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Plasma Esterified F_2-Isoprostanes and Oxidative Stress in Newborns: Role of Nonprotein-Bound Iron

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ABSTRACT: Nonprotein-bound iron (NPBI) and F_2-isoprostanes, reliable markers of oxidative stress, are increased in plasma of newborns and inversely correlated to the gestational age. Because NPBI represents a pro-oxidant stimulus in plasma, we test the hypothesis that the entity of lipid peroxidation is related with NPBI concentrations. Plasma levels of free, esterified, and total F_2-isoprostanes were investigated in relation to NPBI levels in 59 newborns and 16 healthy adults. The pro-oxidant role of iron was ascertained *in vitro*, by measuring all the forms of F_2-isoprostanes after incubation with ammonium iron sulfate. Significant positive correlations were found between NPBI and total as well as esterified F_2-isoprostanes in plasma of the newborns. The addition of ammonium iron sulfate induced a marked increase in all the forms of F_2-isoprostanes after 2 hours of incubation. The higher NPBI concentration, the higher F_2-isoprostanes levels. An increase NPBI dose dependent in total F_2-isoprostanes formation was observed in dialyzed low density lipoprotein from adult plasma. The results clearly show that once NPBI is generated, whatever its source, it is capable of inducing oxidative stress. NPBI-induced oxidative stress may contribute to the morbidity in preterm infants that are particularly susceptible to free radical damage. **(*Pediatr Res* 63: 287–291, 2008)**

Reactive oxygen species are thought to be responsible for a variety of diseases of preterm infants, such as bronchopulmonary dysplasia, retinopathy of prematurity, hypoxic/ischemic encephalopathy (1), and are considered to play a key role in some maternal pregnancy diseases and fetal development (2–5). Free radical (FR) damage in the neonate may result from both accelerated FR production and low antioxidant levels (6,7). Among the possible pro-oxidant factors in fetus and newborns, the presence of "free iron," plasma nonprotein-bound iron (NPBI), potentially redox active (8–11) must be considered. In perinatal period and especially in premature infants, low levels of transferrin, decreased transferrin iron-binding capacity, and low levels of ceruloplasmin and ferroxidase activity may contribute to the appearance of NPBI in amniotic fluid and in plasma (6,7). NPBI has been shown to be related to the severity of the postasphyxial injury and alteration of neurodevelopmental outcome until the second year of age (9,12). Significantly different levels of NPBI have been also found correlated (13) to the intraerythrocyte concentrations of NPBI.

F_2-isoprostanes, a series of prostaglandin F_2-like compounds formed *in vivo* and *in vitro* by FR-catalyzed peroxidation of phospholipids-bound arachidonic acid (a pathway which is independent of the cyclooxygenase pathway), are considered nowadays as the most reliable markers of oxidative stress (14–15). They are initially formed *in situ* on phospholipids (esterified isoprostanes) of membrane cells and released in a free form in plasma (16). Because they are less reactive than other lipid peroxidation products, such as lipid hydroperoxides and aldehydes, free F_2-isoprostanes can be found more easily in plasma and urine.

We previously found significantly higher levels of plasma-free F_2-isoprostanes in cord blood from newborns, compared with healthy adults. Plasma-free F_2isoprostane levels were inversely correlated with the gestational age (17). No correlation was found between plasma F_2-isoprostanes in their free form and NPBI. At that time, the other forms of F_2-isoprostanes total (sum of free and esterified), and esterified were not considered.

Recently, Salahudeen *et al.* (18) demonstrated that the i.v. iron infusion in patients on hemodialysis does not change plasma levels of free F_2-isoprostanes, whereas significantly increasing plasma-esterified isoprostanes, which allegedly derive from the peroxidation of plasma lipids. They hypothesized that free and esterified F_2-isoprostanes originate mainly from generalized or plasma compartmentalized lipid peroxidation, respectively, and argued that lipids in plasma could be exposed to a higher concentration of iron during i.v. infusion than lipids in peripheral tissues. Dani *et al.* (19) found a significant increase in NPBI plasma levels after packed red blood cells transfusion in newborns, but it was not associated with significant changes in markers of oxidative stress.

The present study aims: 1) to evaluate the levels of plasma-free, esterified, and total F_2-isoprostanes in relation to NPBI concentrations in newborns at birth; 2) to test the hypothesis that the entity of lipid peroxidation is related with NPBI concentrations.

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Abbreviations: FR, free radicals; **NPBI,** non protein-bound iron

MATERIALS AND METHODS

Subjects. Fifty-nine newborns, (30 terms and 29 preterms), and 16 healthy adults as control group, were the subject of the study (for clinical characteristics see Table 1).

Blood samples were obtained from the umbilical vein, immediately after cord clamping and from the cubital vein adults.

The study was approved by the Human Ethics Committee of the Medical Faculty of Siena University. Informed written parental consent was obtained before enrolment of each infant.

Platelet poor plasma was obtained from heparinized blood by centrifugation at 2400*g*. For F_2-isoprostane determination, butylated hydroxytoluene (90 μM) was added to plasma as an antioxidant and aliquots were stored under nitrogen at −70°C until analysis (up to 3 mo).

NPBI and free isoprostanes were measured in all subjects. Total and esterified isoprostanes were measured in 11 preterm and 11 term newborns out of 59 when plasma amount allowed.

Extraction and purification procedure for plasma-free F_2-isoprostane determination. Plasma (1 mL) was spiked with $PGF_{2\alpha}$-d_4 (500 pg in 50 μL ethanol; Cayman, Ann Arbor, MI) as an internal standard. To each sample, 2 mL of acidified water (pH 3.0) was added. After an equilibration time of 15 min at 4°C, the extraction and purification procedure was carried out. It consisted in two solid phase separation steps: after acidification, each sample was applied on a C_{18} cartridge followed by a NH_2 cartridge (Waters, Milford, MA) according to the method of Nourooz-Zadeh *et al.* (20).

Extraction and purification procedure for plasma total (sum of free plus esterified) F_2-isoprostane determination. Five hundred microliters of 1 M KOH were added to plasma (1 mL) according to Nourooz-Zadeh *et al.* (20), with little modifications. After incubation at 45°C for 45 min, the pH was adjusted to 3.0 by adding HCl (1 M, 500 μL). Subsequently, plasma was spiked with tetradeuterated $PGF_2\alpha$ (500 pg in 50 μL of ethanol) and the two solid phase separation steps, as referred for free F_2-isoprostanes, were performed.

Derivatization procedure and gas chromatography—tandem mass spectrometry analysis for F_2-isoprostanes. Both for free F_2-isoprostanes and for total F_2-isoprostanes, the carboxylic group was derivatized as pentafluorobenzyl ester whereas the hydroxy groups were converted to trimethylsilyl ethers (20,21). Briefly, the pentafluorobenzyl esters were prepared by adding 40 μL of pentafluorobenzyl bromide (10% in acetonitrile) (Fluka Chemical Corp., Milwaukee, WI) and 20 μL of diisopropylethylamine (10% in acetonitrile) (Sigma Chemical Co., St. Louis, MO) to the purified dried samples. After a reaction time of 45 min at 40°C, the solvent was removed and the trimethylsilyl ethers were prepared by adding 50 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Fluka Chemical Corp.) and 5 μL of diisopropylethylamine (10% in acetonitrile). The samples were kept at 45°C for 1 h. The solvent was then removed and the samples were reconstituted in 50 μL of undecane containing 10% of *N,O*-bis(trimethylsilyl)trifluoroacetamide.

The determinations were carried out by gas chromatography/negative ion chemical ionization tandem mass spectrometry analysis according to our reports (18,21). Briefly, the derivatized samples were injected (2 μL), into the gas chromatograph (ThermoFinnigan Instrument) in the splitless mode (splitless time 2 min). The gas chromatography was programmed from 175°C (3 min) to 270°C at a rate of 30°C/min. The temperature of the injector was 250°C; the transfer line was heated to 280°C and the ion source to 200°C. The carrier gas was ultrapure helium and it was maintained at a constant flow of 1 mL/min. Methane was used as reagent gas (flow of 1.2 mL/min). The collision energy used was 1.3 eV. The measured ions were m/z 299 and m/z 303 derived from the typical ions (m/z 569 and m/z 573) produced from 15-F_{2t}-IsoP, also referred as 8-iso $PGF_{2\alpha}$ or 8-epi $PGF_{2\alpha}$, (the most represented F_2-isoprostane isomer) and the tetradeuterated derivative of $PGF_2\alpha$, respectively. The interassay and the intra-assay coefficients of variability were 10.9% and 12.8%. The detection limit of the authentic $PGF_{2\alpha}$ compounds extracted from plasma samples was 10 pg/mL.

Plasma NPBI. Plasma NPBI measurement was performed essentially according to Singh *et al.* and Paffetti *et al.* (22,23). A large excess of nitrilotriacetic acid (NTA), which is a low-affinity ligand for iron and complexes the iron nonspecifically bound to plasma protein, but does not remove that bound to transferrin or ferritin, was added to plasma. The Fe-NTA complex was ultrafiltered and quantified using an HPLC procedure in which on-column derivatization with a high-affinity iron chelator (3-hydroxy-1propyl-2-methyl-pyridin-4-one, CP22; Aldrich, Sigma Chemical Co., Aldrich, St. Louis, MO) was 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.

In vitro model was performed in two steps: 1) plasma incubation with ammonium iron sulfate; 2) low density lipoprotein (LDL) isolation and iron-mediated oxidation.

Plasma incubation with ammonium iron sulfate. The experimental model was carried out to test plasma peroxidation in presence of ammonium iron sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$), as a source of free iron (24). Ammonium iron sulfate hexahydrate was added to newborn and adult plasma at 50 μM concentration and the plasma was incubated at 37°C for 2 h. At the end of incubation total, esterified and free F_2-isoprostanes and NPBI were determined.

LDL isolation and iron-mediated oxidation. Because plasma esterified F_2-isoprostanes allegedly are those still attached to plasma lipoproteins, plasma LDL was isolated from adult plasma and incubated in the presence of Fe-NTA, as source of free iron, instead of ammonium iron sulfate. This was required because although in plasma, ammonium iron sulfate may be redox cycling active because of the presence of physiologic reductants (GSH, cysteine, *etc.*), with isolated and completely dialyzed LDL, a form of iron able to spontaneously redox cycling was necessary.

Healthy adult blood was collected in presence of ethylene diamine tetra acetic acid (1 mg/mL) and plasma was obtained by centrifugation at 2400*g*. LDL was prepared according to Minotti *et al.* (25), with minor modification. Briefly, LDL was isolated by a 110-min ultracentrifugation at 182,000*g* in a swing rotor (Beckman, SW65), using a discontinuous gradient obtained by overlayering 48% (wt/vol) sucrose (0.8 mL), 0.4 M NaCl-fortified plasma (1.2 mL), and 0.67 M NaCl (2 mL). After recovery from the midlower part of the gradient, LDL (measured density 1.056 ± 0.006 g/mL) was dialyzed against phosphate buffered saline, pH 7.4 and resuspended in the same buffer at the protein concentration of 0.3 mg protein/mL, as determined by Bradford method (26), and used immediately for the LDL oxidation experiments.

LDL oxidation was carried out by incubation in the absence or in the presence of 1–50 μM Fe-NTA. Fe-NTA was prepared by complexation of ammonium iron sulfate and NTA in a molar ratio of 1:7 (27). The LDL oxidation process was followed by monitoring the conjugated diene absorption at 234 nm, as reported by Ramos *et al.* (28). Total F_2-isoprostane formation was also evaluated, by the same procedure used for plasma samples.

These experiments were carried out only in adults, because of the limited (for ethical reasons) neonatal blood sampling.

Statistical analysis. Results were expressed as mean ± SE. The Kolmogorov-Smirnov test, *t* tests, and linear correlation were used for statistical

Table 1. *Clinical characteristics of the newborns at birth*

	Term newborns	Preterm newborns
Number	30	29
Gestational age (wk)	38.2 ± 0.2 (37 to 41)	33.6 ± 0.4* (28 to 36)
Birth weight (kg)	3.1 ± 0.1 (2.4 to 4.5)	2.0 ± 0.1* (1.1 to 2.7)
Male	14	11
Female	16	18
Vaginal delivery (*n*)	6	0
Elective caesarean section (*n*)	24	20
Emergency caesarean section (*n*)	0	9
Apgar score at 5 min	9.8 ± 0.1 (9 to 10)	8.6 ± 0.3* (5 to 10)
pH	7.30 ± 0.01 (7.22 to 7.39)	7.28 ± 0.02 (7.09 to 7.39)
Base deficit (μmol/mL)	−3.7 ± 0.5 (−8 to 2.3)	−3.9 ± 0.7 (−15.0 to −0.1)

Data are shown as mean ± SE, minimum and maximum in brackets.

* $p < 0.001$ compared with term newborns.

analysis of the data with STATA SE 8.2 software (Stata Corporation College Station, TX).

RESULTS

Clinical characteristics of the newborns are described in Table 1. Total F_2-isoprostanes were significantly higher in term and especially in preterm newborns compared with adults (normal distribution of the sample by Kolmogorov-Smirnov test; Table 2). Esterified F_2-isoprostanes were higher in preterm newborns compared with adults, whereas no significant difference was seen between term newborns and adults (Table 2). Free F_2-isoprostanes were significantly higher in term and especially in preterm newborns compared with adults (Table 2).

Free and total F2-isoprostanes significantly decreased with advancing gestational age (Table 2).

NPBI was present in about 90% of term and in all preterm babies studied, and was significantly higher in preterm than in term babies (Table 2). Moreover, plasma NPBI was inversely correlated to the gestational age ($r = -0.521$; $p < 0.005$).

A positive correlation between NPBI and total and esterified plasma F_2-isoprostanes was found (Fig. 1). No correlation was seen between NPBI and free F_2-isoprostanes.

In vitro *model.* The addition of 50 μM ferrous ions in plasma of newborns (Table 3) induced a clear cut increase in all the forms (total, esterified, and free) of F_2-isoprostanes and such an increase was correlated to the amount of NPBI found in the plasma at the end of the incubation (Fig. 2). The amount of NPBI was stable during the incubation: NPBI concentration measured at 30 min was the same as of that measured at the end of the incubation (14.6 μM). This final concentration, (14.6 μM), lower than that of the initial addition (50 μM), may be related to the chelating activities of plasma iron-binding proteins.

Because the plasma of newborns has different ability than adults to generate F_2-isoprostanes (higher levels of pro-oxidants or decreased antioxidant levels, or both), the effects of NPBI on adult plasma were also studied. The addition of 50 μM ferrous ions in plasma of healthy adults induced an increase in all forms of F_2-isoprostanes (Table 3), and such increase significantly correlated with the amount of NPBI at the end of the incubation ($r = 0.905$, $p < 0.005$; $r = 0.909$, $p < 0.005$; $r = 0.831$, $p < 0.005$; for total, esterified, and free F_2-isoprostanes, respectively).

When LDL were isolated from adult plasma and incubated in the presence of Fe-NTA, an increase in total F_2-isoprostanes was observed (Table 4). Such an increase was significant, compared with the LDL incubated without iron, from 5 μM Fe ions onwards (Table 4). A similar experiment with newborn LDL was not possible because of the small amount of plasma obtainable from the newborns.

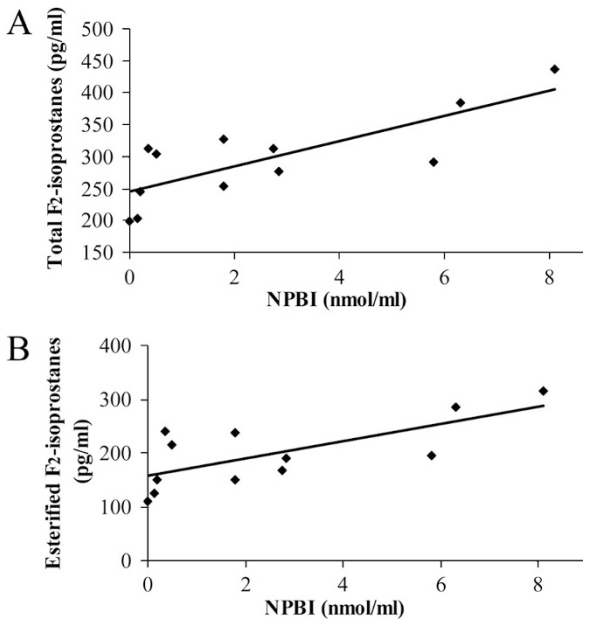

Figure 1. Correlation between plasma NPBI and total F_2-isoprostanes (*A*) and between plasma NPBI and esterified F_2-isoprostanes, $y = 19.689x + 244.96$; $r = 0.788$; $p < 0.001$ (*B*) at birth, $y = 16.205x + 156.59$; $r = 0.07079$; $p < 0.005$.

Figure 3 shows the kinetic of the formation of conjugated dienes with different Fe ions concentrations. An increase in 234 nm absorption with increasing to Fe ions concentration can be observed.

DISCUSSION

Iron is the most abundant transition metal in the body and an essential factor for growth and well being of almost all living organisms. Iron deficiency during early development of the brain has been related to behavioral alterations including deficits in learning and memory mediated by the hippocampus (29). We recently found that NPBI concentration in amniotic fluid increases from 14–15 wk to 17–18 wk and then regresses positively with gestational age (30), suggesting its essential role in the earliest phases of life for the normal neurologic development.

Table 2. *Plasma nonprotein-bound iron (NPBI), total, esterified, and free F_2-isoprostanes in term and preterm newborns and adults*

	NPBI (nmol/mL)	Plasma total F_2-isoprostanes (pg/mL)	Plasma-esterified F_2-isoprostanes (pg/mL)	Plasma-free F_2-isoprostanes (pg/mL)
Term newborns	1.1 ± 0.2 (30)	257 ± 19* (11)	161 ± 21 (11)	83 ± 4§ (30)
Preterm newborns	3.9 ± 0.4† (29)	359 ± 18† (11)	227 ± 14‡ (11)	138 ± 9‖ (29)
Adults	0.7 ± 0.1 (16)	184 ± 12 (8)	141 ± 10 (8)	42 ± 2 (8)

Results are shown as mean ± SE.
* $p < 0.005$ *versus* adults.
† $p < 0.001$ *versus* term newborns, $p < 0.01$ *versus* adults.
‡ $p < 0.01$ *versus* term newborns, $p < 0.001$ *versus* adults.
§ $p < 0.001$ *versus* adults.
‖ $p < 0.001$ *versus* term newborns and adults.

Table 3. *NPBI and total, esterified, and free F_2-isoprostanes in plasma of term newborns and adults incubated with ammonium iron sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$)*

	Plasma, no ammonium iron sulfate added (0 time)	Plasma, no ammonium iron sulfate added (2 h incubation)	Plasma, plus ammonium iron sulfate (2 h incubation)
NPBI (nmol/mL)			
Term newborns	1.4 ± 0.6 (3)	1.6 ± 0.6 (3)	14.6 ± 0.9* (3)
Adults	1.2 ± 0.3 (3)	1.9 ± 0.3 (3)	9.2 ± 2* (3)
Total F_2-isoprostanes (pg/mL)			
Term newborns	263 ± 14 (3)	274 ± 6 (3)	442 ± 11* (3)
Adults	207 ± 9 (4)	217 ± 5 (4)	319 ± 27† (4)
Esterified F_2-isoprostanes (pg/mL)			
Term newborns	174 ± 12 (3)	179 ± 6 (3)	307 ± 15* (3)
Adults	150 ± 11 (3)	170 ± 3 (3)	232 ± 19‡ (3)
Free F_2-isoprostanes (pg/mL)			
Term newborns	89 ± 3 (3)	95 ± 3 (3)	135 ± 6* (3)
Adults	50 ± 5 (3)	48 ± 4 (3)	87 ± 10§ (3)

Plasma was added with 50 μM ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) and incubated at 37°C for 2 h. At the end of incubation total and free F_2-isoprostanes and NPBI values were determined as reported in Materials and Methods. Results are shown as mean ± SE.

* $p < 0.001$.

† $p < 0.005$.

‡ $p < 0.05$.

§ $p < 0.02$ *versus* no ammonium iron sulfate added 2 h incubation and 0 time.

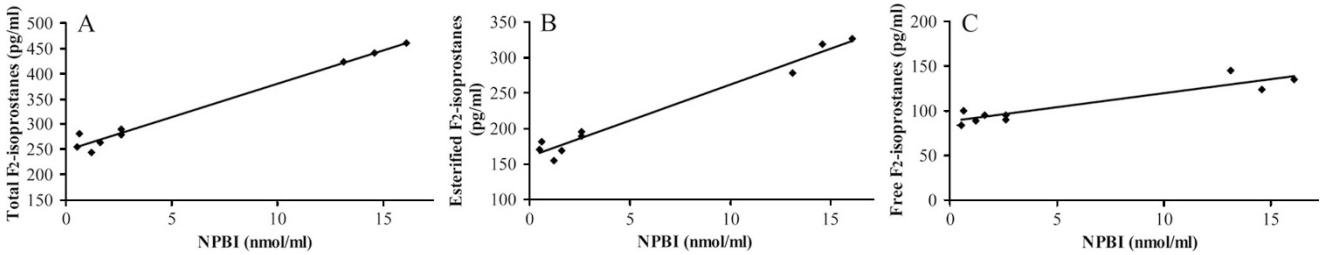

Figure 2. Correlation between NPBI and total F_2-isoprostanes (*A*), between NPBI and esterified F_2-isoprostanes, $y = 13.21x + 248.8$; $r = 0.99$; $p < 0.001$ (*B*), between NPBI and free F_2-isoprostanes, $y = 10.077x + 160.88$; $r = 0.986$; $p < 0.001$ (*C*) in term newborn plasma incubated with 50 μM ammonium iron sulfate $y = 3.1328x + 87.919$; $r = 0.929$; $p < 0.001$.

However, iron is a double-edged sword and whereas the body can tolerate moderate iron depletion, iron excess is a potentially greater threat. In large quantities and unleashed to protein, iron can be toxic, and because of its pro-oxidative function in the presence of oxygen, it can cause subtle tissue damage that can have long-term consequences. We previously demonstrated that NBPI is the best early predictive marker of impaired neurodevelopmental outcome in hypoxic babies (13).

In view of the possibility that redox active iron is responsible for lipid peroxidation and F_2-isoprostane increase, we investigated *in vitro* and *in vivo* the relationship between these two parameters.

We found significant correlations (Fig. 1) between NPBI concentration and total as well as esterified F_2-isoprostanes in plasma suggesting that a source of redox active iron is the main cause of F_2-isoprostane formation in newborns. These correlations let us also to hypothesize that the origin of lipid

Table 4. *Total F_2-isoprostane levels in LDL suspension incubated in presence of iron (1, 3, 5, 10, and 50 μM)*

	Total F_2-isoprostanes (pg/mg protein)
0 Time (not incubated)	43 ± 10
Control	45 ± 7
Fe 1 μM	50 ± 11
Fe 3 μM	51 ± 4
Fe 5 μM	83 ± 6*
Fe 10 μM	155 ± 21*†
Fe 50 μM	486 ± 94*‡

The desalted lipoproteins were resuspended in phosphate-buffered saline, pH 7.4 at a protein concentration of 0.3 mg/mL. Iron was added as Fe-NTA complex which was prepared by complexation of ammonium iron sulfate and NTA in a molar ratio of 1:7 (see Material and Methods section). In the table are reported the Fe concentration. The incubation time in both control and Fe-incubated samples was 60 min. Results are shown as mean ± SE.

* $p < 0.01$ *versus* control.

† $p < 0.05$ *versus* Fe 5 μM.

‡ $p < 0.02$ *versus* Fe 10 μM.

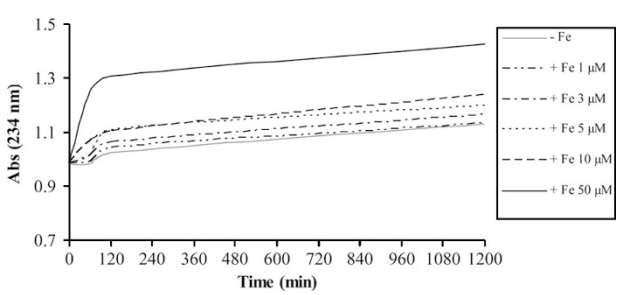

Figure 3. Effect of NPBI (Fe ions 1, 3, 5, 10, and 50 μM) on kinetics of LDL oxidation. The oxidation was followed by recording the 234 nm absorption. Conjugated diene *versus* time curves are reported.

peroxidation are plasma lipoprotein, from which esterified F_2-isoprostanes are likely to be derived. On the other hand, the lack of correlation between NPBI and free F_2-isoprostanes in plasma sheds a light on the origin of free F_2-isoprostanes from additional sources besides plasma lipid, as previously suggested by Salahudeen *et al.* (18).

The present article firstly reports that NPBI in plasma is primarily involved in the formation of esterified F_2-isoprostanes. This data are fundamental to better understand the mechanism by which free iron explains its activity.

The correlation found in the *in vitro* studies between plasma Fe ions (redox active iron) and total, esterified, and free F_2-isoprostanes (Fig. 2) confirms the major role of iron in F_2-isoprostanes formation. *In vitro* the formation of free F_2-isoprostanes (besides total and esterified ones) is correlated to the amount of iron present in the system (Fig. 2), whereas in plasma of newborns free F_2-isoprostanes are not correlated with NPBI (Fig. 1). This discrepancy is likely due to the fact that *in vivo* free F_2-isoprostanes may be formed from other sources besides plasma lipids.

Esterified F_2-isoprostanes return to normal concentration of adults at the 37th gestational week, whereas total and free F_2-isoprostanes are higher than those of the adults even at the 39th gestational week, as a consequence of a long-lasting oxidative stress (31).

The possibility that plasma lipids contribute to F_2-isoprostane formation and that peroxidation of plasma lipids is responsible for esterified F_2-isoprostane production with iron involvement is confirmed by the *in vitro* studies of iron-induced LDL oxidation evaluated both as conjugated dienes and as F_2-isoprostane production. In our record of cases, blood products were not used during surgery for caesarean section and operating room times were not longer than 5 min in all cases. Thus, it is unlikely that a modification of NPBI levels occurred because of the high frequency of emergency cesarean section. This finding is in agreement with our previous reports, which showed no statistically significant differences between the plasma F_2-isoprostanes of newborns with vaginal delivery and those with caesarean section (17). In summary, the results of this study clearly show that once NPBI is generated, whatever its source, it is capable of inducing oxidative stress.

Considering the proposed role of oxidative stress in preterm newborn morbidity with respect to the higher risk of FR damage in these babies, these results may shed light on diseases of the newborn such as bronchopulmonary dysplasia and retinopathy of prematurity, phenotypes clearly associated with oxidative stress.

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