Histol Histopathol (2015) 30: 117-124
DOI: 10.14670/HH-30.117

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

The effect of inhaled nitric oxide on the carrageenan-induced paw edema

Carly Faria Coelho[1], Rodolfo P. Vieira[2], Patrícia Sardinha Leonardo Lopes-Martins[2], Simone Aparecida Teixeira[3], Alexandre Urban Borbely[4], Irene Maria Gouvea[3], Lucio Frigo[5] and Rodrigo Álvaro Brandão Lopes-Martins[1,6]

[1]Laboratory of Pharmacology and Experimental Therapeutics, Department of Pharmacology, Institute of Biomedical Sciences ICB1, University of Sao Paulo USP, Cidade Universitária, [2]Nove de Julho University, UNINOVE, [3]Laboratory of Pharmacology of Free Radicals, Department of Pharmacology, Institute of Biomedical Sciences ICB1, University of Sao Paulo USP, Cidade Universitária, [4]Laboratory of Endometrium Biology, Departament of Cellular and Developmental Biology ICB1, University of Sao Paulo USP, Cidade Universitária, [5]Biological Sciences and Health Center, Cruzeiro do Sul and [6]University, São Paulo - SP, Brazil University of Mogi das Cruzes - UMC, Núcleo de Pesquisas Tecnológicas - NPT, Mogi das Cruzes, SP, Brazil

Summary. Inhaled nitric oxide therapy reaches not only pulmonary vessels, but also other vasculatures, presenting anti-inflammatory effects. Therefore, this study investigated the effects of inhaled nitric oxide on a mice model of carrageenan-induced paw edema. Paw edema was induced in male Swiss mice (20-30 g) by subplantar injection of carrageenan (0.05 ml of a 1% suspension in 0.9% saline). The evaluation of time-course edema (mililiter) was measured by plethysmometry until 12 h following carrageenan administration. Thirty minutes after carrageenan injection, some groups received inhaled nitric oxide (300 ppm at variable doses and times) or Indometacin (INDO 5 mg/Kg, v.o), while others received sildenafil (1 mg/Kg, i.p) or rolipram (3 mg/Kg, i.p.) with or without inhaled nitric oxide. Paws were assessed for edema levels by plethysmometry, mieloperoxidase activity and histological analysis. Inhaled nitric oxide significantly reduced carrageenan-induced paw edema, mieloperoxidase activity and inflammatory infiltrate, although similar results were also observed in sildenafil and rolipram treated groups. In addition, significant effects between inhaled nitric oxide with pharmacological therapy was observed. Inhaled nitric oxide presents anti-inflammatory effects on carrageenan-induce paw edema, as observed through reduced edema, mieloperoxidase activity and neutrophil infiltration, indicating that inhaled nitric oxide therapy goes beyond lung vascular effects.

Key words: Inflammation, Inhaled nitric oxide, Carrageenan, Paw edema, Mieloperoxidase

Offprint requests to: Rodrigo Alvaro Brandão Lopes-Martins, B.Sc., Ph.D., Laboratório de Farmacologia e Terapêutica Experimental, Departamento de Farmacologia, Instituto de Ciências Biomédicas - ICB 1, University of Sao Paulo - Av. Lineu Prestes, 1524, São Paulo - SP, Brazil. e-mail: rmartins@icb.usp.br

Introduction

The introduction of inhaled nitric oxide (iNO) for the treatment of many pulmonary injuries, as hypertension of the newborn, adult respiratory distress syndrome (ARDS), lung reperfusion injury (Botha et al., 2007) and after cardiac surgery has been one of the most significant advances in recent intensive care (Porta and Steinhorn, 2008; Matamis et al., 2012). Inhaled NO is qualified as "microselective therapy" once it dilates only the vessels directly adjacent to the alveolar units under ventilation (Lunn, 1995). However, some evidences support that iNO reaches vasculatures beyond the pulmonary bed, by forming nitric oxide (NO) complexes, which are transported in blood stream to distant sites and promoting anti-inflammatory effects (Mathru et al., 2007; Ibrahim et al., 2012; Torok et al., 2012).

In a very current study, it was concluded that inhaled NO, was able to mobilize from bone marrow endothelial

progenitor cells into the circulation and attenuates damage of lung alveolar-capillary barrier in ARDS reducing severity of this disease (Qi et al., 2012).

NO is an important endogenous regulatory molecule, which possess both pro-inflammatory and anti-inflammatory properties in mammals (Raychaudhuri et al., 1999). The biosynthesis of NO from L-arginine is a metabolic pathway for the regulation of cell function and communication. It is catalysed by NO synthase in mammalian cells, which are currently classified as either constitutive or inducible. The constitutive isoforms (cNOS; types I and III), found in endothelial cells and neurons, are Ca^{++}/calmodulin-dependent, whereas the inducible NO synthase (iNOS; type II) found in macrophages and other cell types is Ca^{++}/calmodulin-independent (Sautebin et al., 1998; Moncada and Higgs, 1991). The iNOS-derived NO plays a key role in host defence mechanisms, as cytotoxic molecule for invading microorganisms and tumour cells, and is involved in pathological vasodilatation and tissue damage. NO and nitrite interacted with neutrophil myeloperoxidase (MPO) to stimulate oxidative reactions during inflammation (Eiserich et al., 1998). NO synthase type II (iNOS) is induced by cytokines and microbial products, producing large amounts of NO for a prolonged period of time (Nussler and Billiar, 1993).

The carrageenan-induced rat paw edema is characterized by an early phase (1-2h) brought about by the release of histamine, 5-hydroxytryptamine and bradykinin followed by a late phase (3-4h) mainly sustained by prostaglandin release (Di Rosa et al., 1971; Di Rosa and Willoughby, 1971) and accumulation of neutrophils at the inflammatory site (Lyons, 1995). The accumulation of leukocytes at the inflammatory site results from the interaction between leukocytes and endothelial cells (Kubes et al., 1991; Granger and Kubes, 1994). It has been reported that endogenous NO inhibits leukocyte adhesion (Kubes et al., 1991) through interference with the classical inflammatory cascade of events involved in leukocyte recruitment (De Caterina et al., 1995). The literature is controversial if nitric oxide (NO) releasing during inflammatory process has a pro- or inhibitory effect on neutrophil migration. Secco et al (2003) recently demonstrated that during inflammation, NO released by either constitutive NOS (cNOS) or iNOS down-modulates the neutrophil migration (Secco et al., 2003). This NO effect seems to be a consequence of decreased rolling and adhesion of the neutrophils on endothelium and also the induction of apoptosis in migrated neutrophils. Although the functional analogy of NO to Endothelium-derived Relaxing Factor remains controversial, medical use of exogenous NO gas by inhalation has grown exponentially in pulmonary hypertension, hypoxaemia, inflammation and edema (Troncy et al., 1997). In the perspective of these findings, this study aimed to investigate the effect of inhaled nitric oxide in the classical experimental model of mice paw edema induced by carrageenan injection.

Material and methods

Experimental procedures

All experimental procedures were approved by the Ethical Committee of the Institute of Biomedical Sciences - University of São Paulo. The experiments were carried out on male Swiss mice weighting between 20-30 g, with food and water "*ad libitum*" provided by Central Animal House of the Research and Development Department (ICB-USP). All mice were placed in a common box and randomly divided into groups of six.

Mice received a subplantar injection of carrageenan (0.05 ml of a 1% suspension in 0.9% saline; Sigma Chemical Co., St Louis, MO, USA) in the left hind paw under brief anesthesia with halothane (10 seconds).

Volumetric measurements of the mice paw edema

The volume of the paw edema (ml) was measured in each animal using a plethysmometer (plethysmometer 7150, Ugo Basile, Italy) with a precision of two decimal places. For carrageenan-injected groups, measurements were made immediately before and after 1^{st}, 2^{nd}, 3^{rd}, 4^{th} h and 12^{th} h of injection. The increase in paw volume (ml) was calculated by subtracting the basal volume from the final volume.

Treatments

For this study, 5 different experimental sets were developed.

The first experiment, the animal groups, which were submitted for the carrageenan or saline injection, inhaled air, as a control, or NO (300 ppm) at variable doses, thirty minutes after the subplantar injections.

In the second experimental set, as the best NO dose had been found in the group of animals which received NO, we submitted these animals for different times of inhalation, which were 2, 5 and 10 min after the carrageenan injection.. In the third experimental set, we submitted the animals for multiple administrations of iNO (30 min, 4 hs ½ and 8 hs ½ after carrageenan injection), using the best NO dose and the best time of inhalation, which had already determined in previous sets of experiments..

In the fourth and fifth set of experiments, in order to try to discover in which way inhaled NO was acting, we used sildenafil (1 mg/Kg, i.p) or rolipram (3 mg/Kg, i.p.), just after the carrageenan subplantar injection associated or not with NO (300 ppm) at the best dose, time and posology found.

At the end of experiments, after 4 or 12 hours, depending on the experiment, the animals were sacrificed with CO_2 overexposure and the injected paws were excised to determine mieloperoxidase activity and histological analysis by microscopy.

Tissue myeloperoxidase activity

Myeloperoxidase (MPO) is a well-known enzyme, mainly released by activated neutrophils, characterised by powerful pro-oxidative and proinflammatory properties (Loria et al., 2008).

The assay was performed as previously described in the literature (Bradley et al., 1982). Briefly, tissue samples were homogenized with a Polytron homogenizer (5-15 sec, at a setting of 4, using 3ml of phosphate-buffered saline (PBS) containing 0.5% of hexadecyltimethylammonium bromide and 5 mM EDTA, pH 6.0). The homogenized samples were sonicated (6-10 sec at 40 Hz) and then centrifuged at 3000 g for 30 min. The MPO activity in the supernatants was assayed by measuring the change in absorbance at 460 nm resulting from the decomposition of H_2O_2 in the presence of o-dianiside.

Histological analysis and neutrophils counting

Mice paw were immediately removed and immersed in a 4 % phosphate buffered paraformaldehide solution for 48 h. Specimens were dehydrated and embedded in paraffin prior the 5 μm microtome sections. Histological sections were collected in glass slide and hematoxyilin-eosin stained. Neutrophil counting and photographs were carried out in a Nikon-YS100 photomicroscope. A single blinded cell counting was carried out in each slide selecting 10 aleatory microscope fields in the subplantar mice paw region at 400x magnification.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Variance data in the text are given as 95% confidence intervals (95% CI). All data were statistically evaluated by analysis of variance (ANOVA), followed by the Newman-Keuls-Student's test. Analyses of group comparisons were performed with unpaired Student's t-tests. Values at $p<0.05$ were considered to be statistically significant.

Results

Effect of inhaled NO on the carrageenan-induced paw edema in mice - Testing different doses

The subplantar injection of carrageenan, into the left hind paw of mice, caused a significant edema, which has achieved the maximum value of volume (0,179 ml) at 4th hour of edema development. Saline, control group, was not able to develop significant edema (0,004 ml).

Different doses of inhaled NO were used, as therapeutic goal, but only the lowest doses (13.6 and 41.6 ppm) showed significant effect, reducing paw edema (0.111 ml and 0.113 ml, respectively), as shown in Fig. 1, panel A, similar to the anti-inflammatory effect of indomethacin (0.091 ml) when compared with carrageenan + air. This way, it was established the best dose, 13.6 ppm, for the subsequent experiments.

Determination of the best time of NO inhalation

Following the same pattern of the experiment above, in the Fig. 1, panel B, we can observe that carrageenan is a potent phlogistic agent, as previously described (Winter et al., 1962). Saline was not able to trigger any inflammation response and indomethacin (5 mg/Kg), via i.p., reduced paw edema since 2nd hour.

As the lowest dose of inhaled NO (13.6 ppm) was significant in reducing paw edema, lower times of inhalation were also tested. However, only 10 min of inhalation of NO showed a significant antiinflammatory effect (0.116 ml) when compared with 5 or 2 min of inhalation (0.135 and 0.140 ml respectively).

Determination of posology for inhaled NO

After the determination of the best dose of inhaled NO (13.6 ppm during 10 min of inhalation), multiple administrations of inhaled NO was tested.

Fig. 1, panel C shows that all 3 groups of variable inhaling NO administrations (1; 2 or 3 administrations of inhaled NO) presented reduced paw edema. The indomethacin treated group presented reduced paw edema since the 2nd hour. The third administration occurred after the 8th hour, since that edema measurements occurred longer than in other experiments. Of note, there were no significant differences between the number of iNO expositions (1, 2 or 3) reducing edema among groups treated with one, two or three administrations of NO. For example, the averages of the paw volumes in the group of animals which inhaled NO only once were 0.101 ml (4th hour) and 0.147 ml (12th hour). The averages of the paw volumes in the group of animals which received iNO twice were 0.109 ml (4th hour) and 0.151 ml (12th hour); and 0.109 ml (4th hour) and 0.133 ml (12th hour) were the averages of the paw volumes in the group of animals which received iNO three times.

Effect of the concomitant treatments of inhaled NO and phosphodiesterase V inhibitor, Sildenafil

The subplantar injection of carrageenan caused a significant inflammation response (0.094 ml) and saline did not cause any huge modifications on the paw volume (0.001 ml).

Comparing with these groups, Fig. 2, panel A shows that inhaled NO as much as sildenafil alone or the concomitant administration of both treatments significantly decreased carrageenan-induced paw edema. But it is interesting to note that there was a significant difference among the groups, where sildenafil is more potent in reducing edema (0.035 ml) than inhaled NO alone (0.051 ml) and an additive effect occurred, much more potent (0.016 ml), with the concomitant

administrations of both inhaled NO and sildenafil.

Effect of the concomitant treatments of inhaled NO and phosphodiesterase IV inhibitor, Rolipram

Fig. 2, panel B, shows the same pattern of response of the experiment mentioned above.

Although inhaled NO like rolipram alone could decrease edema significantly (0.051 ml and 0.029 ml, respectively), rolipram administrated alone presented more potent antiinflammatory effect than inhaled NO alone. However, a very significant additive effect was

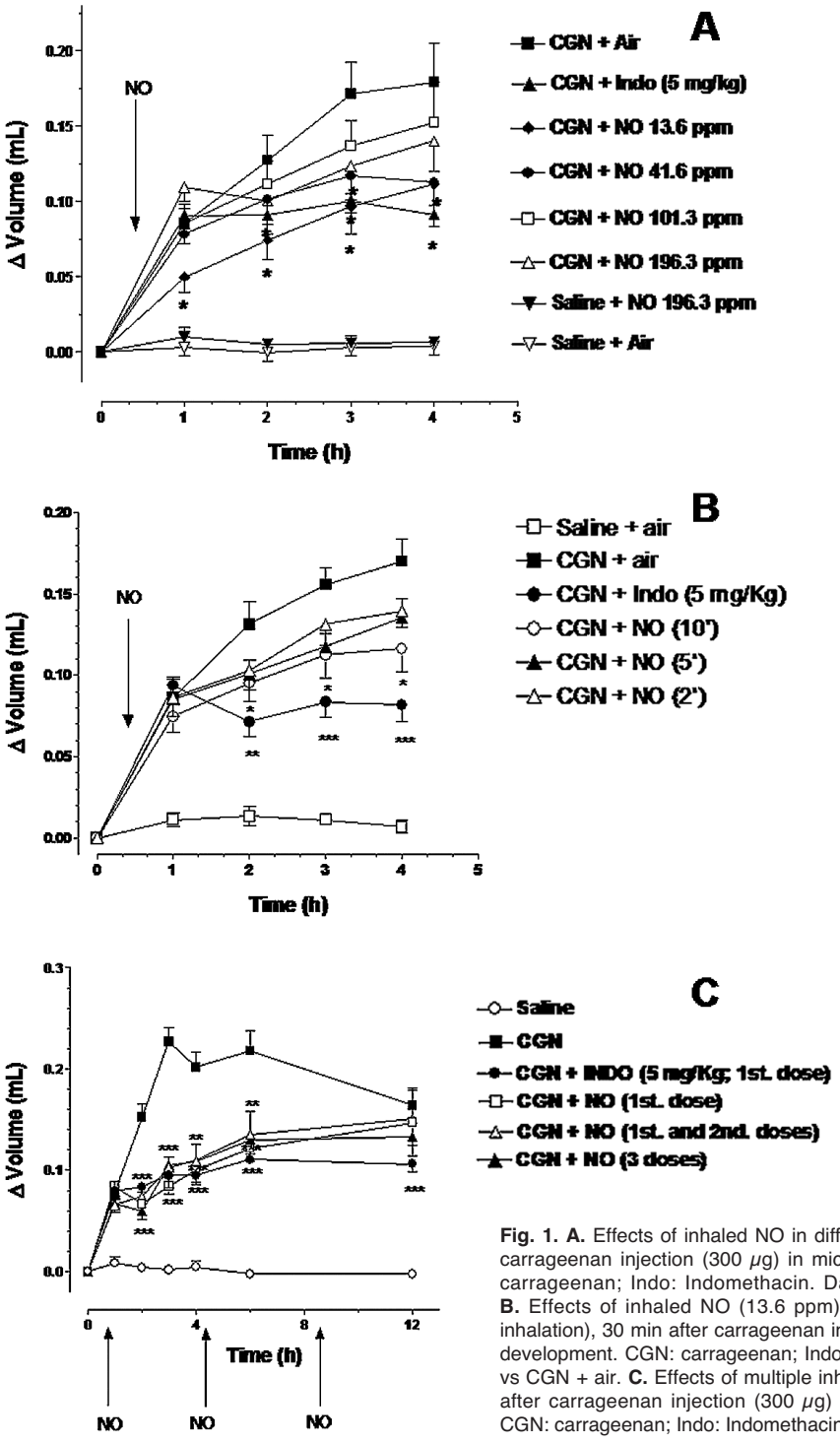

Fig. 1. A. Effects of inhaled NO in different doses, during 10 min of inhalation, 30 min after carrageenan injection (300 μg) in mice hind paw on temporal edema development. CGN: carrageenan; Indo: Indomethacin. Data are mean ± SEM, n=8 * P<0,05 vs CGN + air. **B.** Effects of inhaled NO (13.6 ppm) during variable inhalation times (2, 5 or 10 min of inhalation), 30 min after carrageenan injection (300 μg) in mice hind paw on temporal edema development. CGN: carrageenan; Indo: Indomethacin. Data are mean ± SEM, n=6 * P<0.05 vs CGN + air. **C.** Effects of multiple inhaled NO administrations (30 min, 4 hs ½ and 8 hs ½) after carrageenan injection (300 μg) in mice hind paw on temporal edema development. CGN: carrageenan; Indo: Indomethacin. Data are mean ± SEM, n=6 * P<0.05 vs CGN + air.

observed when both treatments were administrated together (0.013 ml).

Effect of inhaled NO on neutrophil accumulation on mice paw edema

Fig. 3 A shows that carrageenan caused a significant increase on MPO activity, an indicator of neutrophils accumulation, while saline injection did not result in any signficant change. Considering that MPO activity is an indirect way to quantify neutrophils on the inflammatory site, histological slides of mice paws were prepared to allow the confirmation of the presence of these cells. The results showed that inhaled NO, as well as indomethacin, significantly reduced the cell migration to the inflammation site observed after 4 hours of carrageenan injection. Fig. 3 B shows as well as panel A, that inhaled NO and indomethacin could decrease the neutrophils migration to the inflamed paws when compared with positive control group, carrageenan.

Photomicrography of histological slides of mice paws after the inoculation with carrageenan or saline

Histological slides were confectioned to illustrate the *in situ* conditions of the hind paws in the groups. In the first photomicrograph (Fig. 4, panel A) the organization of subplantar paw region image after saline injection can be observed. Muscle bundle fibers can be observed in the upper margin and a distinct epimisium separate it from the underlying connective tissue. The connective tissue shows no evident signs of inflammation with scant

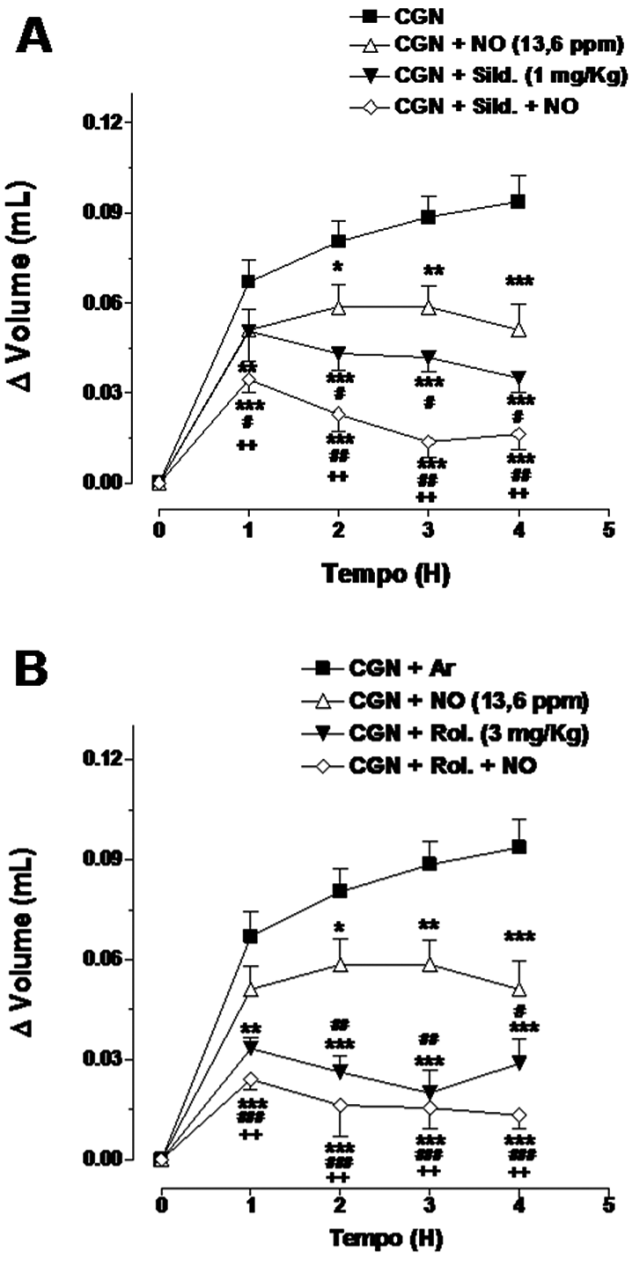

Fig. 2. A. Effects of inhaled NO administrations (13.6 ppm during 10 min), 30 min after carrageenan injection (300 μg), sildenafil (1 mg/Kg; i.p.) and its associations on temporal paw edema development in mice. CGN: carregeenan; Sild.: sildenafil. Data are mean ± SEM, n=5 *P<0.05 vs CGN + air. **B.** Effect of inhaled NO administrations (13.6 ppm during 10 min), 30 min after carrageenan injection (300 μg), rolipram (3 mg/Kg; i.p.) and its associations on temporal paw edema development in mice. CGN: carrageenan; Rol.: rolipram. Data are mean ± SEM, n=5 *P<0.05vs CGN + air.

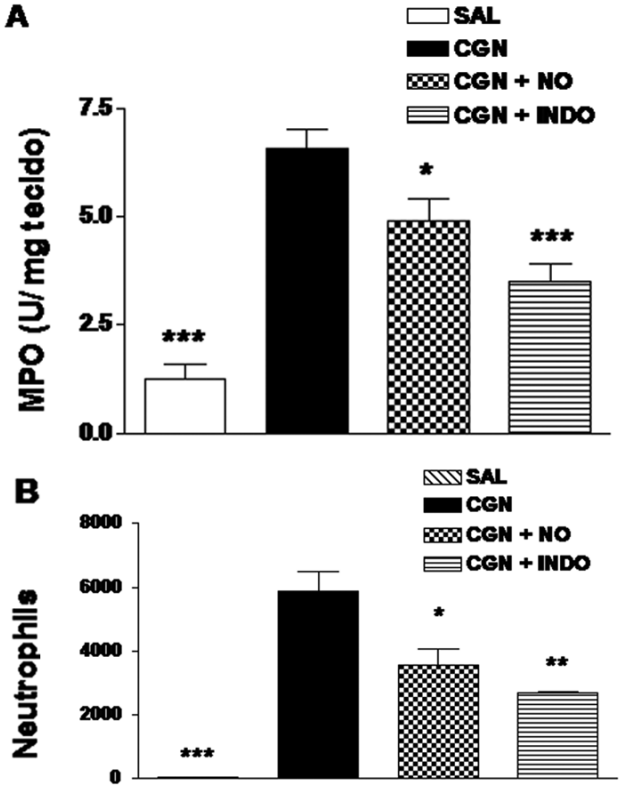

Fig. 3. Effect of inhaled NO on neutrophil migration on paws. **A.** Measurement of MPO activity on mice hind paw, after 4 hours of carrageenan (300 µg) - induced edema. **B.** Neutrophils counting in mice paws histological slides after 4 hours of carrageenan (300 µg) - induced edema. CGN: carrageenan; INDO: indomethacin; SAL: saline. Data are mean ± SEM, n=5 *P<0.05 vs CGN + air.

inflammatory cells, absence of edema and haemorrhage. Panel B, shows an intense presence of polymorfonuclear cells, specifically neutrophils, of the inflammatory infiltrate and edema characterized by ample irregular spaces in connective tissue extra-cellular matrix of the carrageenan group. Carrageenan-injected inhaled NO group (panel C) presented a reduced cell infiltration and edema when compared with carrageenan group. The reduction of cell infiltration was lesser than indomethacin-treated group. Furthermore, edema reduction seems more evident in this group as compared to indomethacin-treated group. Carrageenan-injected treated with indomethacin group (panel D), presented a decrease the neutrophils migration, discrete reduced edema as compared to carrageenan-injected only group. However, the most striking characteristic was the great number of red blood cells dispersed in connective tissue extra-cellular matrix (haemorrhage), present in animals of this group.

Discussion

The present study shows for the first time that inhaled NO present anti-inflammatory effects in a classical model of carrageenan-induced paw edema, as demonstrated through reduced paw volume, reduced MPO activity and reduced neutrophils accumulation. These results clearly reinforce the concept that therapy

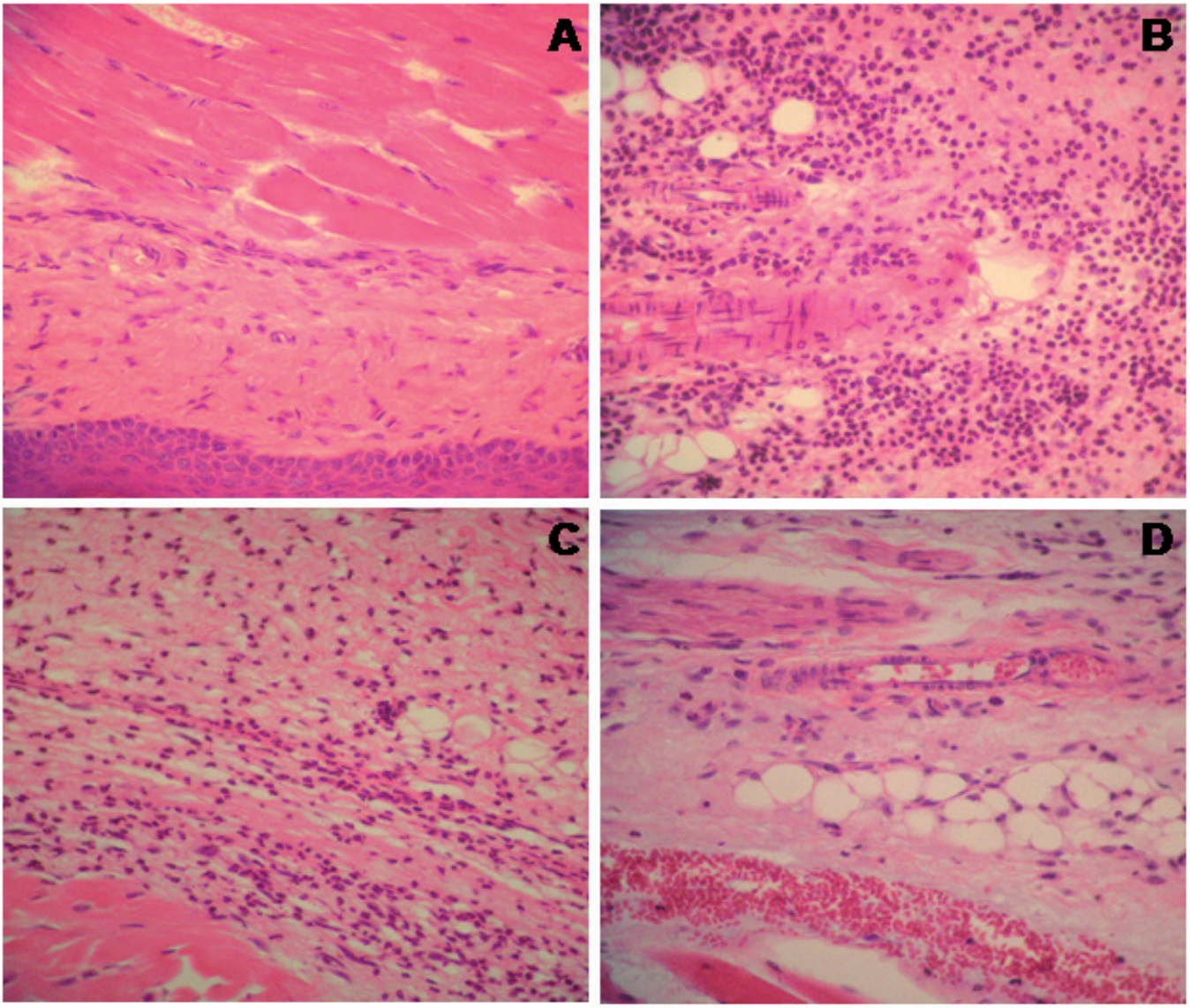

Fig. 4. Representative photomicrographs of histological slides stained with hematoxylin and eosin of hind paws of Control group (**A** - saline injected), Carrageenan group (**B** - carrageenan injected), Carrageenan + iNO group (**C** - carrageenan injected + inhaled NO) and Carrageenan + indomethacin group (**D** - carrageenan + indomethacin 5mg/kg). x 100.

with inhaled NO reaches not only the lungs, but also present systemic effects.

Clinical use of iNO was introduced in 1991 and remains highly used until now. It has been shown to reduce pulmonary arterial hypertension and improve oxygenation with concentrations of 18 and 36 ppm in patients with acute respiratory distress syndrome (ARDS) (Frostell et al., 1991; Pepke-Zaba et al., 1991; Malhotra et al., 2011).

Inhalation of NO using doses between 5 and 80 ppm was demonstrated to reduce also pulmonary hypertension in sheep (Yu et al., 2009). Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) presents acute hypoxemia and bilateral lung infiltrates on radiography without left atrial hypertension and are characterised by inflammation of the alveolar-capillary membrane triggered by various insults (Bigatello and Hellman, 2003).

Nitric oxide is considered a selective pulmonary vasodilator and has been suggested to present anti-inflammatory properties (Gitto et al., 2012). We investigated the possible anti-inflammatory effect of iNO, using, for this purpose, the classical experimental model or carrageenan-induced mice paw edema.

In agreement to the literature, as we can observe in Fig. 1, inhaled nitric oxide was able to reduce carrageenan-induced mice paw edema with the most significant effect at the lowest dose employed of 13.6 ppm. The second experimental set up demonstrated that, at the previously selected dose of 13.6 ppm, 10 minutes of inhalation presented significant inhibition of mice paw edema. Two and five minutes of a unique inhalation session reduced, but not significantly the paw edema.

In order to determinate an adequate posology of nitric oxide inhalation in our experimental model, we submitted the animals to 1, 2 or 3 sessions of NO inhalation. The inhalation sessions were applied 30 minutes, 4 hours and 30 minutes and 8 hours and 30 minutes after carrageenan injection. Fig. 1, panel C shows the results until 12 hours after carrageenan administration. We could observe that mice paw edema remained significantly reduced until de 12th hour after 3 sessions of nitric oxide inhalation.

In another set of experiments, we tested the possible additive effect of sildenafil, a type 5 phosphodiesterase inhibitor on the observed anti-inflammatory effect of inhaled nitric oxide in mice paw edema. As observed in Fig. 2 A, the combination of sildenafil and inhaled nitric oxide was able to produce an additive anti-inflammatory effect than sildenafil or nitric oxide alone. In addition, the type IV phosphodiesterase inhibitor rolipram presented similar effect (Fig. 2B).

It is well known that the carrageenan-induced paw edema as well as other rat paw edema models has an important participation of neutrophil accumulation at the paw (Ezeamuzie and Njoku, 1992; Houle et al., 2005).

In order to investigate the effects of inhaled nitric oxide on the neutrophil accumulation at the mice paw, we performed direct cell counting in hematoxylin-eosin stained plates of the mice paw as well as the myeloperoxidase activity at the paw tissue as a biochemical indicator of neutrophil accumulation and functional activity. Fig. 3 (AB) demonstrates that NO inhalation was able to significantly reduce neutrophil accumulation in both protocols. According to Kubes et al., inhaled nitric oxide is able to inhibit the expression of adhesion molecules and consequently reducing cell migration to the inflammatory site (Kubes et al., 1991; Kubes and Granger, 1992; Kubes, 1993).

In our work, besides the anti-edematogenic effect, inhaled nitric oxide was able to prevent or reduce significantly, neutrophil infiltration to the mice paw, decreasing inflammatory signs.

To confirm our findings, we performed the histological analysis and cell counting in mice paws. We can observe in carrageenan-injected mice that inhaled NO reduced cell infiltration and edema when compared to only carrageenan-injected group (Fig. 4, panel C-B). In addition, the anti-inflammatory effects of inhaled NO, characterized by reduced paw edema volume, MPO activity and neutrophils infiltration was lesser than the carrageenan-injected indomethacin-treated group (Fig. 4, panel D).

These results demonstrate that the beneficial effect of inhaled nitric oxide therapy goes beyond the initially described pulmonary vascular effects.Moreover, iNO do not act only by cGMP and cAMP ways, because when associated with sildenafil and rolipram, phospho-diaterases inhibitors V and IV respectively, iNO showed an additional anti-inflammatory effect reducing paw edema.

Acknowledgements. This study was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo).

References

Bigatello L.M. and Hellman J. (2003). Inhaled nitric oxide for ARDS: searching for a more focused use. Int. Care Med. 29, 1623-1625.

Botha P., Jeyakanthan M., Rao J.N., Fisher A.J., Prabhu M., Dark J.H. and Clark S.C. (2007) Inhaled nitric oxide for modulation of ischemia-reperfusion injury in lung transplantation. J. Heart Lung Transplant. 26, 1199-1205.

Bradley P.P., Priebat D.A., Christensen R.D. and Rothstein G. (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol. 78, 206-209.

De Caterina R., Libby P., Peng H.B., Thannickal V.J., Rajavashisth T.B., Gimbrone M.A. Jr, Shin W.S. and Liao J.K. (1995). Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J. Clin. Invest. 96, 60-68.

Di Rosa M. and Willoughby D.A. (1971). Screens for anti-inflammatory drugs. J. Pharm. Pharmacol. 23, 297-298.

Di Rosa M., Giroud J.P. and Willoughby D.A. (1971). Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J. Pathol. 104, 15-29.

Eiserich J.P., Patel R.P. and O'Donnell V.B. (1998). Pathophysiology of nitric oxide and related species: free radical reactions and

modification of biomolecules. Mol. Aspects Med. 19, 221-257.

Ezeamuzie I.C. and Njoku A.C. (1992). The role of neutrophils in acute and chronic inflammation in rats. Afr. J. Med. Sci. 21, 23-28.

Frostell C., Fratacci M.D., Wain J.C., Jones R. and Zapol W.M. (1991). Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. Circulation 83, 2038-2047.

Gitto E, Pellegrino S, Aversa S, Romeo C, Trimarchi G, Barberi I., Calabró M.P., Salpietro C.D. and Reiter R.J. (2012). Oxidative stress and persistent pulmonary hypertension of the newborn treated with inhaled nitric oxide and different oxygen concentrations. J. Matern. Fetal Neonatal Med. 25, 1723-1726.

Granger D.N. and Kubes P. (1994). The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. J. Leukoc. Biol. 55, 662-675.

Houle S., Papez M.D., Ferazzini M., Hollenberg M.D. and Vergnolle N. (2005). Neutrophils and the kallikrein-kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. Br. J. Pharmacol. 146, 670-678.

Ibrahim Y.I., Ninnis J.R., Hopper A.O., Deming D.D., Zhang A.X., Herring J.L. Sowers L.C., McMahon T.J., Power G.G. and Blood A.B. (2012). Inhaled nitric oxide therapy increases blood nitrite, nitrate, and s-nitrosohemoglobin concentrations in infants with pulmonary hypertension. J. Pediatr. 160, 245-251.

Kubes P. (1993). Nitric oxide-induced microvascular permeability alterations: a regulatory role for cGMP. Am. J. Physiol. 265, H1909-19015.

Kubes P. and Granger D.N. (1992). Nitric oxide modulates microvascular permeability. Am. J. Physiol. 262, H611-615.

Kubes P., Suzuki M. and Granger D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc. Natl. Acad. Sci. USA 88, 4651-4655.

Loria V., Dato I., Graziani F. and Biasucci L.M. (2008). Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. Mediators Inflamm. 2008, 135625.

Lunn R.J. (1995). Inhaled nitric oxide therapy. Mayo Clin. Proc. 70, 247-255.

Lyons C.R. (1995). The role of nitric oxide in inflammation. Adv. Immunol. 60, 323-371.

Malhotra R., Hess D., Lewis G.D., Bloch K.D., Waxman A.B. and Semigran M.J. (2011). Vasoreactivity to inhaled nitric oxide with oxygen predicts long-term survival in pulmonary arterial hypertension. Pulm. Circ. 1, 250-258.

Matamis D., Pampori S., Papathanasiou A., Papakonstantinou P., Tsagourias M., Galiatsou E., Koulouras V. and Nakos G. (2012). Inhaled NO and sildenafil combination in cardiac surgery patients with out-of-proportion pulmonary hypertension: acute effects on postoperative gas exchange and hemodynamics. Circ. Heart Fail. 5, 47-53.

Mathru M., Huda R., Solanki D.R., Hays S. and Lang J.D. (2007). Inhaled nitric oxide attenuates reperfusion inflammatory responses in humans. Anesthesiology 106, 275-282.

Moncada S. and. Higgs E.A. (1991). Endogenous nitric oxide: physiology, pathology and clinical relevance. Eur. J. Clin. Invest. 21, 361-374.

Nussler A.K. and Billiar T.R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. J. Leukoc. Biol. 54, 171-178.

Pepke-Zaba J., Higenbottam T.W., Dinh-Xuan A.T., Stone D. and Wallwork J. (1991). Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. Lancet 338, 1173-1174.

Porta N.F. and Steinhorn R.H. (2008). Inhaled NO in the experimental setting. Early Hum. Dev. 84, 717-723.

Qi Y., Qian L., Sun B., Liu L., Wu P. and Sun L. (2012). Inhaled NO contributes to lung repair in piglets with acute respiratory distress syndrome via increasing circulating endothelial progenitor cells. PLoS One 7, e33859.

Raychaudhuri B., Dweik R., Connors M.J., Buhrow L., Malur A., Drazba J., Arroliga A.C., Erzurum S.C., Kavuru M.S. and Thomassen M.J. (1999) Nitric oxide blocks nuclear factor-kappaB activation in alveolar macrophages. Am. J. Respir. Cell Mol. Biol. 21, 311-316.

Sautebin L., Ialenti A., Ianaro A. and Di Rosa M. (1998). Relationship between nitric oxide and prostaglandins in carrageenan pleurisy. Biochem. Pharmacol. 55, 1113-1117.

Secco D.D., Paron J.A., Oliveira S.H., Ferreira S.H., Silva J.S. and Cunha F.Q. (2003). Neutrophil migration in inflammation: nitric oxide inhibits rolling, adhesion and induces apoptosis. Nitric Oxide 9, 153-164.

Torok J.A., Brahmajothi M.V., Zhu H., Tinch B.T., Auten R.L. and McMahon T.J. (2012). Transpulmonary Flux of S-Nitrosothiols and Pulmonary Vasodilation During Nitric Oxide Inhalation: Role of Transport. Am. J. Respir. Cell Mol. Biol. 47, 37-43.

Troncy E., Francoeur M. and Blaise G. (1997). Inhaled nitric oxide: clinical applications, indications, and toxicology. Can. J. Anaesth. 44, 973-988.

Winter C.A., Risley E.A. and Nuss G.W. (1962). Carrageenan-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 111, 544-547.

Yu B., Volpato G.P., Chang K., Bloch K.D. and Zapol W.M. (2009). Prevention of the pulmonary vasoconstrictor effects of HBOC-201 in awake lambs by continuously breathing nitric oxide. Anesthesiology 110, 113-122.

Accepted July 29, 2014