered.

The hypoxia-induced increase in iron release is consistent with the observation[42] that during the hypoxic incubation of partially oxygenated hemoglobin superoxide radical is formed, the production of which coincides with autoxidation of hemoglobin. The hypoxic autoxidation of hemoglobin involves predominantly an inner-sphere mechanism with an electron transferred from Fe (II) to bound oxygen leading to oxidized hemoglobin and superoxide radical.[42] If similar events are going to occur in hypoxic newborn erythrocytes, the subsequent aerobic incubation could find an environment favourable to induce redox cycling of iron, with further increase in formation of reactive oxygen species and release of iron.

As mentioned above, the presence of NPBI in plasma is difficult to be explained. As is known, in plasma iron is bound to transferrin in the ferric form (Fe^{3+}). In healthy adults, transferrin is only 20/35% saturated and the conversion of ferrous to ferric iron is facilitated by the ferroxidase activity of caeruloplasmin.[43] In plasma of newborns, particularly premature babies, the presence of NPBI could be explained by the low level of circulating transferring[27,44] and caeruloplasmin.[45] However, Evans *et al.*[27] showed that plasma NPBI is detectable, despite the coexistence of transferrin iron-binding capacity. Dorrepal *et al.*[29] observed that NPBI was significantly elevated in severely asphyxiated infants. They suggested that lowering of the plasma pH, as occurs during ischemia,[46] enables transferrin to liberate its iron. On the other hand, we have observed (unpublished results) that in newborns there is no correlation between the levels of plasma NPBI and transferrin, suggesting again that the presence of NPBI does not depend exclusively upon the low levels of this protein. In the present study, the significant correlation between iron release in the erythrocytes and NPBI, the demonstration that both are higher in hypoxic newborns and both are correlated with pH or BE cord blood values suggest that plasma NPBI derives, at least in part, from the red cells. This suggestion is strengthened by the demonstration that iron released after aerobic incubation is able to diffuse from the erythrocytes into the incubation medium, in which it can be recovered roughly at the 66% level of that detected into the cells. It should be observed that the concentration of DCI in the incubation medium is indicative of its diffusion across the erythrocytes membrane, but not of the total amount diffused. In fact, the solubility of iron (ferric form) in salt solutions (such as the buffer) is extremely low,[39] and unless ligands capable of forming multiple coordination points for stable binding are available, it forms insoluble aggregates from which it cannot be extracted by DFO.[47]

In conclusion, our studies seem to indicate that in erythrocytes of newborns the release of iron is higher than in those of adults, and that such release is higher in hypoxic than in non hypoxic babies. Such release could explain, at least in part, the appearance of plasma NPBI, even if this latter event needs further delucidations.

A number of studies of Hebbel and coworkers[18,48–50] have shown that iron decompartmentalization occurs in thalassemic and sickle red cells and that iron can be found associated with the cytoplasmic surface of the membrane, in which several discrete iron compartments (denatured hemoglobin, free heme, molecular iron, etc.) can be demonstrated.

In particular, molecular iron[50] would be able to cycle between ferric and ferrous states and thereby participate in redox reactions. Our observation that part of the released iron diffuses from the erythrocytes into the incubation medium does not exclude that a considerable part of the delocalized iron remains associated to the membrane. Probably this membrane associated iron can be chelated by DFO thus accounting for the "free iron" level that we found in the cells.

With regard to the potential sources of iron release, our previous studies[10] carried out with reconstituted systems of erythrocyte lysate containing ghosts and different fractions of erythrocyte cytosol, incubated in the presence of oxidants, strongly suggested that iron is released from hemoglobin. The study of the mechanism of release (oxidation and degradation of heme moiety) was beyond the purpose of the present investigation. It must be considered, however, that it has been shown[51–53] that iron release occurs after hemoglobin autoxidation, formation of reactive oxygen species and loss of normal tetragonal symmetry around heme iron. In our studies, measurement of heme moiety before and after 24 h of incubation did not show significant decrease in both adult and newborn erythrocytes. Also the spectrophotometric analysis[54] of the ultrafiltered (protein free) supernatant fraction of an erythrocyte lysate to detect possible presence of heme or porphirin gave negative results. Negative results were also obtained with the spectrofluorimetric analysis[53] of the same ultrafiltrate to detect possible heme degradation products. Further studies are necessary to clarify these points. In any case the amount of released iron is minimal as compared to that present in erythrocyte hemoglobin. Other sources of iron release such as ferritin or transferrin cannot account significantly for the iron release since these compounds are present in very low amount in erythrocytes.

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