Synergistic antioxidant and anti-inflammatory action of N-acetylcysteine in portal hypertensive gastropathy in rats

ⓘ Francielli Licks[1], ⓘ Renata Minuzzo Hartmann[2], ⓘ Elizângela Schemitt[2], ⓘ Josieli Raskopf Colares[3], ⓘ Camila Marques[4],
ⓘ Henrique Fillmann[5], ⓘ Norma Possa Marroni[1,2]

[1]Program in Biological Sciences: Physiology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil; [2]Program in Medicine: Medical Sciences, UFRGS; Porto Alegre, RS, Brazil; [3]Program in BioHealth, Universidade Luterana do Brasil (ULBRA), Canoas, RS, Brazil; [4]Health Science Education Program, Faculdades Pequeno Príncipe, Curitiba, PR, Brazil; [5]School of Medicine, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

Abstract

Background and Aim: Portal hypertension (PH) is a syndrome associated with cirrhosis and characterized by a progressive increase in portal pressure, with consequent compensatory vascular dilation. Gastric vascular changes associated with oxidative and nitrosative stress characterize the clinical presentation of portal hypertensive gastropathy (PHG). In addition, the inflammatory process is considered an aggravating factor for severity by contributing to gastric tissue injury. The aim of this study was to investigate the synergistic anti-inflammatory and antioxidant action of N-acetylcysteine (NAC) in the stomach of rats with PH.

Materials and Methods: Eighteen Wistar male rats were used in this experimental protocol and were divided into three groups with six in each group: sham-operated (SO), partial portal vein ligation (PPVL), and PPVL + NAC. Treatment with NAC at a dose of 10 mg/kg (i.p.) was initiated on day 8 after surgery and continued for 7 days. We evaluated the expression of iNOS, NQO-1, HSP-90, and SOD by Western blot, as well as nuclear factor-kappa B (NF-κB) and tumor necrosis factor (TNF)-α staining by immunohistochemistry, in the rat stomach.

Results: The PPVL group exhibited increased expression of HSP-90, iNOS, SOD, and NQO-1 when compared with controls. NAC reduced the expression of all studied proteins. Similarly, NF-κB and TNF-α staining was increased in PPVL animals versus controls and reduced in PPVL + NAC versus PPVL animals, respectively.

Conclusion: These results suggest the effectiveness of NAC as a dual anti-inflammatory and antioxidant in animals with experimental PHG induced by partial ligation of the portal vein.

Keywords: Inflammation; N-acetylcysteine; oxidative stress; portal hypertension.

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Introduction

The portal system is responsible for leading blood from the intra-abdominal portion of the gastrointestinal tract, pancreas, spleen, and gallbladder to the liver sinusoids via the terminal branches of the portal vein.[1]

Pre-hepatic abnormalities (such as splenic vein thrombosis), intrahepatic abnormalities (such as cirrhosis), and post-hepatic conditions (such as Budd-Chiari syndrome) may all influence blood pressure within the portal system. The only shared characteristic of the aforementioned examples is the emergence of an anatomical barrier to blood flow. This obstacle triggers a compensatory mechanism to reduce blood pressure, promoting the development of hyperdynamic collateral circulation. This condition, characterized by a progressive increase of pressure in the portal system, is called portal hypertension (PH).[2]

The resulting bypass of blood flow from the site of obstruction directly into the systemic circulation triggers serious complications, such as ascites, hepatic encephalopathy, and gastrointestinal bleeding, with the latter accounting for the high mortality rate of 50% among patients with PH.[3]

Microcirculatory alterations in the gastric mucosa as a result of the characteristic vasodilation of HP were described by McCormack et al. in 1985.[4] Several different terms have been used to describe these changes, such as inflammatory gastritis, gastric mucosal vasculopathy, portal hypertensive mucosa, and portal hypertensive gastropathy (PHG).[5]

PHG is characterized by vasodilation secondary to obstruction of gastric blood flow, and nitric oxide (NO) is the main mediator in this vascular activation pathway.[6] NO is an endothelium-derived relaxing factor produced from L-arginine and molecular oxygen, in a process catalyzed by enzymes in the nitric oxide synthase (NOS) family. The three major NOS isoforms, neuronal NOS (nNOS and NOS1), inducible NOS (iNOS and NOS_2), and endothelial NOS (eNOS and NOS_3), are described in the literature, with well-established roles. NOS_2 can be expressed in various cell types, and it plays an important role in inflammatory diseases and septic shock. NOS_3 is expressed in endothelial cells and acts on endothelial smooth muscle relaxation.[7] All three isoforms have been associated with PH; however, the most influential isoforms in this syndrome are NOS_2 and NOS_3.[8] NOS_3 is closely related to several factors and proteins that have positive and negative impacts on its production. Proteins that positively influence the production of this enzyme include the positive regulator molecular chaperone heat shock protein 90 (HSP90).[9]

Shear stress and vascular endothelial growth factor (VEGF) also act on the pathway that stimulates the production of NOS_3, and both have been studied previously in investigations conducted by our research group.[10,11]

NOS_2 produces a relatively high level of NO. This isoform is expressed in response to stimulation from lipopolysaccharides or inflammatory cytokines, and its expression is modulated by transcriptional mechanisms. Nuclear factor-kappa B (NF-κB) is the primary mediator for the induction of iNOS, which, in turn, is activated mainly by tumor necrosis factor (TNF)-α and oxidative stress.[8]

In PH, overproduction of NO via NOS_2 and NOS_3 determines its reaction with the superoxide anion radical (O_2^-°), leading to the formation of peroxynitrite ($ONOO^-$). This, in turn, is a highly reactive species that can contribute to cellular damage, thus aggravating the overall clinical picture.[12]

The inflammation characteristic of PH affects gastric regeneration and defense, thus increasing the risk of bleeding.[13] Currently, one hypothetical treatment strategy involves the direct reduction of proinflammatory chemokines and cytokines, which contribute to the progression of PH.[14]

Thus, treatment with both antioxidant and anti-inflammatory action would represent a promising candidate for minimizing the consequences of PHG. Previous work published by our research group has demonstrated the antioxidant properties of N-acetylcysteine (NAC), a molecule used extensively in clinical practice, in an experimental model of PH.[11] The aim of this study was to evaluate the synergistic action of NAC as an antioxidant and anti-inflammatory in PHG.

Materials and Methods

Ethics

All procedures were carried out in accordance with current Brazilian legislation for the practice of scientific research using animals (Law 11.794 of October 8, 2008, 2013 Brazilian Guidelines for the Care and Use of Animals for Scientific and Educational Purposes, and 2013 CONCEA Guidelines for the Practice of Euthanasia) and followed the recommendations of the Principles of Care for Laboratory Animals formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Animal Groups and Experimental Protocols

The animals used in this study were obtained through vivarium, in accordance with institutional specifications. Throughout the experiment, animals were kept in plastic bin-type cages (47 × 34 × 18 cm) lined with wood shavings, under a 12-h light/dark cycle, at a temperature of 22 ± 4°C. They had access to water ad libitum and were fed commercially available rodent chow (Purina®–Nutripal, Porto Alegre, RS, Brazil), approximately 16 g/day.

On the first day of the experiment, the Wistar male rats were randomly divided into three groups (n=18 animals): sham-operated (SO) (n=6), partial portal vein ligation (PPVL) (n=6), and PPVL + NAC (n=6). All animals were weighed (±250 g) and anesthetized with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Once a proper plane of anesthesia had been achieved, a midline laparotomy was performed, and the bowel was gently retracted with a gauze pad soaked in saline. In group SO, only manipulation of the portal vein was performed,

whereas in the other groups, the vein was ligated for experimental induction of PH. Briefly, the portal vein was isolated with 3-0 silk thread, partially blocked using a 20G needle placed in front of the vessel, and tied off. After ligation, the needle was removed, thus leaving the vein partially obstructed. Bowel loops were gently replaced into the abdomen, 10 mL of saline solution infused the abdominal cavity, and the muscle closed with running sutures. The experimental model used in this study was first described by Sikuler et al.[15] in 1985 and induces pre-hepatic PH.

After the surgical procedures, animals were kept in individual cages. Metamizole was administered for postoperative analgesia, with the first dose given via intramuscular injection (200 mg/kg) and subsequent doses given orally (500 mg/kg every 8 h for 72 h).

Seven days were allowed to elapse for the establishment of PHG before starting treatment. NAC (Sigma Chemical Co., St. Louis, MO, USA; CAS registry number 616-91-1) was administered intraperitoneally at a dose of 10 mg/kg, in agreement with previous studies.[11,12] The drug was dissolved in 0.6 mL of saline (0.9% NaCl), and the same volume was administered to groups that received vehicle only (SO and PPVL). Treatment was continued for 7 days, thus completing the full 15-day experimental period.

Euthanasia and Collection of Tissue Samples

On day 15, animals were again weighed and anesthetized with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Once adequate anesthesia had been achieved, a midline laparotomy was performed, and the stomach was resected for analysis. One portion of the stomach was frozen at -80°C, and a second fragment was fixed in 10% buffered formalin for 24 h. Then, 3-mm sections were cut from the paraffin block using a rotary microtome.

Western Blot

Western blot analysis was performed using cytosolic extract prepared from stomach homogenates. The protein concentration of each sample was measured by the Bradford method. Then, lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked by submerging in Tris–buffered saline and 0.05% Tween (TTBS) with 5% nonfat dry milk for 1 h at room temperature and probed overnight at 4°C. The following antibodies were used: mouse polyclonal antibody [NOS_2 (sc-7271), Santa Cruz Biotechnology, Santa Cruz, CA, USA]; mouse monoclonal antibody [HSP90 (sc-101494), Santa Cruz Biotechnology, Santa Cruz, CA, USA]; goat monoclonal antibody [NQO1 (sc-16464), Santa Cruz Biotechnology, Santa Cruz, CA, USA]; goat monoclonal antibody [SOD (sc-8637), Santa Cruz Biotechnology, Santa Cruz, CA, USA], at 1:200 dilution with TTBS in 5% nonfat dry milk; and anti-β-actin (42-kDA) antibody (Sigma Aldrich, St. Louis, MO, USA) at 1:1000 dilution with TTBS in 5% nonfat dry milk. After overnight incubation, the membranes were washed with TTBS and incubated for 1 h at room temperature with secondary goat anti-mouse IgG-HRP sc-2005, donkey anti-goat antibody (sc-2020, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:4000). Protein detection was performed via chemiluminescence using a commercial ECL kit (Amersham Pharmacia Biotech, Little Chalfont, England). The density of the specific bands was quantified with imaging densitometry software (Scion Image, Maryland, MA, USA).[16]
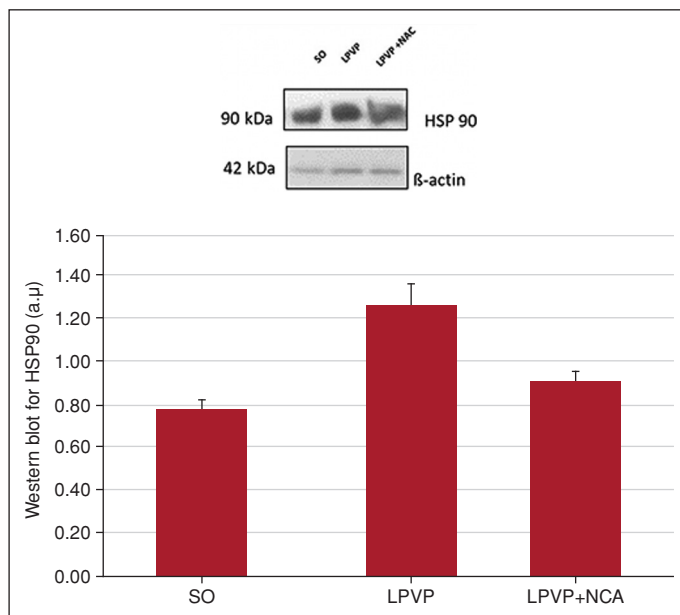
Figure 1. Western blot for HSP90. Effects of PPVL and NAC administration on HSP90, quantified by the Western blot technique.

*: P<0.01; **: P<0.01 (n=6); SO: Sham-operated group; PPVL: Partial portal vein ligation group; PPVL + NAC: Partial portal vein ligation group plus N-acetylcysteine treatment.
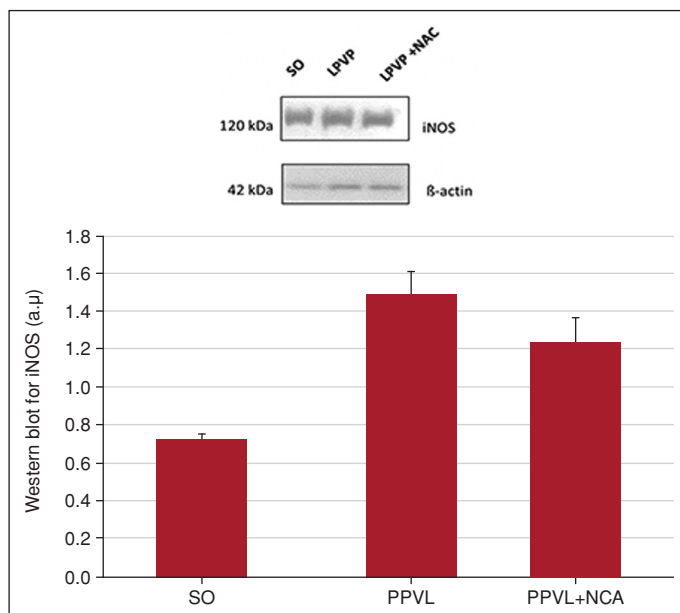

Figure 2. Western blot for iNOS. Effects of PPVL and NAC administration on iNOS, quantified by the Western blot technique.

*: P<0.01; **: P<0.05 (n=6).

Immunohistochemistry

The expression of NF-κB and TNF-α in stomach tissue was analyzed using immunohistochemical techniques. Buffer at 100°C was used for antigen retrieval, and endogenous peroxidase activity was blocked by incubation in absolute methanol. Slides were then incubated with rabbit polyclonal antibody (NF-κB [sc-9072], 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat monoclonal antibody (TNF-α [sc-1351],
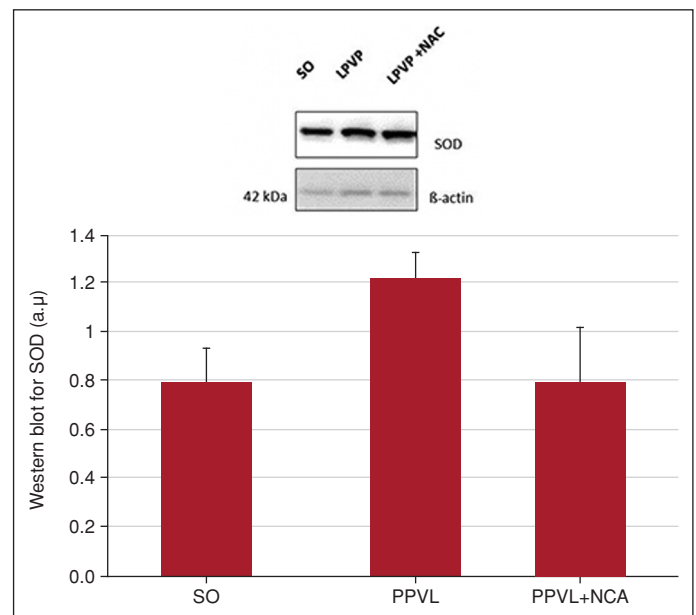

Figure 3. Western blot for SOD. Effects of PPVL and NAC administration on SOD, quantified by the Western blot technique.

*: P<0.05; **: P<0.05 (n=6).

1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by washing with buffer and incubation with secondary goat anti-rabbit IgG-HRP (sc-2004) for 30 min at room temperature. The slides were analyzed under a microscope equipped with a digital camera, and images were captured using Image-Plus software (Media Cybernetics, Bethesda, MD, USA). Quantification of staining for both markers was performed by digital image analysis in Adobe Photoshop® CS3 Extended 10.0, by counting the number of pixels stained. The level of expression was determined by multiplying the average density of the image by the percentage of positively stained areas (those colored brown).[17]

Statistical Analysis

Analysis of Variance was used to evaluate the quantitative data, followed by the Student–Newman–Keuls test for the analysis of multiple comparisons. The significance level was set at 5% (p<0.05). All data are presented as mean ± SE. The GraphPad InStat version 3.0 program for Windows was used.

Results

Western Blotting Analysis

Analysis of HSP90 protein expression revealed a significant increase in the PPVL group compared with controls (p<0.01). NAC effectively reduced these values in the LPVP + NAC group (p<0.01) (Fig. 1).

When evaluating the expression of iNOS, a decrease was observed in the LPVP + NAC group (p<0.05) and a significant increase in the LPVP group (p<0.01) (Fig. 2).

NAC was able to substantially reduce SOD values in the treated group, up to control levels (p<0.05). In the LPVP group, SOD was overexpressed when compared with the SO group (p<0.05) (Fig. 3).

In the analysis of NQO1 protein expression, NAC significantly reduced the values in the LPVP + NAC group when compared with the LPVP
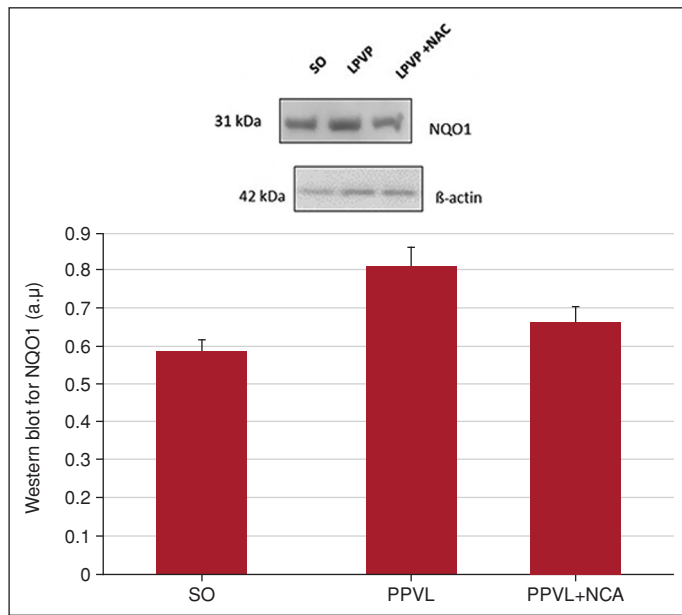
Figure 4. Western blot for NQO1. Effects of PPVL and NAC administration on NQO1, quantified by the Western blot technique.
*: $P<0.05$; **: $P<0.05$ (n=6).

group ($p<0.05$), which had a significantly increased expression when compared with the control group ($p<0.05$) (Fig. 4).

Immunohistochemistry

Animals in the PPVL group showed a significant increase in positive staining for NF-κB as compared with controls ($p<0.01$). NAC was effective in reducing this immunoreactivity in the PPVL + NAC group ($p<0.01$) (Fig. 5).

The same pattern of expression was observed in the analysis of TNF-α. In the PPVL group, there was intense staining as compared with the SO group ($p<0.01$); in the PPVL + NAC group, this immunoreactivity was reduced ($p<0.01$) (Fig. 6).

Discussion

PH, often described as a complication of chronic liver disease, is characterized by increased blood pressure in the portal system, splanchnic vasodilation, and subsequent formation of portosystemic collaterals.[18]

The stomach is one of the main sites affected by this marked increase in vascular diameter; accordingly, gastropathy is one of the most frequent complications of PH. In practice, gastric lesions are typically located at the fundus and superior portion of the body of the stomach[19] and are characterized by a mosaic-like pattern, with or without cherry-red spots.[20] Still, the release of inflammatory mediators and consequent gastric inflammation appears to contribute to the development and worsening of gastropathy of PH.[21]

The involvement of NO in the development of hyperdynamic collateral circulation was originally proposed in 1991 by Vallance and Moncada.[22] This hypothesis has since been confirmed by several studies that demonstrated NO as the main mediator of vascular abnormalities in PH.[9,23] In previous investigations, our research group found increased expression of two NOS isoforms predominantly involved in the activation of NO synthesis (eNOS and iNOS), using an animal model of PH.[10,24] An increase in eNOS expression is already detectable in
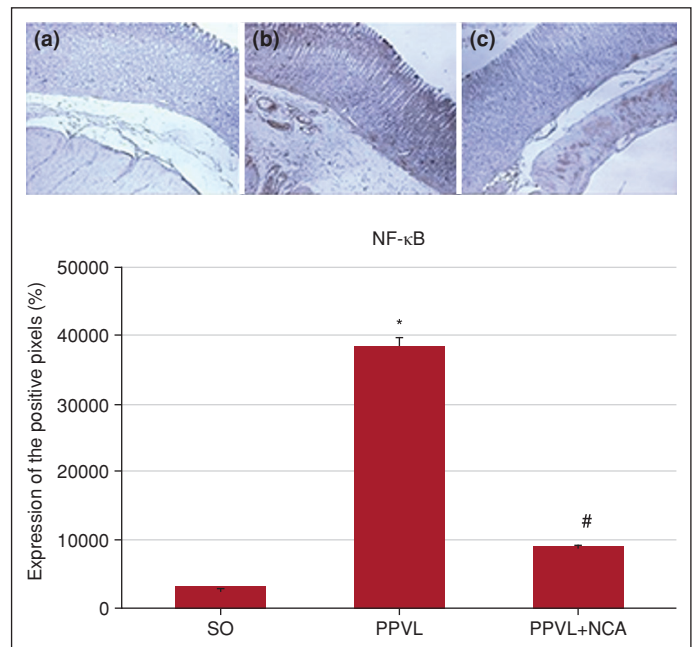

Figure 5. Immunohistochemical staining for NF-κB. Effects of PPVL and NAC administration on NF-κB expression. **(a)** SO group, **(b)** PPVL group, and **(c)** PPVL + NAC group.
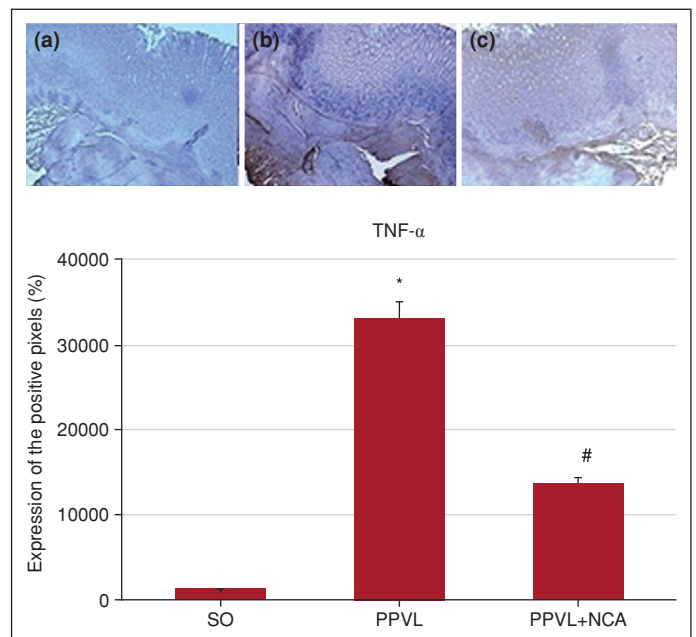*: $P<0.001$ (n=6).

Figure 6. Immunohistochemical staining for TNF-α. Effects of PPVL and NAC administration on TNF-α expression. **(a)** SO group, **(b)** PPVL group, and **(c)** PPVL + NAC group.
*: $P<0.001$ (n=6).

the early stages of PH induced by carbon tetrachloride (CCl4),[25] and eNOS appears to be the major enzymatic pathway for NO synthesis.[26] Agonists in the eNOS activation pathway include shear stress, VEGF, and HSP90, among others. Furthermore, inflammatory cytokines such as TNF-α appear to influence NO production by eNOS.[9]

Another modulator of NO upregulation is VEGF, which is activated by increased portal pressure. Through its angiogenic activity, VEGF ultimately contributes to increased blood flow in the splanchnic territory and to local vasodilation.[27] In the present study, animals subjected to the same experimental model had increased expression of HSP90 when compared with controls (Fig. 1), which demonstrates the influence of this chaperone in the eNOS activation pathway and confirms previous results published by Ai et al.,[28] in which HSP90 was overexpressed in the endothelium of mesenteric vessels in PPVL animals.

HSP90 in PH exerts an agonist effect on NO production and vasodilation via eNOS.[29] NAC was able to reduce the expression of HSP90 in the model tested herein. Therefore, the chosen treatment appears to fully modulate the studied pathway, reducing the levels not only of VEGF but also of HSP90, thereby contributing to a reduction in vasodilation mediated by eNOS (Fig. 1).

In PH, iNOS is also upregulated. The inducible form of NOS is activated during disease progression and may even be regulated by NF-κB. Stimulation of iNOS expression occurs along with the activation of the innate and adaptive immune systems, infiltration by polymorphonuclear leukocytes, and recruitment of lymphocytes. The latter produce large amounts of NO and cytokines that modulate the inflammatory process, such as TNF-α.[8] Our results showed higher expression of iNOS in PPVL animals (Fig. 2). This enzyme is overexpressed in the gastric mucosa of animals with PHG. Our results suggest that gastric inflammation is clearly established in this experimental model, which is consistent with the literature.[24]

NAC has modulatory activity at different stages of the inflammatory and phagocytic process, stimulating immune functions and reducing levels of proinflammatory cytokines. Furthermore, it inhibits the production of NO via iNOS.[30] In this study, animals treated with NAC exhibited reductions in iNOS expression in the stomach and, therefore, an attenuation of gastric inflammation (Fig. 2). The same phenomenon was reported in a previous study using an animal model of PH induced by dimethylnitrosamines.[31]

This finding was reaffirmed in our analysis of NF-κB and TNF-α immunoreactivity in gastric tissue: both were significantly increased in PPVL animals as compared with controls (Figs. 5, 6). NF-κB is currently considered a typical proinflammatory signaling molecule, due to its action on the expression of proinflammatory genes, chemokines, cytokines, and adhesion molecules.[32] In the cytoplasm, this factor is bound to its inhibitory protein, IκB; only after phosphorylation and degradation by specific protein kinases, such as the IκB kinase complex, does translocation of NF-κB to the nucleus become possible.[33] The stimulus for NF-κB translocation occurs via neurotransmitters such as glutamate, viral and bacterial products, reaction products (via iNOS), increased intracellular calcium levels, and oxidative stress.[34]

TNF-α, in turn, is a cytokine with an important role in the activation of NF-κB. It participates in a wide spectrum of biological activities, including inflammation, apoptosis, and cell growth and differentiation. By extracellular stimuli, TNF causes phosphorylation of IκB and subsequent release of NF-κB, which can then act on target genes in the cell nucleus.[34]

In the literature, the anti-inflammatory properties of NAC have been associated with its ability to modulate the immune response and its actions on leukotriene and prostaglandin metabolism, among other mechanisms.[35] All of these effects are related to the nature of NAC as a donor source of –SH groups, inducing the synthesis of glutathione (GSH), which increases cellular protection against oxidative stress and inflammatory processes. NAC also acts to reduce reactive oxygen species produced by leukocytes.[36]

NAC inhibits the activation of NF-κB and also blocks TNF-α by reducing its affinity for the TNF receptor.[37] It is well known that not only the inflammatory process but also oxidative stress is an aggravating factor in chronic liver disease.[30] Prolonged NO synthesis at high levels may induce oxidative stress due to the production of peroxynitrite (ONOO–), a powerful oxidant. This compound, generated by the NO and peroxynitrite reaction, can cause oxidative and nitrosative damage to proteins, lipids, and DNA.[38] The presence of a high concentration of peroxynitrite in the gastric mucosa of animals subjected to the PPVL model of PH was previously demonstrated by immunohistochemistry.[11] Yet, in the same experimental model, an increase in the thiobarbituric acid reactive substances and NO levels and a reduction in the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) have been demonstrated, results that suggest increased oxidative stress.[39]

In the present study, we evaluated the possibility that NAC would act synergistically as a dual antioxidant and anti-inflammatory. To determine its antioxidant role, we used the Western blot technique to assess the expression of SOD and NADPH quinone oxidoreductase-1 (NQO1). SOD is an antioxidant enzyme responsible for catalyzing the dismutation of superoxide ($O_2^{-\circ}$) into hydrogen peroxide (H_2O_2). Increased levels of this enzyme are indicative of increased $O_2^{-\circ}$ production and hence of oxidative stress.[30,40] In the present study, expression of this enzyme was significantly increased in PPVL animals as compared with controls, and NAC was able to reduce such overexpression (Fig. 3). The reactive oxygen species can be removed from tissue by endogenous antioxidants such as thiols, components which are found, e.g., in GSH.[41] As a precursor of this molecule, NAC acts as an antioxidant and can thus be beneficial in disorders aggravated by oxidative stress. In this study, NAC had a clear antioxidant effect, as demonstrated in the analysis of SOD expression (Fig. 3) in the PPVL + NAC group. Levels of this enzyme returned to near-control values, demonstrating a reduction in oxidative stress and, consequently, in the expression of the SOD enzyme.

Another antioxidant molecule, which is less effective than SOD but acts as a direct scavenger of the superoxide anion, is NQO1.[39] The NQO1 gene encodes enzymes responsible for cell protection, which catalyze the reduction of electrons and prevent the formation of reactive oxygen species.[42] Increased expression of NQO1 was demonstrated in PPVL animals (Fig. 4) when compared with controls. This finding is probably related to the cellular response to oxidative stress caused by inflammation,[42] and NAC showed a synergistic antioxidant and anti-inflammatory activity in reducing expression of this marker in the gastric mucosa of PPVL + NAC animals (Fig. 4).

From the findings of this study, we conclude that the experimental model of PPVL successfully induces PH with subsequent inflammation and oxidative stress in the gastric mucosa. Furthermore, we suggest that a synergistic antioxidant and anti-inflammatory effect of NAC was able to attenuate the damage caused by these processes, contributing to the integrity of gastric tissue and mitigating the injuries caused by this experimental model.

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