Early Inhaled Nitric Oxide Improves Oxidative Balance in Very Preterm Infants

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ABSTRACT

Inhaled nitric oxide (iNO) improves oxygenation in premature infants, but concern has been raised about its potential oxidative toxicity. We designed this study to assess the oxidative balance in premature infants who were exposed to low dose iNO and the relationship with their clinical outcome on day 28 of life. A total of 274 infants who were <32 wk gestation were randomized at birth to receive 5 ppm of iNO if they presented with hypoxemic respiratory failure. Nonhypoxemic infants were studied as the reference group. Blood samples were withdrawn 24 h apart, within the first 4 d of life, to assess malondialdehyde (MDA) concentration as oxidative stress marker and total plasmatic glutathione (GSH), intraerythrocyte GSH peroxidase, and GSH reductase activities as antioxidant defenses. After 24 h, the rise in MDA was blunted in the iNO group compared with controls and was close to the reference infants. Conversely, GSH was more stable in the iNO group, when there was no difference for the GSH peroxidase and GSH reductase activities. On day 28, Oxygen dependence was linked with a higher increase in MDA as was the risk for death, whereas intraventricular hemorrhage was associated with a higher initial drop in GSH. Early low-dose iNO in hypoxemic preterm infants improves oxidative balance and seems to be clinically beneficial up to day 28 of life. (*Pediatr Res* **57**: 637–643, 2005)

Abbreviations
aAO$_2$, arterio-alveolar ratio in oxygen
BPD, bronchopulmonary dysplasia
F$_{IO_2}$, fraction of inspired oxygen
GA, gestational age
GPX, glutathione peroxidase
GR, glutathione reductase
GSH, glutathione
HRF, hypoxemic respiratory failure
iNO, inhaled nitric oxide
IUGR, intrauterine growth retardation
IVH, intraventricular hemorrhage
MDA, malondialdehyde
OR, odds ratio
Pao$_2$, arterial oxygen pressure
ROI, reactive oxygen intermediates

Since 1992, several randomized trials have shown that inhaled nitric oxide (iNO) significantly improved oxygenation in term or near-term infants, with a significant reduction in the use of extracorporeal membrane oxygenation (1–6). In 1997, Skimming *et al.* (7) were the first to report a positive response of 23 premature infants with respiratory distress syndrome to low-dose iNO (5 ppm). Since then, a few clinical trials involving a small number of premature infants have shown that the initial improvement in oxygenation seemed to be transient (8–10) because no improvement in mortality or in morbidity had been demonstrated (11). However, most of these studies

were considering iNO therapy in premature infants who had severe hypoxemic respiratory failure or refractory hypoxemia (8,10,11) or were at an advanced stage of respiratory insufficiency (12). Most of them used a high dosage of iNO (10–20 ppm).

Finally, concerns have been raised about specific side effects of this new molecule that could worsen long-term outcome after an initial improvement in this high-risk population (13–15). Besides the risk for increased brain hemorrhage as a result of a prolonged bleeding time (16), neonatologists remain cautious in the use of this treatment because NO behaves as a "chameleon" molecule for the oxidative balance. Endogenous NO is incriminated in the cascade leading to cerebral lesions in perinatal experimental models of hypoxo-ischemic injuries (17). In addition, iNO may interact with oxygen to form nitrogen dioxide and peroxynitrites, which would enhance pulmonary toxicity. This is a main concern when dealing with

premature infants, who already are unable to face oxidant stress (18,19). However, at low dosage and in experimental models, NO is described as antioxidant at the lung level, able to counteract oxidative stress and enhance surfactant function (20,21). Low doses of iNO also seem to improve oxygenation in premature infants with chronic lung disease (11,12) without alteration of their inflammatory markers. Thus, a policy of treatment aiming to get a beneficial and sustained protective effect of iNO in preterm infants should take into account their fragile oxidative balance. Therefore, we designed this study, as part of a large multicenter trial studying the safety and efficacy of early low-dose iNO (22), to assess the oxidative balance of very premature infants according to their respiratory status and the effect of iNO on this balance.

METHODS

Study population. This prospective, randomized, controlled trial was conducted in an academic level III neonatal unit and approved by the "Comite Consultatif de Protection des Personnes soumises à la Recherche Biomedicale" and by our Institutional Review Board. Inclusion criteria were gestational age (GA) <32 wk at birth and <48 h of life, with written informed consent of the parents. Exclusion criteria were initial refractory hypoxemia, thrombocytopenia <50,000/mm^3, or the presence of major fetal abnormality.

Study design. Randomization was stratified by GA (<28 wk and 28–31 wk GA) and kept blind until 6 h of age with optimal care performed according to standardized written protocols. Subsequently, up to 48 h after birth, when the infants presented with hypoxemic respiratory failure (HRF) defined as the need for mechanical ventilation with a fraction of inspired oxygen (Fio$_2$) >0.4 and had an arterio-alveolar O$_2$ ratio [aAO$_2$; aAO$_2$ = arterial oxygen pressure (Pao$_2$)/713 × Fio$_2$ − arterial partial pressure of CO$_2$] <0.22 but no refractory hypoxemia (defined as Po$_2$ < 50 and Pco$_2$< 50 mm Hg for Fio$_2$ = 1.0), the allocation was disclosed as "control" or "iNO" group. For both HRF groups, one blood sample (1 mL) was withdrawn at the time of randomization (T$_1$) through a central line, and a second sample (T$_2$) was drawn 24 h later for biologic analysis. iNO was delivered from a certified 225-ppm cylinder (Air Liquide Sante International, Paris, France) with a compact unit including a pressure regulator and a low-flow meter (0.1–1.5 L/min). NO and nitrogen dioxide were continuously monitored at the T-piece with electrochemical cells (Polytron, DrägerAG, Lübeck, Germany). A dose of 5 ppm of iNO was first used for 1 h in the iNO group, and then subsequent dosage was determined according to aAO$_2$ response. As soon as the response was positive (defined as an aAO$_2$ increase >0.22), iNO was decreased to 2 ppm for 2 h and then weaned according to blood gas examination; when the response was intermediate (aAO$_2$ remaining <0.22 but increasing by at least 25%), iNO was left at 5 ppm for 2 h and the response was re-evaluated every 2 h thereafter. Finally, when the infants showed no response, iNO was increased up to 10 ppm for 2 h and then re-evaluated. In case of treatment failure, iNO was weaned after 4 h. If during these 48 h the infants did not present with HRF ("reference group"), then randomization was kept blind until analysis, usual optimal care was performed, and two blood samples (T$_1$ and T$_2$) were drawn 24 h apart during the first 72 h of life.

Biologic analysis. All blood samples were processed immediately and frozen at −20°C and −80°C until final biochemical analysis, blinded for the allocation group.

Malondialdehyde (MDA) plasmatic concentration, the marker of oxidative stress, was assessed by colorimetric method (Kit MDA Sobioda). This measurement was based on the reaction of MDA with two molecules of thiobarbituric acid, after heating at 95°C, to form a pink fluorescent complex at 553 nm (23,24). Results were expressed in nmol/g of serum protein.

Total plasmatic glutathione (GSH) was assessed by enzymatic assay by a method based on the spectrophotometric monitoring of GSH-mediated reduction of 5,5'-dithio-bis (2-nitrobenzoic acid) at 412 nm (25). Plasmatic GSH, chosen as antioxidant substrate, was expressed in nmol/L.

Intraerythrocyte glutathione peroxidase (GPX) activity was determined by enzymatic assay following the oxidation of reduced GSH, in the presence of glutathione reductase (GR), by measuring the decrease of NADPH concentration at 340 nm. Results were expressed in μmol of oxidized NADPH per min/g Hb (26).

Intraerythrocyte GR activity acts as a GSH regenerator by catalyzing the reduction of GSH disulfite in the presence of NADPH (27), which is oxidized

to NADP$^+$. The decrease in absorbance at 340 nm was measured (Kit RANDOX), and results were expressed in μmol · min^{-1} · g Hb^{-1}.

Data collection and clinical outcome measures. History of toxemia, gestational diabetes, premature rupture of membranes, chorioamnionitis, maternal-fetal infection, and the use of antenatal steroids was recorded carefully following a standardized review of the maternal medical records. We prospectively recorded placental abruption; mode of delivery; newborn characteristics; episodes of illness; all events that occurred at birth and within the first 28 d of life, such as the incidence and severity of intraventricular hemorrhage (IVH) and periventricular leukomalacia thanks to weekly head ultrasound examination; and the prevalence of oxygen dependence on day 28. The primary measure of outcome of the clinical trial was defined as survival on day 28 without any respiratory support, oxygen supplementation, or IVH greater than grade I (28).

Statistical analysis. Categorical variables were compared among the three groups by either χ^2 or Fischer exact test. Continuous variables were compared by two-way ANOVA for one or two parameters and completed by Tukey pair-wise comparisons test when global test was positive. We considered $p <$ 0.05 as significant.

The relationship between MDA or GSH and the clinical outcome on day 28 was studied by ANOVA when normality of the data was achieved. Otherwise, we used Kruskal-Wallis test to measure differences of a single variable across two or more independent groups of cases. When linearity of the data was achieved, MDA and GSH were categorized using 5 nmol/g protein and 0.1-nmol/L intervals, respectively, and odds ratio (OR) were calculated. Data are presented as mean ± SEM or median (25th–75th quartiles). Statistical analysis was performed with SAS 8.2 software (SAS Institute, Cary, NC).

RESULTS

Population. From July 1999 to February 2001, 274 premature infants received care in our unit. After exclusion for parental refusal or exclusion criteria ($n = 22$), lack of biologic sampling ($n = 6$), or protocol violation ($n = 6$), 240 infants were studied and distributed within three subgroups: reference ($n = 164$), HRF-control ($n = 39$) and HRF-iNO ($n = 37$; Fig. 1). Demographic and baseline characteristics before randomization are presented in Table 1. Mean GA was significantly higher in the reference group in comparison with both HRF groups. There was no statistical difference among the three groups for birth weight, sex distribution, incidence of maternal toxemia, chorioamnionitis, severe intrauterine growth retardation, or the incidence of cesarean section. We noticed a higher rate of antenatal steroid use for the reference group *versus* the
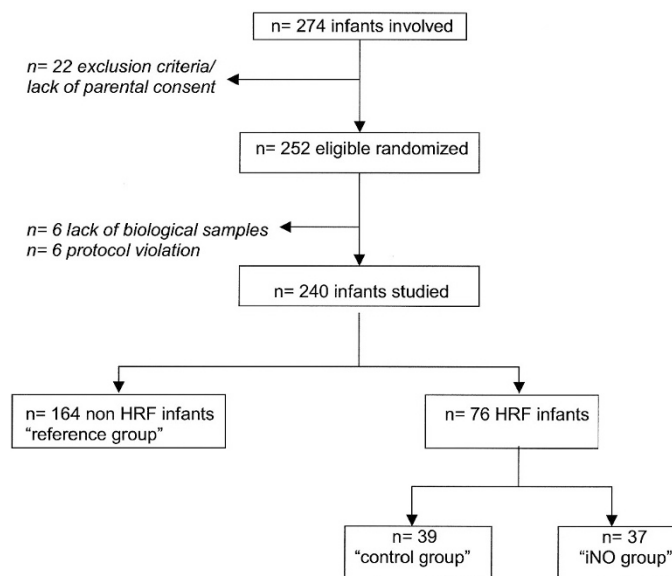

Figure 1. Trial profile.

Table 1. *Demographic and baseline characteristics of the infants*

Characteristics	Reference [$n = 164$ (%)]	Control-HRF [$n = 39$ (%)]	iNO-HRF [$n = 37$ (%)]	p value
GA (wk)	28.9 ± 0.1	27.9 ± 0.4	27.3 ± 0.4	<0.001*†
Birth weight (g)	1206 ± 026	1102 ± 054	1083 ± 058	NS
Male sex	78 (48)	22 (56)	17 (46)	NS
Antenatal steroids	134 (82)	26 (67)	24 (65)	0.02*†
Toxemia	25 (15)	6 (15)	3 (8)	NS
Chorioamnionitis	4 (2)	2 (5)	0 (0)	NS
Severe IUGR	38 (23)	6 (15)	4 (11)	NS
Cesarean section	94 (57)	22 (56)	16 (43)	NS
Hyaline membrane disease	97 (59)	39 (100)	35 (94)	<0.001*†
CRIB score				
<6	124 (76)	23 (59)	18 (49)	<0.01*†
5–10	17 (10)	11 (28)	14 (38)	
>10	3 (2)	5 (13)	4 (11)	

IUGR, intrauterine growth retardation.

* p value significantly different between reference and control HRF.

† p value significantly different between reference and iNO HRF.

control and the iNO groups, whereas there was no difference within these two HRF groups (Table 1). The incidence of hyaline membrane disease was almost twice as much in both HRF groups than in the reference group. The incidence of poor postnatal adaptation assessed by the neonatal CRIB score (29) was higher in both HRF groups than in the reference group (Table 1). At the time of randomization, F_{IO_2} requirement was significantly higher to start with in the iNO group than in the control group (Table 2).

Protocol. In our study, median duration of iNO administered according to the protocol was 35.1 h (21.7–53.9). There was no significant difference for the postnatal age at T_1 between the two hypoxemic groups (14.1 ± 1.4 *versus* 15.9 ± 1.8 h in controls, respectively). It was significantly higher, however, in the reference group (29.4 ± 0.8 h; $p < 0.01$). We verified within this group that there was no effect of time for the studied variables except a minimal effect on MDA (between 1 and 2% over 96 h). Twenty-four hours after entering the randomization (T_2 for biologic sampling), there was a significant drop in the F_{IO_2} requirement in the iNO group (-20.4 ± 4.4 *versus* $-7.4 \pm 3.7\%$ for the controls; $p < 0.01$).

Biologic results. MDA. At the time of randomization (T_1), MDA levels were not significantly different among the three groups (Table 2). At T_2, MDA increased moderately in the reference population (1.63 ± 0.79; median, 1.4 [-3.7 to 6.5]) as in the iNO HRF group [2.20 ± 2.57; 4.6 (-2.6 to 9.7)], whereas during this 24-h period, HRF controls increased significantly their MDA level [12.11 ± 2.67; 8.4 (4.4–20.6); $p < 0.05$; Fig. 2A].

Total plasmatic GSH. At the time of randomization (T_1), GSH levels were not significantly different among the three groups (Table 2). At T_2, GSH level decreased moderately in the reference population [-0.025 ± 0.014; -0.020 (-0.080 to 0.060)] in comparison with the iNO group [-0.080 ± 0.027; -0.055 (-0.120 to -0.010)] and the HRF control [-0.105 ± 0.029; -0.070 (-0.210 to -0.000)], where the drop reached statistical significance *versus* the reference group ($p < 0.05$; Fig. 2B).

Table 2. *Effect of early iNO on the F_{IO_2} requirements and the oxidative balance in premature infants*

Parameters	Reference group	Control-HRF	iNO-HRF
F_{IO_2} at T_1 (%)	23.61 ± 0.38*†	51.97 ± 3.08*‡	62.32 ± 3.44†‡
	($n = 164$)	($n = 39$)	($n = 37$)
MDA at T_1	47.80 ± 1.04*	41.80 ± 1.92*	48.50 ± 2.59
GSH at T_1	0.941 ± 0.015	0.958 ± 0.033	0.945 ± 0.034
GPX at T_1	10.86 ± 0.22	11.06 ± 0.49	11.07 ± 0.38
GR at T_1	11.85 ± 0.19	11.61 ± 0.40	11.17 ± 0.40
ΔF_{IO_2} at T_2 (%)	-0.77 ± 0.40†	-7.36 ± 3.72‡	-20.42 ± 4.42†‡
	($n = 151$)	($n = 33$)	($n = 36$)
Δ MDA at T_2	$+1.63 \pm 0.79$*	$+12.11 \pm 2.67$*‡	$+2.20 \pm 2.57$‡
	($n = 138$)	($n = 30$)	($n = 34$)
Δ GSH at T_2	-0.025 ± 0.014*	-0.105 ± 0.029*	-0.08 ± 0.027
	($n = 137$)	($n = 31$)	($n = 34$)
Δ GPX at T_2	$+0.06 \pm 0.12$†	$+0.36 \pm 0.21$	$+0.90 \pm 0.25$†
	($n = 143$)	($n = 31$)	($n = 34$)
Δ GR at T_2	$+0.58 \pm 0.13$	$+0.48 \pm 0.41$	$+0.81 \pm 0.31$
	($n = 143$)	($n = 31$)	($n = 34$)

Results expressed as mean $\pm$ SEM, Δ value = $T_2 - T_1$.

* p value significantly different ($p \leq 0.05$) between reference and control HRF.

† p value significantly different between reference and iNO HRF and ‡between control HRF and iNO HRF.
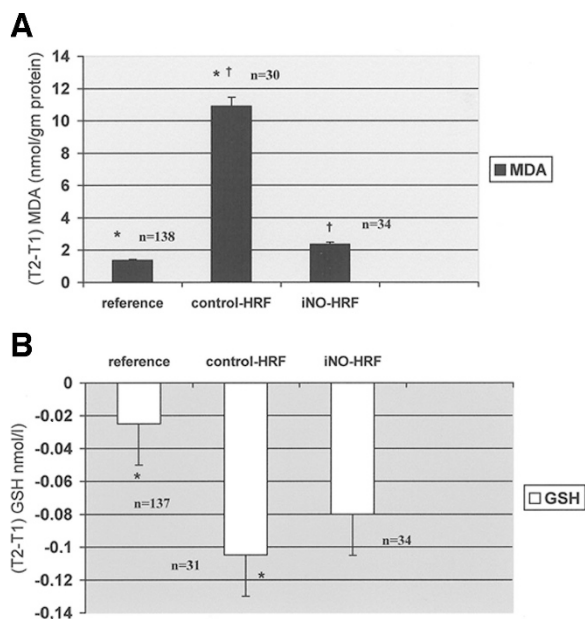
A

B

Figure 2. Oxidative stress balance over 24 h within the three groups of infants. (*A*) MDA changes. (*B*) Total plasmatic GSH changes. *p value significantly different (<0.05) between "reference and control HRF" and † between "control HRF and iNO HRF". "n" = number of biological values.

Intraerythrocyte GPX activity. At the time of randomization (T_1), the GPX activity was not significantly different among the three groups. At T_2, there was a significant increase ($p < 0.05$) in GPX activity for the iNO group [0.90 ± 0.25; 0.65 (−0.10 to 1.40)] in comparison with the reference group [0.06 ± 0.12; 0.10 (−0.40 to 0.70)], when the rise in GPX was not significantly different for the HRF controls [0.36 ± 0.21; 0.00 (−0.30 to 1.00)].

Intraerythrocyte GR activity. At the time of randomization (T_1), the GR activity was not significantly different among the three groups. At T_2, the moderate rise in GR activity (Table 2) remained statistically not different among the three groups.

Clinical outcome on day 28. On day 28 of life, survival rate was 92% in the reference group *versus* 69% in the control group and 59% in the iNO group. Fifty-five percent of the infants in the reference group *versus* 23% in the control group and 19% in the iNO group reached the primary measure of outcome ($p < 0.0001$ for the reference group *versus* the control and iNO groups, respectively), a composite value to describe "intact survival." The incidence of O_2 dependence on day 28 was different among the three groups, with a significantly

higher incidence for the HRF controls, when this incidence was not different between the reference population and the HRF infants who were treated with iNO (Table 3). The incidence of patent ductus arteriosus was significantly higher in both HRF groups in comparison with the reference group (Table 3). There was no statistical difference among the three groups for the incidence of IVH of any grade or the incidence of significant brain lesion (greater than IVH grade 1 and/or periventricular leukomalacia and/or hydrocephaly; Table 3).

Correlations between F_{IO_2} changes and early oxidative stress. For MDA, the oxidative stress marker, we found a positive correlation with F_{IO_2} requirements ($r = 0.158$, $p = 0.02$). Moreover, MDA changes over 24 h ($T_2 - T_1$ values) were also correlated with F_{IO_2} changes within the same period of time ($r = 0.148$, $p = 0.03$). For GSH, the antioxidant substrate, we found that the drop in GSH level over 24 h was correlated with the initial F_{IO_2} requirements ($r = 0.182$, $p = 0.01$).

Relationship between early oxidative stress and clinical outcome on day 28. We found no significant relationship between the early oxidative stress marker evaluated at T_1 and the postnatal clinical outcome. However, MDA evolution, over a 24-h period ($T_2 - T_1$) was significantly associated with the risk for death, respiratory, and/or neurologic outcome on day 28.

For MDA, we found by univariate analysis that the magnitude of the early increase ($T_2 - T_1$) was significantly associated with the risk for death within the first 28 d of life ($p = 0.009$), with the combined risk for death or having worrisome brain lesion ($p = 0.004$) or developing an oxygen dependence on D28 ($p = 0.003$; Table 4). For GSH, we found by univariate analysis that the magnitude of the early drop ($T_2 - T_1$) was significantly associated with the risk for developing a significant IVH (>grade I) during the first month of life ($p = 0.03$).

DISCUSSION

In our study, iNO was not associated with an increased oxidative stress in very preterm infants. On the contrary, we observed a significant improvement in the oxidative balance of very premature infants within the first days of life. Premature infants are highly susceptible to injury from reactive oxygen intermediates (ROI), and tissue injury occurs when ROI from endogenous and exogenous sources exceed the already limited antioxidant capacities (17,30). Therefore, it seemed necessary to study the impact of iNO on both sides of the oxidative balance. Plasmatic MDA, a byproduct of cell membrane lipid

Table 3. *Clinical outcome on day 28 of life among the three groups of infants*

Clinical outcome on day 28	Reference [$n = 164$ (%)]	Control-HRF [$n = 39$ (%)]	iNO-HRF [$n = 37$ (%)]	*p* value
Survival	151 (92.1)	27 (69.3)	22 (59.5)	<0.001*†
Oxygen dependence	40 (26.5)	15 (55.6)	8 (36.4)	0.0099*
PDA incidence	37 (22.6)	16 (41.0)	18 (48.6)	0.001*†
Cumulative incidence of IVH at last HUS examination (all grades)	16 (10.5)	5 (17.2)	7 (25.9)	0.07
Significant brain lesion	9 (5.9)	2 (7.4)	2 (9.1)	NS
Necrotizing enterocolitis	31 (18.9)	4 (10.2)	4 (10.8)	NS

PDA, patent ductus arteriosus; HUS, head ultrasound; "significant brain lesion," occurrence of any IVH >grade I and/or periventricular leukomalacia and/or hydrocephaly.

* *p* value significantly different between reference and control HRF.

† *p* value significantly different between reference and iNO.

Table 4. *Relationship between the initial oxidative stress and the clinical outcome of the infants on day 28*

Clinical outcome on day 28 of life	No. of infants evaluated	Δ (T2−T1) MDA (mean ± SEM)	*p* value	OR Δ MDA (95% CI)
Death				
No	169	+2.42 ± 0.74	0.009	1.23
Yes	33	+7.74 ± 3.36		(1.04–1.44)
Death + total neuro				
No	156	+2.20 ± 0.75	0.004	1.21
Yes	46	+6.91 ± 2.60		(1.04–1.39)
Oxygen dependence				
No	109	+0.99 ± 0.95	0.003	1.24
Yes	60	+4.99 ± 1.11		(1.04–1.47)
		Δ (T2−T1) GSH (mean ± SEM)		OR Δ GSH (95% CI)
Any IVH > grade I				
No	153	−0.038 ± 0.011	0.03	0.045
Yes	23	−0.126 ± 0.044		(0.003–0.570)

OR, odds ratio; CI, confidence interval; Death + total neuro on day 28 death and/or neurologic permanent abnormality at HUS examinations (HIV >grade I and/or periventricular leukomalacia and/or hydrocephaly).

$p < 0.05$: significant association between the clinical risk on day 28 death, death and/or neurologic abnormality, O_2 dependence, or IVH and Δ (T2−T1) evolution of biological markers of oxidative stress.

peroxidation as a result of ROI, has been accepted as a valid indicator of oxidative stress in the neonate (31,32). In our study, we were able to demonstrate a direct link between the levels of oxygen exposure and the magnitude of MDA rise in very premature infants. iNO led to a significant decrease in early F_{IO_2} requirements, and the MDA levels of the HRF infants who were treated with iNO were not significantly different from the reference population after 24 h, when those of the HRF controls were significantly rising. However, the number of HRF infants was too small to show a direct impact of iNO on MDA levels, and we can only speculate that MDA level stabilization was obtained thanks to the important drop in F_{IO_2} requirement (−20% over 24 h). A rapid improvement in oxygenation after iNO in premature infants is described in several studies (7,14), but the originality of our prospective controlled study was to obtain this response with very low doses (≤5 ppm) (33) over a short period of time, when others were using from 5 (11), to 10 (6), and often 20 ppm (7,8,10).

On the other side of the oxidative stress balance, we chose total plasmatic GSH as a biologic marker of the antioxidant defense pool, able to detoxify hydrogen peroxide and other peroxides in a reaction catalyzed by GPX. GR is the main enzyme able to restore intracellular GSH levels. Several clinical data have shown that plasmatic GSH concentrations are low in premature infants and inversely proportional to GA (34,35). A further early depletion as a result of the adjunction of a new oxidant molecule would be worrisome. With the clinical settings of our study, the sick infants who were treated with low-dose iNO had a trend toward a less severe depletion in GSH levels than the sick control subjects and were not significantly different from the reference group. Because NO does not have any direct impact on the GSH metabolism, we can speculate that iNO, by reducing early F_{IO_2} requirements and ROI, indirectly decreases the need for scavenger use. Thus, by reducing the magnitude of both oxidant production and antioxidant depletion, iNO that is administered early in the postnatal course (14–16 h of life) and at a small dosage (≤5 ppm) in hypoxemic premature infants seems to be able to blunt the oxidative cascade triggered by high initial oxygen concentrations.

Because this study was an ancillary part of a larger, prospective, safety-efficacy controlled trial of early iNO in hypoxemic very premature infants (22), we were able to benefit from the collection of all postnatal data of these infants. This gave us clues to answer the yet unsolved question (30,35): does the biologic stabilization of the early oxidative stress have any sustained clinical benefits in this high-risk population? On day 28 of life, when most of these infants have reached a stable clinical condition and the inflammatory processes triggered by oxidative stress has been identified, we found a significant relationship between clinical outcome and early oxidative balance. The early increase of MDA plasmatic levels was significantly associated with the risk for death during the first month of life (OR = 1.23) as well as the risk for death and/or severe neurologic abnormalities (OR = 1.21). The significant relationship between early MDA increase and the risk for having an oxygen dependence on day 28 (OR = 1.24) is consistent with previous publications (30,31,36–39). Data indicating that early lipid peroxidation occurs in infants who subsequently develop bronchopulmonary dysplasia (BPD) came from Pitkanen *et al.* (37) in 1990. They also demonstrated that increased concentration of exhaled pentane and ethane occurred early and at higher levels in preterm than in term infants, indicating a higher oxidative stress in the former group (37,38). Inder *et al.* (31) found a positive correlation between lipid peroxidation measured by MDA-thiobarbituric acid in 22 very low birth weight infants and the number of days with high F_{IO_2} levels. MDA-thiobarbituric acid values were ~50% higher in the infants who developed BPD (31). Ogihara *et al.* (32) also found a positive correlation between an elevated level of several aldehydes, products of lipid peroxidation in 13 preterm infants, on the first day of life, and the risk for BPD. At 4–6 d of life, no more significant correlation was found, suggesting

that early oxidative stress has a stronger impact on subsequent clinical outcome (32). Finally, Weinberger *et al.* (40), in a study on 25 premature infants, confirmed that elevated urinary MDA in the first days of life was correlated with a risk for oxygen radical disease.

For the antioxidant defenses, we found a link between the magnitude of early depletion in plasmatic GSH and the risk for developing IVH within the first 28 d of life (OR = 0.045). To our knowledge, this is the first time such a relation has been established in very premature infants. Most of the studies focused on the impact of low antioxidant defenses on the incidence of BPD. Neonates are short in antioxidant defenses, on both their extracellular (41,42) and intracellular part (42,43). Moreover, in preterm animals, in contrast with term, induction of antioxidative enzymes does not occur after oxidative stress (41,44). Randomized trials of antioxidant intervention in premature infants, with strong rationale and good study design, failed to demonstrate any improvement on BPD incidence (36,45–47). In our study, we speculate that low-dose iNO probably did not act as an antioxidant by itself but by reducing the oxidative stress as a result of high oxygen concentration allowed a less dramatic consumption of GSH stores.

NO is a free radical that may be oxidized or reduced depending on its concentration and the presence of other oxidants such as oxygen. Hyperoxic exposure of rat pups up-regulates both inducible and endothelial NO synthase and therefore increases the concentration of NO and subsequently peroxynitrite as well (48). This compound is highly toxic for preterm infants. Reports regarding the effect of NO in oxidative stress, however, are conflicting (43). In experimental studies, several authors found a worse clinical outcome or surfactant dysfunction as a result of lipid peroxidation or damage to surfactant protein, but they often used very high doses of NO (40–100 ppm) combined with high and stable F_{IO_2} levels (80–100%) (19,43,49). Because of these data, wide use of iNO therapy in preterm infants is unwarranted by most neonatologists (13–15,50).

In our study, we continuously monitored by transcutaneous Pa_{O_2} the infants' response to iNO, and we aimed to wean both iNO and oxygen as quickly as possible keeping Pa_{O_2} between 50 and 65 mm Hg. Because of the continuous monitoring of iNO (to ensure constant dosage) and of NO_2 (to verify safety), it was difficult to blind the administration of the gas with the design of this study. However, the duration of treatment was short (35 h on average), the infants were treated following standardized written protocol, and the clinicians in charge of the infants on day 28 were not aware of the allocation. In addition, randomization remained blinded for biologic analysis as well as for head ultrasound examination. Therefore, a bias related to unmasked administration of NO on biologic data or neurologic outcome is unlikely. Our strategy of treatment of lowering F_{IO_2} as soon as possible by continuous follow-up of Pa_{O_2} may explain the positive results on the oxidative stress balance as well as on clinical outcome, in comparison with experimental models using the combined and toxic combination of high iNO and F_{IO_2} levels without any decrease according to the animal response (48,49). In addition, experimental data suggest that iNO might have direct antioxidant properties

reducing the inflammatory reaction in the lungs (51). Kinsella *et al.* (11) were the first to use 5 ppm of iNO in a prospective trial on 80 very sick premature infants who were ≤32 wk for at least 7 d. They showed, besides an early positive response on oxygenation, no change in the mortality rate but a significant decrease in the need for mechanical ventilation and the incidence of chronic lung disease. Many studies that have evaluated the effect of iNO in premature infants at an advanced stage of respiratory failure (12) failed to demonstrate an effect on mortality and/or outcome (8–11). In those studies, all infants did not respond to iNO, whereas the outcome was improved in the responders (22). Noteworthy, the first study to demonstrate a positive effect of iNO has treated infants early, with a low severity of respiratory disease (52). In this study, Schreiber *et al.* (52) used 10 ppm of iNO in 105 infants who were <34 wk and compared the incidence of chronic lung disease or death with 102 control subjects. They found a positive effect of iNO despite the low severity of initial HRF disease assessed by a mean oxygenation index of 6.8. In our study, we considered iNO therapy only in infants who required >40% F_{IO_2} and enrolled 76 HRF infants among whom the infants randomly assigned to receive iNO (n = 37) were slightly sicker to start with than control subjects (Table 1). Despite these worse initial conditions, this subgroup was able to respond to low-dose iNO, to decrease its early oxidative stress, and to have a relatively fair clinical outcome in comparison with most of the other studies (14,52). Therefore, one could speculate that the lack of significant results on the outcome in most of the studies with iNO might be related to the policy of treatment used in these studies.

CONCLUSION

Our study demonstrates that low doses of iNO administered soon after birth in very preterm infants who have moderate hypoxemic respiratory failure is associated with a decrease in their oxidative stress. We speculate that this impact of iNO occurs thanks to an early, rapid, and sustained decrease in F_{IO_2} requirements, at a postnatal age when the deleterious cascade of oxidative/inflammatory injuries can be reversed.

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