Effects of Inhaled Nitric Oxide on Inflammation and Apoptosis After Cardiopulmonary Bypass*

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**Background:** Cardiopulmonary bypass (CPB), a procedure often used during cardiac surgery, is associated with an inflammatory process that leads to lung injury. We hypothesized that inhaled nitric oxide (INO), which has anti-inflammatory properties, possesses the ability to modulate lung cell apoptosis and prevent CPB-induced inflammation.

**Methods:** Twenty male pigs were randomly classified into four groups: sham, sham plus INO, CPB, and CPB plus INO. INO (20 ppm) was administered for 24 h after anesthesia. CPB was performed 90 min into INO treatment. BAL fluid and blood were collected at time 0 (before CPB), at 4 h after beginning CPB, and 24 h after beginning CPB (T24).

**Results:** At T24, BAL interleukin (IL)-8 levels and neutrophil percentages were elevated significantly in the CPB group. At T24, INO reduced IL-8 concentrations and attenuated the increase of neutrophil percentage in the CPB-plus-INO group. Nitrite-plus-nitrate (NOx) concentrations were decreased significantly in groups without INO. Moreover, animals treated with INO showed higher rates of pulmonary apoptosis compared to their respective control groups except for the sham-plus-INO group, in which they were diminished.

**Conclusion:** These results demonstrate that NOx production is reduced after CPB, and that INO acts as an anti-inflammatory agent by decreasing neutrophil numbers and their major chemotactic, IL-8. INO also increases cell apoptosis in the lungs during inflammatory conditions, which may explain, in part, how it resolves pulmonary inflammation.

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**Key words:** cardiopulmonary bypass; endothelial nitric oxide synthase; inhaled nitric oxide

**Abbreviations:** ALI = acute lung injury; ANOVA = analysis of variance; CPB = cardiopulmonary bypass; eNOS = endothelial nitric oxide synthase; IL = interleukin; INO = inhaled nitric oxide; iNOS = inducible nitric oxide synthase; NF = nuclear factor; NO = nitric oxide; NOx = nitrate plus nitrite; T0 = time before cardiopulmonary bypass; T4 = 4 h after beginning cardiopulmonary bypass; T24 = 24 h after beginning cardiopulmonary bypass; TNF = tumor necrosis factor; TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

Cardiopulmonary bypass (CPB) induces a systemic inflammatory response via many factors, including the exposure of blood to nonphysiologic surfaces and conditions, surgical trauma, ischemia-reperfusion of the organs, changes in body temperature, and release of endotoxin.

The effects of CPB on proinflammatory tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, and anti-inflammatory IL-10 cytokines have been measured, with many studies showing an increase in plasma and BAL cytokines after CPB. IL-8, a potent chemoattractant for neutrophils, is involved in neutrophil influx and in the release of their cytotoxic products, which include free radical species, proteolytic enzymes, and eicosanoids that subsequently cause acute lung injury (ALI). Neutrophils...
normally have a short half-life (< 24 h), with aging cells undergoing apoptosis (programmed cell death). This mechanism is the most efficient way of removing these cells without release of their cytotoxic products, limiting damage to surrounding tissues. Conversely, inhibition of apoptosis of these cells increases their life span, leading to amplification of the inflammatory process.

Nitric oxide (NO) has numerous physiologic and pathophysiologic functions. It can be inhaled as a therapeutic agent. Inhaled NO (INO) is capable of decreasing high pulmonary artery pressure, improving hypoxemia by reducing intrapulmonary shunt, and optimizing ventilation-perfusion matching. INO can also inhibit the inflammatory process by lowering cytokine synthesis and inactivating nuclear factor (NF)-κB, as several cytokines contain a binding site for NF-κB in their promoter regions. NO can also decrease the expression of adhesion molecules, preventing neutrophil adhesion and migration. INO can exert its effect on the lungs, on leukocytes trapped in the pulmonary area, but as it is transported by RBC to the general circulation, INO could have extrapulmonary outcomes.

Studies have demonstrated that NO can inhibit apoptosis induced in several cell types by proinflammatory agents. NO also exhibits a proapoptotic role, which involves inducible NO synthase (iNOS) activation. The source and dose of NO, cell type, and local environment appear to be important determinants of cellular fate. All this knowledge of NO has stimulated much interest in INO as a therapeutic agent against inflammation and to improve pulmonary function. In our study, we assessed whether 20 ppm of INO administered preventively can modulate both inflammation and apoptosis in the lungs of pigs submitted to CPB for 90 min.

**Materials and Methods**

The following experimental protocol was performed with the approval of the institutional animal care committee in compliance with the guidelines of the Canadian Council on Animal Care. The anesthetic method and CPB technique have been described extensively elsewhere. Briefly, healthy male pigs (mean weight, 37.15 ± 2.48 kg [± SD]) were premedicated IM with atropine (0.04 mg/kg), azaperone (4 mg/kg), and ketamine (25 mg/kg), and then anesthetized with 5 μg/kg of fentanyl and 5 mg/kg of thiopental. After intubation with an 8-mm endotracheal tube (Mallinckrodt Company, Mexico City, Mexico), the pigs were placed in the supine position. Anesthesia was maintained by continuous infusion of 5 mg/kg/h of thiopental and 20 μg/kg/h of fentanyl. Muscle relaxation was induced with 0.2 mg/kg of pancuronium followed by intermittent reinjections of 0.1 mg/kg to achieve optimal surgical and ventilatory conditions.

After induction of anesthesia and tracheal intubation, 20 ppm of NO gas was injected cyclically into the inspiratory line during the inspiratory phase by a NO injector for 24 h, and 1,000 ppm NO (Balanced N2 Cylinder; VitalAire Santé Ltée, Montreal, QC, Canada) NO and NO2 concentrations delivered to the animals were monitored with an electrochemical device (Polytron NO/NO2; Drager A.G., Lubeck, Germany). During CPB, NO was also added directly to the gas mixture delivered to the oxygenator.

Two groups of pigs underwent 90 min of CPB with cardiopulmonary cardiac arrest for 75 min. After 90 min, the animals were weaned from CPB (Table 1). Homeostasis was performed after removal of the CPB cannula, and the chest was closed.

The CPB circuit consisted of a membrane oxygenator with a cardiotomy reservoir (Affinity NTSH; Medtronic Minneapolis, MN), filter (Affinity 351; Medtronic), and tubing. A Sarns roller pump (Sarns; Ann Arbor, MI) was used. The circuit was primed with 1,500 mL of lactated Ringer solution (Baxter Corporation, Toronto, ON, Canada), 500 mL of colloidal fluid (Pentaspan; DuPont Pharma; Mississauga, ON, Canada), 1 mEq/kg of sodium bicarbonate, 5,000 IU of heparin, and 200 mL of mannitol.

The animals were ventilated and monitored in an intensive care set-up for 24 h after the beginning of CPB, and then killed. Lung samples were obtained as described below. BAL fluid was collected at time before CPB (T0), 4 h after the beginning of CBD (T1), and 24 h after the beginning of CPB (T24). Animals not submitted to CPB underwent sternotomy alone for 90 min and chest closure without bypass (sham operation).

**Protocol**

The animals were randomized into four groups: (1) the sham group (n = 5) was subjected to sternotomy for 90 min, followed by chest closure without CPB, and monitored until T24; (2) the sham-plus-INO group (n = 5) was submitted to INO administration (20 ppm), 90-min sternotomy, chest closure without CPB, and monitored until T24; (3) the CPB group (n = 5) was subjected to 90 min of CPB and monitored until T24; and (4) the CPB-plus-INO group (n = 5) underwent 90 min of CPB with INO therapy (20 ppm) throughout the experiment and was monitored until T24.

**Measurements**

**BAL**: The BAL method consisted of instilling three separate aliquots (25 mL, 25 mL, and 25 mL) of isotonic sterile saline solution (0.9% NaCl) into a segment of the cranial lobe of either lung, via a flexible fiberoptic bronchoscope introduced through the endotracheal tube and wedged in an airway. During BAL sampling, fraction of inspired oxygen was increased to 100%.

**BAL Analysis**: BAL cells were harvested by centrifugation at 900 revolutions per minute for 8 min at 4°C. The supernatant was aliquoted and preserved at −80°C for further analysis of IL-8 and TNF-α. The pellet was resuspended in 10 mL of RPMI-1640

<table>
<thead>
<tr>
<th>Time</th>
<th>Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:30 AM</td>
<td>Induction of anesthesia, intubation, beginning of mechanical ventilation, start of INO (if used)</td>
</tr>
<tr>
<td>7:45 to 8:30 AM</td>
<td>Catheterization</td>
</tr>
<tr>
<td>8:30 to 10:00 AM</td>
<td>CPB (90 min)</td>
</tr>
<tr>
<td>12:00 noon</td>
<td>Weaning from INO (if used)</td>
</tr>
<tr>
<td>1:00 PM</td>
<td>Weaning from anesthesia and mechanical ventilation</td>
</tr>
<tr>
<td>Summary</td>
<td>Duration of general anesthesia and mechanical ventilation, 5.5 h; duration of CPB, 1.5 h; duration of INO, 4.5 h</td>
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</tbody>
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plus 10% fetal calf serum, and viability was determined by trypan blue exclusion. Cell counts were then adjusted to $1 \times 10^5$/mL.

Total cell count was achieved by the hemocytometer method; differential cytospin slides were produced and stained with Wright-Giemsa for cell differentiation. Cells were counted under a microscope at original $\times 100$ magnification.

**Cytoxin Measurements:** BAL IL-8 and TNF-α concentrations were measured by enzyme-linked immunosassay (Biosource; Camarillo, CA; Pierce Endogen, Woburn, MA), according to the manufacturer protocols.

**Plasma and Lung NO Metabolite Measurements:** Heparinized blood samples were centrifuged at 1,500 revolutions per minute for 10 min at 4°C, and the plasma was stored at –80°C. At T24, the pigs were killed, and their lungs were harvested and stored at –80°C.

Plasma and lung tissue nitrite-plus-nitrate (NOx) levels were quantified by chemiluminescence: NO analyzer (270B; Sievers Instruments; Boulder, CO) and a data acquisition system (Dataq; Dataq Instruments; Akron, OH) as described previously.9

**Apoptosis in Lung Tissue:** Apoptosis in lung tissue samples was measured by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique with fluorescein in situ apoptosis detection kits (ST110 ApopTag-Intergen Company; Purchase, NY), as described in the manufacturer protocol. Briefly, lung sections were mounted on immunologic slides, deparaffinized, and pretreated with proteinase K. Free termini of the 3'-OH end of lung DNA were labeled with digoxigenin-deoxyuridine triphosphate. A peroxidase-binding antibody conjugate (anti-digoxigenin) was added, followed by its staining with fluorescein (substrate). The slides were mounted under glass coveslips and read with a fluorescence microscope. A positive control was pretreated with deoxyribonuclease, and a negative control was incubated without terminal deoxynucleotidyl transferase. At least 10 fields per tissue section were analyzed per pig.

Apoptosis was calculated as the percentage of fluorescence of each field divided by total tissue surface fluorescence. TUNEL labeling was defined as positive when tissue fluorescence intensity was as high as or higher than that of the positive control. All slides were analyzed with Metamorph 4.6 software (Roper Scientific; Tucson, AZ).

**Western Blot Analysis:** Pig lung tissues were snap frozen in liquid nitrogen and stored at –80°C. Protein extracts were prepared by Dounce homogenization of lung tissue in 50 mmol/L Tris (pH 7.6)-HCl lysis buffer containing protease inhibitors (5 mmol/L, ethylenediamine tetra-acetic acid; 1 mmol/L phenylmethylsulfonyl fluoride; 5 μg/mL pepstatin; 5 μg/mL aprotinin; 5 μg/mL leupeptin). Extracts were clarified through centrifugation (14,000g for 10 min at 4°C). The supernatants were quantified for protein concentration by protein assay (Bio-Rad Laboratories; Hercules, CA). Equal amounts (50 μg) of protein were subjected to (10 to 15%) sodium dodecylsulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes (Amersham Biosciences; Buckinghamshire, UK) that were blocked with 5% non-fat dry milk in Tris buffered saline solution containing 0.1% Tween 20, and then washed with Tris buffered saline solution-Tween. The membranes were incubated overnight at 4°C with gentle shaking with primary polyclonal rabbit anti-endothelial nitric oxide (eNOS) antibody, or polyclonal rabbit anti-iNOS antibody (1:1,000 and 1:3,000) dilutions, respectively; Transduction Laboratories) or polyclonal rabbit anti-caspase-3 antibody (5 μg/mL; NeoMarker, supplied by Medicorp; Montreal, QC, Canada), which recognize the native form (32 kd) and processed (20 kd and 18 kd) forms of caspase-3.

After washing, the membranes were incubated with gentle shaking for 2 h at room temperature with goat anti-rabbit IgG-horseradish peroxidase antibody (1:5,000 dilution; Bio-Rad). The membranes were visualized with enhanced chemiluminescence kit (Amersham Biosciences) and detected by photographic film. Bands were quantified with using software (ImageQuant; Molecular Dynamics; Sunnyvale, CA).

**Results**

**Effect of INO on BAL Cell Count, Neutrophil Percentage, and IL-8 Levels**

BAL cell count and neutrophil percentage showed a trend to increase from T0 to T24 in all groups. This increment was significant in the CPB group ($p < 0.05$). However, its magnitude was attenuated in groups receiving INO (Table 2). Neutrophil count was correlated with IL-8 concentration in the CPB group at T24 ($r = 0.66$, $p < 0.05$).

At T24, IL-8 levels were significantly elevated in the CPB group and reduced in the CPB-plus-INO group (Fig 1). No significant variation in the IL-8 between T0 and T4 was noted in all groups. BAL TNF-α concentrations were not detected.

**NOx Concentrations**

In plasma, NOx levels decreased significantly in the sham group at T24, and at T4 in the CPB group (Table 3); however, they were significantly higher at day 5 in CPB and CPB-plus-INO at T0, T4, and T24 compared to T0.

**Statistical Analysis**

All results are expressed as means ± SEM. Comparisons between experimental groups were performed with one-way analysis of variance (ANOVA), followed by the post hoc Tukey test, and comparisons over time within each group were made by ANOVA for repeated measures, followed by the Bonferroni post hoc test. Nonparametric Kruskal-Wallis ANOVA was followed by Dunn post hoc test, where appropriate. Correlations between variables were analyzed by Pearson correlation test. $p < 0.05$ was considered statistically significant.

### Table 2—Change in WBC Count and Neutrophil Percentage in BAL*

<table>
<thead>
<tr>
<th>Groups</th>
<th>T0</th>
<th>T4</th>
<th>T24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham PMN, %</td>
<td>4.94 ± 1.55</td>
<td>2.90 ± 0.86</td>
<td>5.24 ± 1.29</td>
</tr>
<tr>
<td>WBC, × 10^6/mL</td>
<td>5.00 ± 0.43</td>
<td>5.02 ± 1.00</td>
<td>7.52 ± 1.00</td>
</tr>
<tr>
<td>Sham plus INO PMN, %</td>
<td>2.50 ± 0.72</td>
<td>5.04 ± 1.65</td>
<td>4.93 ± 1.63</td>
</tr>
<tr>
<td>WBC, × 10^6/mL</td>
<td>4.60 ± 0.78</td>
<td>7.25 ± 1.32</td>
<td>6.02 ± 1.20</td>
</tr>
<tr>
<td>CPB PMN, %</td>
<td>3.8 ± 1.75</td>
<td>2.25 ± 0.64</td>
<td>11.65 ± 5.50</td>
</tr>
<tr>
<td>WBC, × 10^6/mL</td>
<td>5.94 ± 1.02</td>
<td>6.04 ± 0.92</td>
<td>12.14 ± 2.12</td>
</tr>
<tr>
<td>CPB plus INO PMN, %</td>
<td>1.79 ± 0.22</td>
<td>1.60 ± 0.36</td>
<td>5.25 ± 2.27</td>
</tr>
<tr>
<td>WBC, × 10^6/mL</td>
<td>4.52 ± 0.91</td>
<td>7.26 ± 1.08</td>
<td>9.42 ± 1.62</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM. PMN = polymorphonuclear neutrophil. No statistical differences were found in WBC count between groups and within groups.

$fp < 0.05$ vs T0.

$fp < 0.05$ compared to CPB plus INO at the same time point.
T24 in INO groups (171.77 ± 40.77 μmol/L) than T0 (131.9 ± 12.02 μmol/L). In lung tissue, NOx concentration was similar in groups without INO. It was increased in the CPB-plus-INO group compared to the control animals (p < 0.05) [Fig 2].

Effect of INO on Lung Tissue Apoptosis

The percentage of apoptosis was significantly decreased in the sham-plus-INO group vs the sham group (p < 0.05). In the CPB group, the percentage of apoptosis was lower but not statistically significant in comparison to the sham group. Conversely, the percentage of apoptotic cells increased significantly in the CPB-plus-INO group (p < 0.05) compared to the CPB group (Fig 3, 4).

Western blot analysis with an anti-caspase-3 antibody, which recognizes procaspase-3 and the active form, showed the expression of the processed form of caspase-3 in all groups. The expression of activated caspase-3 revealed that the cells were in apoptosis (Fig 5), confirming the results obtained with the TUNEL technique.

eNOS

Western blot analysis was performed to identify the mechanism of decreased NOx production. eNOS protein demonstrated similar levels in all groups (Fig 6). No iNOS protein was detected in the lungs of all pigs.

DISCUSSION

The present study showed a CPB-induced decrease of endogenous NO production, and the data

Table 3—Plasma NOx Levels*

<table>
<thead>
<tr>
<th>Plasma NOx Levels, μmol/L</th>
<th>T0</th>
<th>T4</th>
<th>T24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>28.20 ± 4.68</td>
<td>26.60 ± 3.00</td>
<td>12.8 ± 1.87†</td>
</tr>
<tr>
<td>CPB</td>
<td>76.76 ± 21.64</td>
<td>54.47 ± 21.50*</td>
<td>54.27 ± 19.7†</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM.
†p < 0.05 vs T0.
* p < 0.05 vs T4.
indicate that INO delivered early and at a low dose (20 ppm) had a beneficial effect on inflammation. This occurs by reducing cytokine synthesis and by attenuating neutrophil activation and migration into the alveolar spaces. However, INO promotes apoptosis in inflammatory conditions. Increasing apoptosis could be one of the mechanisms by which INO leads to the resolution of lung inflammation.

The positive aspect of NO is that plays an important role in the physiologic modulation of coronary artery tone and myocardial function. However, increased formation of NO within the myocardium can also have detrimental and negative effects, contributing to the pathophysiology of myocardial dysfunction in ischemic heart diseases. If the increased production of NO is well in balance with a moderate increase in oxygen radicals, then NO will exert beneficial effects. However, if the oxygen radicals are produced in excess of NO as in prolonged ischemic injury, then deleterious effects will be induced.

Inhaled NO is the first vasodilator to produce truly selective, potent pulmonary vasodilation that is independent of endothelial cell function. Therefore, it has been employed in the preoperative, perioperative, and postoperative assessment of pulmonary hypertension. Although many studies demonstrate...
a clear benefit in patient outcome with INO use, several safety concerns remain, including unpredictable and nonsustained responses to INO and a clinically significant rapid increase in pulmonary vascular resistance on its acute withdrawal.

ALI after CPB has been attributed to the CPB-associated pulmonary and systemic inflammatory response syndrome. Several studies have already demonstrated that CPB can induce ALI and may also cause ARDS. These effects are neutrophil related. A significant relationship between neutrophil influx and IL-8, a potent chemoattractant mainly released by neutrophils, has been noted in many inflammatory conditions.

In our study, CPB caused neutrophil activation and infiltration, reflected by an increase in BAL IL-8 levels and neutrophil percentage in comparison to the control group. Our results are consistent with previous data that showed heightened in TNF-α, IL-6, and IL-8 production after CPB. The effects of INO in ALI and ARDS have been indicated in many studies, which suggest that INO reduces pulmonary hypertension and improves oxygenation. INO can also inhibit transendothelial migration of activated neutrophils, and preserve alveolar-capillary integrity after acute insults. The inflammatory process associated with CPB has been observed in conjunction with a decrease of arterial oxygen. A study by Kotani and associates has demonstrated a significant correlation between an increase in the number of neutrophils, IL-8, and elastase concentrations in BAL fluid, with changes in PaO₂/fraction of inspired oxygen and intrapulmonary shunt. This may explain at least in part how INO improves oxygenation after CPB.

In INO groups, BAL analysis revealed a decrease of IL-8 concentration and a trend toward reduction of neutrophil number. This effect could occur by inhibiting NF-κB, a multiprotein complex that regulates a variety of genes, including those of IL-8, TNF-α, and several adhesion molecules. Accordingly, INO may prevent the production of proinflammatory cytokines because the genes of these molecules contain, in their promoter regions, the binding sites for NF-κB.

In our study, we have also observed a decrease of plasma NOx concentrations in the sham and CPB groups. Our findings are similar to those of other studies that have demonstrated a reduction of endogenous NO production after CPB or in swine models of endotoxemia. This alteration in NO production may reflect endothelial dysfunction, even if we did not find any significant difference in eNOS protein expression between the sham and CPB groups. The dysfunction probably affects eNOS activity but not gene expression. Furthermore, during CPB, substrate or cofactor availability may play a role in lowering NO production. This result implies that INO may replace the loss of endogenous NO.

NO was found to be involved in regulation of the apoptotic process; in fact, NO can promote apoptosis as well as inhibit it. We observed, in this study, both antiapoptotic and proapoptotic roles of INO. These opposing effects seem to depend on the local environment in which NO acts. Hyperoxia and mechanical ventilation have the potential to cause cellular damage, and investigations have shown that hyperoxia can also induce apoptosis. When INO is administered at low concentrations (10 to 20 ppm), it has a protective effect against pulmonary injury. As such, NO can protect against apoptosis induced by proinflammatory agents in cultured endothelial cells. It may, therefore, be able to protect the lungs in vivo against the apoptotic process. Hyperoxia has already been shown to be associated with increased NF-κB activity in vivo. As NO inhibits NF-κB transcription, it can consequently suppress apoptosis. In addition, INO can also subdue apoptosis through inactivation of caspase-3 by S-nitrosylation.

Many studies have disclosed that during CPB, apoptosis of neutrophils is delayed by proinflammatory cytokines or by modified C-reactive protein. In our experiments, the increase of proinflammatory mediators released during CPB probably inhibits apoptosis in these cells.

In the CPB-plus-INO group, INO, with its anti-inflammatory properties, decreased IL-8 concentrations, and this reduction suppressed the potential to inhibit the apoptotic process. Our findings are consistent with a previous study by Chello and associates, who demonstrated that the decline of neutrophil apoptosis is mainly a consequence of increased plasma IL-8 concentrations.

NO possesses the ability to act directly (leading to DNA damage) or indirectly (through reactive nitrogen species). Indeed, NO can interact with superoxide anion produced by inflammatory cells, leading to peroxynitrite formation, a potent oxidant that can induce apoptosis.

The apoptosis process could participate in the removal of inflammatory cells and may be one of the mechanisms limiting tissue injury. Even if our study did not allow us to identify which kind of cells undergo apoptosis, neutrophils are a major source of IL-8 and the principal cause of inflammation. Inducing apoptosis of neutrophils would reduce the inflammation, prevent tissue lesions, and facilitate recovery. Our limitation of this study is that we did not establish in which cells the apoptotic process occurs, but the outcome of pigs receiving inhaled NO was better than those without NO (reflected by...
hemodynamics parameters data not shown). And this argues on apoptosis ongoing on inflammatory cells rather than on endothelial cells or pneumocytes.

The TUNEL technique could be associated to false-positive findings. Nevertheless the TUNEL technique is still reliable and the most common technique widely used in tissue apoptosis detection. Prochazkova et al demonstrated a positive correlation between the TUNEL technique and others techniques.

INO has been shown to evoke selective pulmonary vasodilation and contrasts markedly with IV-administered vasodilators that provoke dilation also in nonventilated areas of the lung and systemic circulation. NO diffusing into the bloodstream can rapidly react with oxyhemoglobin to form methemoglobin and nitrate. The presence of molecules that could conserve and stabilize NO bioactivity, which might regulate a regional blood flow and oxygen delivery, has been reported.

It is clear that the mechanism by which INO leads to inflammation resolution remains to be clarified, and additional investigations are needed to discover if weaning from INO will cause rebound inflammation. Answering these questions is a crucial step before proceeding to clinical trials.

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