Differential effects of inhaled nitric oxide and hyperoxia on pulmonary dysfunction in newborn guinea pigs

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The objective of this study was to differentiate between the toxicity of NO and hyperoxia and to determine whether NO protects against or augments O2 toxicity, using a newborn guinea pig (GP) model, which is known to be highly susceptible to hyperoxia-induced lung injury (4). We hypothesized that in newborn GPs, inhaled NO and combined inhaled NO and hyperoxia would result in less pulmonary dysfunction and have a longer delay in the onset of respiratory distress compared with hyperoxia exposed.

METHODS

This study was approved by the Animal Care and Use Committee at Tripler Army Medical Center. Procedures involving GPs were performed in accordance with the National Institutes of Health (NIH) policies, the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1985), and the Animal Welfare Act and Amendments. Tripler Army Medical Center is accredited by the American Association for Accreditation of Laboratory Animal Care.

Animal Exposure and Handling

One- to two-day-old newborn GPs (Duncan-Hartley albino; Charles River Breeding Laboratory, Wilmington, MA) of either sex were exposed to room air (RA, n = 14), 95% O2 (n = 36), 20 parts per million (ppm) of NO (n = 14), or combined 20 ppm NO and 95% O2 (NO/O2, n = 13) for up to 5 days. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Plexiglas exposure chamber (15 × 18 × 8 in.) was maintained at 24°C with humidity at 70% and with 10–12 air exchanges/h. NO and NO2 levels (<2 ppm) were monitored using a Pulmonox II (Tofield, Alberta, Canada). Newborns were weighed daily and nursed by their mothers for the first 24 h of exposure and then weaned. Pups were then provided food (Purina GP chow;Ralston, St. Louis, MO and Kitten Milk Replacement, Pet-Ag, Hampshire, IL) and water ad libitum.

At the study end point, newborn GPs were sedated with 40 mg/kg intramuscular ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, NJ), and were euthanized with 150 mg/kg ip pentobarbital sodium (Wyeth, Philadelphia, Parke-Davis, Morris Plains, NJ), and were euthanized with pentobarbital sodium to ensure complete collapse of the alveolar spaces as respiration slowed. The trachea was cannulated in situ and connected to a water pressure manometer. A specific series of studies were performed as described below; however, not every study was able to be completed on each animal, and the number of animals used for each experiment varied.

**Experimental Studies**

**Animal characteristics.** We evaluated time to develop signs of respiratory distress rather than survival as a study end point. The GPs were euthanized on day 5 or earlier if they developed signs of moderate respiratory distress (retractions or nasal flaring) coupled with signs of generalized illness (ruffled fur, decreased activity, cyanosis, pallor, or abnormal resting posture).

**Pressure-volume curves.** Static pressure-volume curves (17, 30) to determine lung compliance were performed by inflating the lungs in 5 cmH2O pressure increments from 5 to 35 cmH2O with lung volume measured after 30 s of equilibration at each pressure. Similar volume measurements were obtained for the deflation curve (35 to 0 cmH2O). Pressure-volume curves were plotted to determine lung compliance from 13 RA control GPs, 5 NO-exposed GPs, 13 hyperoxic-exposed GPs, and 11 NO/O2-exposed GPs. The overall changes in inflation volumes after the initial two isovolumetric measurements of the inflation portion of the pressure-volume curve were used to indicate differences in lung compliance among the four groups. The isovolumetric portion of the deflation portion of the pressure-volume curve was used to compare lung volume capacities in the four groups (33).

**Lung histology and bronchoalveolar lavage cell composition.** Lung histology and white cell differential counts were performed as a measure of inflammation and lung injury. Lung sections were fixed by infusing 10% buffered Formalin under constant pressure. The lungs were then sectioned and stored in Formalin for histopathology. The sections were embedded in Ameraffin (Stephens Scientific, Riverdale, NJ) and stained with hematoxylin and eosin. A four-point, severity-based scoring system (0, no involvement; 1+, mild involvement; 2+, moderate involvement; and 3+, severe involvement) was used to assess seven individual characteristics (11, 22). The characteristics included inflation pattern, inflammation, alveolar fibrin, hyaline membrane, alveolar hemorrhage, interstitial edema, and alveolar edema. Bronchoalveolar lavage (BAL) was performed with three 1-ml aliquots of normal saline, which were then pooled and centrifuged at 2,000 rpm for 10 min at 4°C. White cell differential counts were obtained after Giemsa staining of cytocentrifuge cell samples and processed within 1 h of the experiment. The pathologist was not informed of the exposure group.

**Proteolytic activity and total protein from BAL.** Secreted proteinase activity was assayed as a measure of inflammatory response and total protein as a measure of capillary leak. The supernatant from the centrifuged samples described above were aliquoted and frozen at −70°C for later analysis. Secreted proteinase studies and total protein were performed using frozen samples of BAL fluid by a blinded investigator. The BAL fluid was centrifuged at 5,000 g for 5 min, and duplicate 100-μl aliquots of the supernatant were placed in 96-well microtiter plates and warmed to 37°C for 10 min. Chymotryptic, trypsin, and elastolytic activity were measured by the hydrolysis of substrates N-succinylanlalanylalanyl-prolyl-phenylalanyl-p-nitroanilide (Sigma Chemical, St. Louis, MO), N-benzoyl valylglycylarginyl-p-nitroanilide (Sigma Chemical), and N-succinylanlalanylalanyl-prolylvalyl-p-nitroanilide (Bachem, Torrance, CA), respectively, using methods previously described (25, 32). To correct for pigmentation in the lung lavage fluid samples, a control plate was set up containing duplicate aliquots of lavage samples incubated with buffer and no p-nitroanilide substrate. Microplates were kept at 37°C, and the absorbance at 405 nm was read with a microplate reader (Thermomax; Molecular Devices, Menlo Park, CA) at baseline and 18 h after the substrate was added. Protein levels were determined by the Bradford assay.

**Statistical Analysis**

Data analysis was performed using commercial software packages (SigmaStat, Jandel, San Rafael, CA; JMP, Statistical Analysis System Institute, Cary, NC; and Number Cruncher Statistical System, Kaysville, UT). Normality of data distribution was tested by the Kolmogorov-Smirnov test. A log-rank Gehans-Wilcoxon test was used to compare latency intervals to onset of respiratory symptoms. Pressure-volume statistical comparisons were made using two-way analysis of variance (ANOVA) with orthogonal contrasts of inflation pressures (10–35 cmH2O) and the isovolumetric deflation pressures (35–10 cmH2O) between the four treatment groups. Cell count, histology, and total protein were analyzed by one-way ANOVA and Dunn’s multiple comparison procedure. Proteolytic activity was analyzed by one-way ANOVA and Newman-Keuls multiple comparison procedure. \( P < 0.05 \) was considered significant, and values were expressed as means ± SE or as median with range if the values were not normally distributed.

**RESULTS**

**Animal Characteristics**

All groups had similar weights (RA, 96.1 ± 2.5 g; O2, 97.6 ± 2.5 g; NO, 107.0 ± 3.8 g; and NO/O2, 104.9 ± 2.4 g). The hyperoxic-exposed GPs showed poor weight gain (0.1 ± 0.05 g/day) and developed signs of respiratory distress (retractions, nasal flaring) coupled with signs of generalized illness (ruffled fur, decreased activity, pallor, or abnormal resting posture) at a range of 1–5 days (median 4.0, Fig. 1). The GPs exposed to NO and RA gained weight similarly (NO, 9.9 ± 1.5 g/day; RA, 13.3 ± 1.8 g/day) and had no signs of respiratory distress. The NO/O2-exposed group showed poor weight gain (−1.9 ± 1.9 g/day) but did not develop signs of respiratory and generalized illness until 3–5
days (median 5.0) of exposure. The increased latency interval for the onset of respiratory distress compared with O2 exposure was evident with a statistically significant shift in the latency interval over the 5-day observation period (Fig. 1).

**Pressure-Volume Curves**

Hyperoxia- and NO/O2-exposed GPs had reduced lung volumes compared with NO- and RA-exposed GPs (Fig. 2, orthogonal contrast of isovolumetric portion of deflation curve, \( P < 0.01 \)), indicating a reduced functional lung capacity. GPs receiving O2 alone or in combination with NO also exhibited a decreased rate of change in volume with increasing inflation pressure, indicating decreased lung compliance in these animals. This decreased lung capacity and lung compliance were due to the O2 exposure as NO exposure alone did not alter the pressure-volume curves compared with RA exposure. Also, NO did not exacerbate the effects of O2 on lung volume capacity or compliance since there was no difference between O2 and NO/O2 groups.

**Lung Histology and BAL Cell Composition**

In comparison to RA and NO, O2- and NO/O2-exposed GPs lung histopathology showed increased but comparable evidence of inflammation, hemorrhage, alveolar fibrin, hyaline membranes, and interstitial edema (Table 1).

BAL from O2-exposed GPs contained increased numbers of polymorphonuclear cells (PMNs) compared with RA- and NO-exposed GPs as seen in Table 2. Furthermore, eosinophils and macrophages were decreased (Table 2).

**Secreted Proteinase Assay and Total Protein From BAL**

As shown in Fig. 3, chymotryptic and tryptic activities were significantly increased in O2- and NO/O2-exposed GPs, compared with NO-exposed and RA control GPs. Of note, elastolytic activity was significantly increased in GPs exposed to NO/O2 compared with RA and NO exposed. It tended to be higher than in O2- treated GPs, but this trend did not achieve significance (\( P < 0.1 \)).

Similarly, O2- and NO/O2-exposed GPs’ BAL showed increased total protein as seen in Fig. 4 (O2, 6.2 ± 1.1 mg/ml vs. RA, 0.2 ± 0.05 and NO, 0.2 ± 0.07 mg/ml; NO/O2, 5.0 ± 1.4 vs. RA, \( P < 0.05 \)).

**DISCUSSION**

Hyperoxia has been reported to cause pulmonary pathology and dysfunction, leading to death in several species (4, 12, 22). In survival studies, Frank et al. (4) showed that newborn GPs are particularly susceptible to hyperoxic exposure because of an inability to increase protective lung antioxidant enzymes, resulting in 50% mortality by 3.6 days. Kelly et al. (14) reported a mortality rate of 21% in term GPs exposed to 95% hyperoxia for 96 h. Because of this susceptibility of the newborn GP, we chose this model to evaluate the effects of NO alone and in combination with hyperoxia exposure, as it would allow improved detection of pulmonary dysfunction if NO-mediated oxidative reactions played an important role in pulmonary toxicity. Indeed, the manifestations of pulmonary dysfunction such as decreased compliance, pulmonary edema, increased PMNs, proteolytic enzymes, and total protein in BAL after hyperoxia exposure found in the present study were consistent with severe lung injury.

The effects of NO alone on pulmonary dysfunction are controversial, with discrepancies between in vivo and in vitro biochemical studies. Rabbits (13) and mice (23) with normal lungs have breathed NO in clinically used concentrations for up to 6 mo without evidence of
lungs injury. However, in vitro studies have demonstrated surfactant dysfunction (10), decreased endogenous NO production (3), increased superoxide and peroxynitrite-mediated cell injury (26), increased membrane lipid peroxidation (26), increased cytokine-mediated cytotoxicity (18), enzyme inhibition (16), increased oxidative DNA damage (34), increased endothelial injury (2), and prevention of leukocyte adherence (6). Despite these concerns regarding NO toxicity, we found that in marked contrast to hyperoxia-exposed GPs, pups exposed to NO alone gained weight similarly to their RA-exposed counterparts and did not develop any signs of acute respiratory distress or evidence of pulmonary dysfunction.

However, the toxicities of NO may be manifested or accentuated by the concomitant use of high O2 concentrations. There are presently a limited number of studies on the influence of combined inhaled NO and hyperoxia exposure on lung injury (7, 9, 21, 28). Gutierrez et al. (9) found increased survival in rats at 144 h with the addition of 7.8 ppm NO to >95% O2. Garat et al. (7) found no effect of either 10 ppm NO or 100 ppm NO on survival in >95% O2. They also reported that in rats exposed to >95% O2 for 40 h, adding 100 ppm NO made no difference in the lung wet-to-dry weight ratios or the appearance of intravenous administered131I-labeled albumin in the alveoli. However, adding only 10 ppm NO decreased both lung wet-to-dry weight ratios and the appearance of intravenously administered131I in the alveoli (7). More recently, Nelin et al. (21) demonstrated in a rat model that inhaled NO increased survival with high O2 exposure of 120 h despite significant weight loss. Taken together, these studies (in rats) suggest that the deleterious effects of hyperoxia can be attenuated by the addition of inhaled NO. In the present study, NO/O2-exposed GPs had an increased latency interval to develop signs of respiratory distress compared with the hyperoxia exposure alone, adding further support to the notion that NO did not produce signs of pulmonary dysfunction.

In regard to the specific techniques of assessment, the NO alone and RA control exposure groups were comparable in pulmonary compliance, lung histology, PMNs, proteinase activity, and total protein in BAL. In contrast, hyperoxia- and NO/O2-exposed groups had decreased pulmonary compliance, increased pulmonary edema, PMNs, proteinase activity, and total protein. Moreover, when NO was combined with hyper-

<table>
<thead>
<tr>
<th>n</th>
<th>Atelectasis</th>
<th>Inflammation</th>
<th>Hemorrhage</th>
<th>Alveolar Fibrin</th>
<th>Hyaline Membrane</th>
<th>Interstitial Edema</th>
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<tr>
<td>RA</td>
<td>6</td>
<td>0.3 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>0.8 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>O2</td>
<td>6</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.9 ± 0.0</td>
<td>2.0 ± 0.0*†</td>
<td>1.2 ± 0.2†</td>
</tr>
<tr>
<td>NO</td>
<td>8</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>0.3 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>NO/O2</td>
<td>13</td>
<td>1.7 ± 0.2*</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.1†</td>
<td>2.4 ± 0.2††</td>
<td>1.7 ± 0.2*††</td>
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Values are means ± SE; n, number of newborn guinea pigs (GPs). FIO2, fraction (95%) of inspired O2; ppm, parts per million; RA, room air control; O2, 95% oxygen; NO, 20 ppm (parts per million) nitric oxide; NO/O2, combined 20 ppm NO and 95% O2. Lung histology was graded on a 0—3 point grading scale with 0 showing no evidence of involvement and 3 showing severe involvement. *P < 0.05 vs. RA-exposed GPs. †P < 0.05 vs. NO-exposed GPs. §P < 0.05 vs. RA exposed GPs. ‡P < 0.05 O2 vs. NO. ††P < 0.05 NO/O2 vs. NO.

Table 2. Bronchoalveolar lavage cell count from newborn GPs after exposure to RA, hyperoxia, NO, and combined NO and hyperoxia

<table>
<thead>
<tr>
<th>n</th>
<th>PMNs ± SE</th>
<th>Eosinophils ± SE</th>
<th>Macrophages ± SE</th>
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<tr>
<td>RA</td>
<td>5</td>
<td>42± 7.1</td>
<td>53.6± 7.1</td>
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<tr>
<td>O2</td>
<td>7</td>
<td>58± 1.3</td>
<td>18.1± 3.9</td>
</tr>
<tr>
<td>NO</td>
<td>8</td>
<td>11± 1.0</td>
<td>42.4± 4.1</td>
</tr>
<tr>
<td>NO/O2</td>
<td>9</td>
<td>27.8± 7.0‡*</td>
<td>28.4± 4.4*‡‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of newborn GPs. PMNs, polymorphonuclear cells. *P < 0.05 vs. RA-exposed GPs. †P < 0.05 vs. hyperoxia-exposed GPs. ‡P < 0.05 vs. NO-exposed GPs. §P < 0.05 vs. NO and hyperoxia-exposed GPs.
oxia, the pulmonary compliance, lung histology, PMNs, proteinase activity, and total protein were not worsened compared with hyperoxia exposure alone, suggesting a similar degree of pulmonary dysfunction. These consistent results suggest that it was hyperoxia exposure that resulted in the pulmonary dysfunction rather than the combined exposure.

Several lines of evidence suggest that this pulmonary dysfunction might be due to lung inflammation and capillary leak and may be the mechanism to explain our findings. First, the hyperoxia- and hyperoxia and NO-exposed animals had increased PMNs when compared with NO exposure alone. However, when lung histopathology was evaluated, hyperoxia-alone and NO/O2-exposed GPs showed comparable evidence of alveolar fibrin, hyaline membranes, and interstitial edema compared with RA- and NO alone-exposed GPs.

Second, secreted proteinase activity can be a useful measure of inflammatory activity because proteinases are among the major mediators of neutrophils, mast cells, and other inflammatory cells in all mammalian species. Enzymatic activity can be monitored without confounding species differences in structure/antigenicity of the proteinases (25, 32). The increased concentrations of PMNs and proteinases in BAL from lungs exposed to O2 alone and NO/O2 provide further evidence that these exposures produce lung inflammation. Because the PMNs and proteinases in BAL in the NO alone exposure group were comparable to RA controls, one can speculate that the increased PMNs and proteinases detected in the combined exposure group may be secondary to the inflammation produced primarily by hyperoxia and support studies in other species (13, 23) that NO concentrations used clinically (20 ppm) may not produce significant lung inflammation. The increased elastolytic activity in the NO/O2-exposed group may suggest that inhibitors of elastase are inactivated by oxidants and not mediated by release (19). However, it should be noted that this is the first study that we are aware of to evaluate proteinase activity from BAL specimens. Therefore, extrapolation of these results to other studies evaluating proteolytic activity in isolated tissues may be difficult (25, 32).

Finally, another manifestation of lung injury is capillary leak resulting in increased total protein concentration in BAL, which provides an estimate of hyperoxia-induced alteration in lung permeability (7). The increased concentration of total protein in BAL from lungs exposed to O2 alone and NO/O2 provide further evidence that these exposures produced an alteration in lung permeability. In contrast, the total protein in BAL in the NO alone exposure group was comparable to RA controls, suggesting that the increased total protein in the combined exposure group may be secondary to an alteration in lung permeability produced primarily by hyperoxia.

In summary, this study showed that 1) hyperoxia exposure resulted in multiple measures of pulmonary dysfunction in newborn GPs, 2) 5-day exposure to NO alone produced no noticeable respiratory effects or pulmonary dysfunction, and 3) short-term exposure (≤5 days) to NO/O2 delayed the onset of respiratory distress but neither exacerbated nor attenuated pulmonary dysfunction compared with hyperoxia exposure alone.

**Perspectives**

This study supports other studies that demonstrate the relative short-term safety of inhaled NO. In contrast to hyperoxia exposures, there was no evidence of lung inflammation or capillary leak with NO alone. When combined NO/O2-exposed GPs were compared with hyperoxia-exposed GPs, increased elastolytic activity was noted although other measures of pulmonary dysfunction were similar, suggesting that inhaled NO does not accentuate hyperoxia-induced pulmonary dysfunction. Moreover, the increased latency interval to develop respiratory distress for the combined exposure group supports the notion of a possible attenuating effect of inhaled NO that may be due to mechanism(s) other than lung inflammation. Perhaps subsequent studies evaluating other individual biochemical parameters may reveal additional differences. Taken together, this may help further define the summation of physiological effects related to the oxidant and antioxidant properties of NO. If these results could be applied to humans, these findings may be important in further understanding pathophysiologic mechanisms that may help with determining management strategies for the use of inhaled NO and supplemental O2.

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