

# Monitoring Circulating Nitric Oxide Levels in Infants with Bronchopulmonary Dysplasia for Disease Activity

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**Background:** Contributing factors in bronchopulmonary dysplasia (BPD) in neonatal infants includes inflammation and oxygen radicals. Nitric oxide ( $\text{NO}_2^-$ ), an important mediator of inflammation can lead to cell injury or induce pro-inflammatory cytokines. Glucocorticoids are known to decrease the transcription of inducible NO synthetase.

**Objective:** To investigate if there are altered serum ( $\text{NO}_2^-$ ) nitrite, a products of NO metabolism levels in very low birth weight infants with BPD and respiratory distress syndrome (RDS) as compared to healthy newborns before and after dexamethasone treatment.

**Methods:** We clinically evaluated neonatal infants and obtained samples from 31 infants diagnosed with BPD (N=15), RDS (N=6), prematurity without respiratory distress syndrome (N=6), and full term (FT) neonates (N=4). Levels of nitrite were measured using the Greiss Reagent, and absorbance was determined at 550nm.

**Results:** The highest levels of  $\text{NO}_2^-$  were obtained in the BPD patients (9-253  $\mu\text{M}$ ), mean = 110  $\mu\text{M}$ , with low APGAR scores at 1 minute from 0 to 5. RDS patients with low APGAR scores at 1 minute from 5 to 7, also had high levels (8-93  $\mu\text{M}$ ), mean=54  $\mu\text{M}$  and patients with prematurity with normal APGAR scores at 1 minute from 6 to 9 had relatively low  $\text{NO}_2^-$  levels (29-76  $\mu\text{M}$ ), mean = 41 $\mu\text{M}$ . Dexamethasone treatment (0.5 mg/kg/d) of 12 hrs. for 3-8 days) in premature infants with low APGAR scores dramatically decreased from 85-253  $\mu\text{M}$  to 10-146 $\mu\text{M}$  ( $p<0.05$ ). In 2 of the severe BPD a rise in NO levels after dexamethasone correlated with severe systemic infection.  $\text{NO}_2^-$  levels in FT patients (21 - 37 $\mu\text{M}$ ), (mean=30  $\mu\text{M}$ ). Control serum samples from 16 infants randomly selected had NO levels of (8-53  $\mu\text{M}$ ), mean = 28  $\mu\text{M}$ ).

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## INTRODUCTION

Bronchopulmonary dysplasia (BPD), a clinical syndrome associated with the use of assisted ventilation and high concentration of oxygen has been redefined based on gestational age <32 or 32 weeks, treatment with oxygen > 21% for at least 28 days plus other criteria for mild, moderate or severe disease [1]. Diagnostic criteria for BPD have previously used the following definitions oxygen dependence beyond 28 days with persistent radiographic changes after mechanical ventilation and oxygen dependence beyond 36 weeks counted postnatal gestational age. The contributors to the pathologic picture of BPD are immaturity, inhaled oxygen, ventilation pressures, endotracheal tube injury, infection, and nutritional deficiencies. The abnormality in pulmonary function is characterized by decreased lung compliance due to areas of fibrosis, over distension and atelectasis. There is increased pulmonary resistance secondary to barotraumas [2]. BPD is frequently complicated with pulmonary edema caused by the increased pulmonary vascular pressure. Infection and the resultant inflammatory response frequently complicate the clinical course of chronic neonatal lung injury. The inflammatory

mediators released by either infection or high-inspired oxygen may aggravate the bronchoconstriction and vasoconstriction to which the lungs of these infants are predisposed. The nutritional deficiencies must be corrected and adequate calorie intake made available to BPD patients in order that new alveolar growth and repair of injured lung tissue proceed.

Inflammation is one of the most important factors because the increased production of mediators lead to exaggerated repair process with remodeling and development of pulmonary fibrosis. Thus, an early intervention with anti-inflammatory agents such as corticosteroids may be important.

Nitric oxide in BPD can be hypothesized to cause lung tissue injury by the following mechanisms: directly by its cytotoxic effects, by macrophage mediated cell death, especially in the presence of gram(-) bacilli tracheal colonization, indirectly, by forming the peroxynitrite which acts as an oxidant as investigated by Saugstad and by acting as a mediator to IL-8 which is an attractant for inflammatory cells [3].

Groneck, *et al.* evaluated tracheobronchial aspirate fluid of BPD in 59 neonates with birth weights under 1200 grams [4]. Fluid was assessed for chemotactic activity, neutrophil counts, concentration of elastase proteinase inhibitor,

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complement C5, leukotriene B<sub>4</sub>, IL-8 and other mediators [4]. As in studies from our division, with nasal lavage fluid or serum from either atopic allergic rhinitis, asthma and cystic fibrosis patients and mononuclear cell cultures from asthmatics, levels of inflammatory chemoattractants such as LTB<sub>4</sub>, IL-8 and NO were significantly higher compared to control neonates without chronic pulmonary disease [5-8]. Thus, an inflammatory reaction is also present in the lungs of preterm neonates prone to develop BPD and increased production of these mediators as seen in chronic asthmatics may lead to exaggeration of the repair process with remodeling and development of pulmonary fibrosis. Thus, early interventions with anti-inflammatory agents such as corticosteroids are essential.

Dexamethasone use in patients who turn out to have BPD cause a marked increase in survival rates [9-11]. Studies done by Geller, *et al.* showed that dexamethasone reduced levels of NO synthesis in macrophages, endothelial cells and hepatocytes by as much as 63% [12]. High levels of NO in airway epithelial cells was also shown to be significantly decreased in asthmatics after corticosteroid administration [13]. It is therefore possible that the efficacy of dexamethasone in BPD is partly due to its non-specific inhibition or reduction of NO levels.

Nitric oxide in BPD can be hypothesized to cause lung tissue injury by its cytotoxic effects, [13,14] by macrophage mediated cell death with endotoxin or, by forming the peroxynitrate which acts as an oxidant<sup>3</sup> or by acting as a mediator to IL-8 which is an attractant for inflammatory cells. Studies from our division have detected TNF in normal mononuclear cell cultures after endotoxin [15] and both NO or NO<sub>2</sub> and TNF in cultured epithelial cells [16].

Despite the beneficial effects corticosteroids on cytokines and chemokines [17] there are well documented adverse effects. Monitoring NO along with other inflammatory cytokines associated with bronchial epithelial cells could be very useful biomarkers of inflammation and response to therapy.

## MATERIALS AND METHODS

### Subjects

Thirty-one patients from the Neonatal Intensive Care Unit were eligible for enrollment if they were less than 24 hours of age, weighing between 609 and 2445 gms at birth, requiring intubation and mechanical ventilation. Patients were stratified as to severity of BPD based on birth weight (609-1089 gms and 1010-1601 gms), prematurity, ventilator and oxygen requirements, whether or not there would be a coexisting medical condition other than BPD (e.g. sepsis, necrotizing enterocolitis, and congenital anomalies.) The correlation between the severity of BPD, gestational age, birth weight, APGAR's scores and oxygen requirement was determined. Patients with RDS and premature infants without respiratory distress (1253 – 3181 gms) were also included in the study as well as full term infants (2520 – 3563 gms) who served as controls. Sixteen patients from the newborn nursery also served as controls. Patients with congenital anomalies (chromosomal, cardiac and pulmonary) were excluded from the study. The diagnosis of BPD was

established by accepted clinical and radiologic findings and by the need of oxygen supplementation at 28 days of age. The same number of patients without oxygen evolving BPD and other medical conditions, but who otherwise needed diagnostic blood sampling to rule out medical conditions were included in the study under the group of healthy newborn infants. The study was approved by the Institutional Review Board. No consent was needed since clinical routine samples for standard blood work was used and no additional blood was necessary. Treatment was the standard of care for very low birthweight infants and included appropriate ventilation support, surfactant and dexamethasone administration on or before 2 weeks of age as well as supportive care. Samples for serum nitric oxide were obtained within the first 24 hours of life. Post dexamethasone nitric oxide levels were obtained within 24 hours post treatment.

### Nitric Oxide Method

Nitric oxide in serum was measured by a standardized colorimetric assay for nitrite (NO<sub>2</sub><sup>-</sup>) as an indirect index of NO production using the Greiss reagent. Briefly, 100 µl of sample was mixed with an equal volume of Greiss reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2.5 % phosphoric acid) and incubated at room temperature for 10 min. Sodium nitrite (NO<sub>2</sub><sup>-</sup>)(Sigma) was used as the standard from 0.156 µM – 20 µM concentration. The absorbance at 550nm was measured in a microplate reader with a correction wavelength of 650 nm. The sensitivity of this assay is <0.5 µM.

### Statistical Analysis

All data was expressed, as the mean between and within group comparisons using student's t test. A p value of < .05 was considered statistically significant. SigmaStat was used to calculate sample size with a power of 0.8 and alpha of 0.05. The power analysis using the available data for nitric oxide measurements in normal vs. BPD/RDS subjects showed that power of the study is at 0.998 with an alpha error of 0.05. Sample size with the available data was sufficient to achieve the minimum power of 0.8.

## RESULTS

Table I illustrates serum NO<sub>2</sub><sup>-</sup> levels in very low birth infants with evolving BPD and RDS as compared to normal newborns. Prematurity, birth weight, ventilator and oxygen requirements is listed for both severe and mild to moderate BPD infants as compared to RDS and premature infants without respiratory distress. The highest NO<sub>2</sub><sup>-</sup> levels were obtained in severe BPD infants from 9-253 µM with the lowest APGAR scores at 1 minute from 0 to 5. The mean NO<sub>2</sub><sup>-</sup> levels for severe BPD = 109.7 µM vs. 61 µM in the mild to moderate group. RDS patients with low APGAR scores at 1 minute from 5 to 7 also had high levels (8-93 µM). The mean NO<sub>2</sub><sup>-</sup> in RDS infants was 54.08 µM. Nitrite levels were found to closely reflect the severity of BPD with respects to oxygen requirements as well as the clinical condition of the infants undergoing treatment with steroids.

**Table 1.** Serum NO<sub>2</sub><sup>-</sup> levels in very low birth infants with evolving BPD and RDS.

Data	Bronchopulmonary Dysplasia		RDS
	Severe	Mild/Mod	
Number of patients Evaluated	8	7	6
Prematurity Mean	24-28 26.1	27-31 29.5	28-34 32
Birth weight (gram) Mean	609-1089 869	1010-1601 1305	1253-2445 1822
Ventilator requirement Mean	3-24 weeks 6.8 weeks	14-18 days 16 days	0-6 days 2.3 days
Oxygen Requirement Mean	5-36 weeks 11.4 weeks	4-5 weeks 4.5 weeks	14-21 days 15.6 days
NO <sub>2</sub> Level (µm) Mean	9-253.4 109.7	8-134.8 61	8.23-39 54.08

Table II illustrates serum NO<sub>2</sub><sup>-</sup> in premature infants without respiratory distress and normal full term control infants. The premature infants without respiratory distress had a mean prematurity of 35 weeks and a mean weight of 2265 grams. The mean NO<sub>2</sub><sup>-</sup> levels in this group was 41.03 µM compared to the full term controls either in the Neonatal Intensive Care Unit or regular newborn nursery which was 29.6µM and 27.6 µM. A trend of decreased mean NO<sub>2</sub><sup>-</sup> levels was also observed in these 3 groups, however, levels were not statistically significant. Figure 1 illustrates the

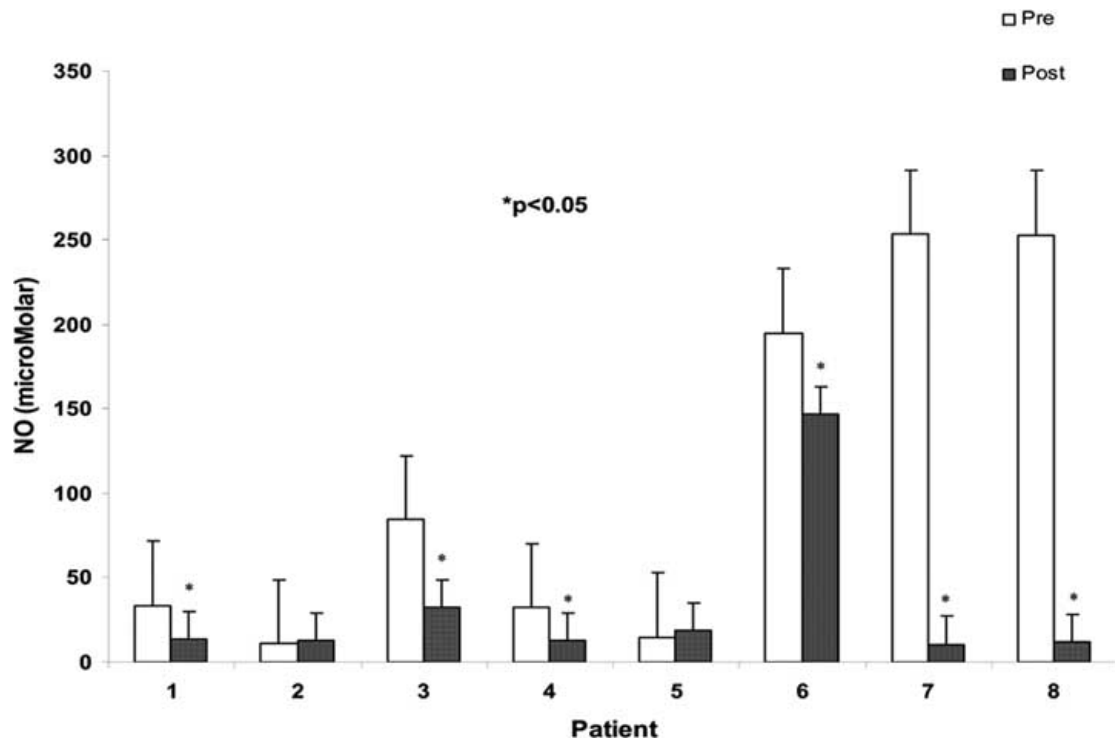
**Table 2.** Serum NO<sub>2</sub><sup>-</sup> levels in premature infants without respiratory distress and normal newborns.

Data	Prematurity without respiratory disorder	Full Term Control	
		NICU	NURSERY
Number of patients Evaluated	6	4	16
Prematurity (weeks) Mean	32-37 35	39-41 40	
Birth weight (gram) Mean	1449-3181 2265	2520-3563 2921	
Oxygen Requirement Mean	0-10 days 3 days	0-2 days 0.2 days	
NO <sub>2</sub> Level (µm) Mean	29.1-76 41.03	21.4-37.3 29.6	8-53 27.6

mean NO<sub>2</sub><sup>-</sup> levels in 8 patients with severe BPD. After treatment with dexamethasone there was a significant decrease in mean NO<sub>2</sub><sup>-</sup> levels in 6 of these patients (p<0.05).

**DISCUSSION**

BPD is a complex disease with multifactorial causes involving oxygen radicals and inflammation [18]. Studies conducted by Gladstone and Levine demonstrated that oxygen radicals and other free radicals lead to protein oxidation injury of the neonatal airway epithelium [19]. Clinical studies have demonstrated that inhaled NO can



**Fig. (1a).** Nitrite levels before and after dexamethasone treatment in eight patients with bronchopulmonary dysplasia.

improve oxygenation and pulmonary hypersensitivity in premature neonates with severe RDS [20]. The main pathologic finding in BPD is cell necrosis [21]. Necrosis eventually results in marked scarring with hyperinflation, squamous metaplasia of large and small airways, increased peribronchial muscle with fibrosis, chronic inflammation and submucosal edema [22]. Thus, the inflammation and increased production of toxic mediators may lead to an exaggerated repair process with remodeling and development of pulmonary fibrosis. Early intervention with anti-inflammatory agents such as corticosteroids is essential. Mammel and associate<sup>23</sup> suggested a wide array of potential mechanisms by which steroid treatment might improve lung function acutely in chronic lung disease of newborns. These include stabilization of cell and lysosomal membranes; increase in surfactant, inhibition of prostaglandin and leukotriene synthesis, decreases in polymorphonuclear cell recruitment in the lung, breakdown of granulocyte aggregates with improvement in pulmonary microcirculation, enhancement of  $\alpha$ -adrenergic activity; and reduction of pulmonary edema. Relaxation of bronchospasm and reduction of bronchial edema, also clearly plays a role in respiratory dependency in BPD [10].

New actions of oxygen radicals have been emphasized and it has been demonstrated that the degree of protein oxidation of the lung of newborn infants is associated with chronic lung injury [3]. Studies in our division have also evaluated superoxide dismutase in asthmatic patients [24]. New insight into the interaction of oxygen radicals with excitatory amino acids and the NO system also increases the possibility to understand and hence prevent oxygen radical injury in the preterm infant as well as adults exposed to an increased load of oxygen radicals [3]. During hypoxia and reoxygenation, excitatory amino acids are released from presynaptic endings, calcium ions enter the neurons, activates nitric oxide synthase or NOS and the production of NO increases [3]. NO also reacts with superoxide and the toxic peroxynitrate radical or ONOO can be formed which may initiate cell injury [3].

Three distinct isoforms of NOS have been isolated and purified in humans [25]. Two of these isoforms, neuronal and endothelial, are constitutive (cNOS), i.e. naturally occurring inside cells. The third isoform is normally absent but its expression is induced in response to external stimuli, such as cytokines and LPS (inducible NOS or iNOS). In addition to its physiological role as a potent vasodilator and neurotransmitter, NO appears to play a critical role in inflammation [26]. NO has a fundamental role in lung growth.

Dexamethasone thought to be effective in BPD has been shown to stimulate antioxidant enzyme activity, decrease neutrophil aggregates improving pulmonary microcirculation and reducing pulmonary edema [27,28].

Betrand evaluated the relation between prematurity, RDS and need for mechanical ventilation [29]. Siblings and mothers of infants with the most significant lung disease had evidence of airway reactivity. Stocker noted early histologic changes in BPD to include patchy loss of cilia along with infiltration of neutrophils and lymphocytes [30]. Of interest,

our division noted  $\text{NO}_2^-$  significantly increases IL-8, a potent neutrophil chemoattract in the airway epithelium [31].

Exhaled NO is considered a surrogate marker of airway inflammation, NO itself may have proinflammatory and anti-inflammatory effects [32]. *In vitro* murine studies suggest that NO promotes the proliferation of Th2 lymphocytes and could shift the balance between Th1 and Th2 cells, and thereby promote airway inflammation [33]. Exhaled breath nitric oxide has been shown to be a non-invasive, sensitive marker of the response to various anti-inflammatory therapies in asthma [34-36]. In our study with a relatively small sample size variations are wide within the BPD and RDS group. Gestational ages and birth weights are different and could account for differences in the mean  $\text{NO}_2^-$  levels.

Thus, in this study in neonatal infants with BPD and RDS,  $\text{NO}_2^-$  levels coincided with disease severity, lower APGAR scores and may be useful as a potential biomarkers or for monitoring clinical responses to corticosteroids and other novel therapeutic agents.

## CONCLUSIONS

Thus in 75% of neonates with BPD and RDS,  $\text{NO}_2^-$  levels coincided with the severity of the disease, lower APGAR scores, and also with infection. Therefore,  $\text{NO}_2^-$  may be utilized as a potential biomarker or used for monitoring clinical response and treatment with corticosteroids.

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