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# THE PROTECTIVE EFFECT OF N-ACETYLCYSTEINE ON APOPTOTIC LUNG INJURY IN CECAL LIGATION AND PUNCTURE-INDUCED SEPSIS MODEL

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ABSTRACT—Apoptotic loss of parenchymal cells may lead to organ dysfunctions in critically ill patients with septic states. As an antioxidant, the protective effects of N-acetylcysteine (NAC) are documented in many experimental and clinical studies. In this experimental study, we investigated the role of chronically used NAC in septic lung injury on a cecal ligation and puncture (CLP) model. To evaluate this, 30 male Wistar rats were randomly divided into four groups as sham (n = 7), CLP (n = 8), sham + NAC (n = 7) and CLP + NAC (n = 8) groups. NAC was administered 150 mg $kg^{-1}$ day through intramuscular route beginning 6 h after the operations and lasting for a period of 1 week. One week later, histopathology and epithelial apoptosis were assessed by hematoxylin-eosin and immunohistochemically by M30 and caspase 3 staining to demonstrate septic lung injury. Additionally, lung tissue myeloperoxidase (MPO) activity, malondialdehyde (MDA), and nitrite/nitrate levels were measured. The MPO activity and MDA levels in lung homogenates were found to be increased in CLP group and the administration of NAC prevented their increase significantly ($P < 0.05$). However, there were no significant differences among the groups regarding nitrite/nitrate levels. The number of apoptotic cells was significantly lower in CLP+NAC group than CLP group, and this finding was supported by M30 and caspase 3 expression in lung ($P < 0.05$). Lung histopathology was also protected by NAC in CLP-induced sepsis. In conclusion, the chronic use of NAC inhibited MPO activity and lipid peroxidation, which resulted in reduction of apoptosis in lung in this CLP model. Because lung tissue nitrite/nitrate levels did not change significantly, organs other than the lungs may be responsible for producing the increased nitric oxide during sepsis. The chronic use of NAC needs further investigation for its possible antiapoptotic potential in septic states besides its documented antioxidant and antiinflammatory effects.

KEYWORDS—N-acetylcysteine, acute lung injury, apoptosis, cecal ligation and puncture, sepsis, myeloperoxidase, nitric oxide, M-30

## INTRODUCTION

One of the common lethal complications of endotoxemia is the acute respiratory distress syndrome (ARDS) and/or acute lung injury (ALI). Traditionally, some treatment models have been used as supportive measures in ARDS and ALI with no significant success (1), but the use of protective ventilation strategies has caused improvement in mortality. For additional therapeutic measures, current research is being directed to prevention or amelioration of the progression of lung injury by exploiting strategies based on the current understanding of the biological mechanisms at the molecular level.

The pathopyhsiology of the lung insult without direct lung injury is complex and may be mainly caused by secondary damage from activated inflammatory cells by the way of the oxidoinflammatory cascade. The role of reactive oxygen species (ROS) in cecal ligation and puncture (CLP)-induced septic lung injury has been shown in a variety of animal models (2, 3). The damaging effects of ROS are mainly determined by the local imbalance between oxidants and antioxidants. As a matter of fact, the results from both experimental and clinical studies have shown that the administration of antioxidants appears to be useful in septic states in which the lungs are mostly affected as remote organs (3, 4).

In sepsis-induced ARDS and/or ALI, the dependent lung regions are the sites of greatest inflammation and injury. There are some experimental studies suggesting that an apoptotic process triggered by oxidative stress is involved in this resulting lung injury (5, 6). There is evidence that apoptotic loss of parenchymal cells may have an association with organ dysfunctions, which seem to be one of the causes for mortality (7). These findings suggest that prevention of parenchymal apoptosis by antioxidants can reduce mortality rates associated with ARDS and/or ALI (8).

One of the popular therapeutic approaches for the inhibition of oxidant-mediated injury is the use of glutathione-modulating agents like thiol or sulfhydryl compounds. Among them, N-acetylcysteine (NAC) is probably one of the most widely investigated agent that serves as a precursor of glutathione and also acts as a direct scavenging agent (9). In addition to its antioxidant properties, NAC also elicits other beneficial effects by demonstrating anti-inflammatory activity such as suppressing cytokine expression/release, inhibiting the adhesion molecule expression, and inhibiting nuclear factor kappa $\beta$ (10–12). However, the role of NAC in the concept of sepsis-induced apoptosis has yet to be elucidated.

In the present study, we aimed to investigate the effects of chronically administered NAC on oxidoinflammatory and apoptotic pathways in CLP-induced sepsis model. To achieve this, lung myeloperoxidase (MPO) content, lipid peroxidation products, and nitrite/nitrate levels were measured as indices of

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lung injury. Additionally, apoptosis and histopathology were evaluated in lung tissue.

## MATERIALS AND METHODS

The experiments described in this article were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. Male Wistar rats, weighing between 200–250 g, were housed at constant temperature with 14/10 h periods of light/dark exposure, respectively. Animals were allowed access to standard rat chow and water *ad libitum* acclimation period of at least 1 week before use in these experiments.

### Experimental sepsis by CLP

Anesthesia was induced by intramuscular administration of ketamine 50 mg $kg^{-1}$ and xylazine 7 mg $kg^{-1}$. After shaving the abdomen and application of a topical disinfectant, a 2-cm midline incision was made below the diaphragm to expose the abdominal organs. After the identification of the cecum, it was ligated below the ileocecal valve without occluding the bowel passage. The cecum was then subjected to a single "through and through" perforation with an 18-gauge needle distal to the point of ligation. The needle was removed and a small amount of stool was extruded from both punctures to ensure patency. After repositioning the bowel, the abdominal incision was closed with 4/0 sterile synthetic absorbable suture (Polyglactin 910, Vicryl, Ethicon Ltd., Edinburg) and skin clips (Ethicon, Somerville, NJ). Sham-operated animals underwent the same procedure except for ligation and puncture of the cecum.

### Experimental protocol

After fasting overnight, 30 rats were randomly divided into four groups. The first group (sham group, n = 7), served as sham-operated received intramuscular saline (0.5 mL/rat) everyday for 1 week. The second group (CLP group, n = 8), which was subjected to cecal ligation and puncture (CLP), received intramuscular (i.m.) saline (0.5 mL/rat) every day for 1 week. The third group (sham + NAC group, n = 7) was given NAC (150 mg $kg^{-1}$) after sham operation, and the fourth group (CLP + NAC group, n = 8) received i.m. NAC (150 mg $kg^{-1}$) after CLP for 1 week beginning 6 h after the operation. All animals received fluid resuscitation.

One week later, rats were anesthetized with i.m. ketamine 50 mg $kg^{-1}$ and xylazine 7 mg $kg^{-1}$ and both lungs were harvested through a midline sternotomy. To evaluate the CLP-induced lung injury and apoptosis, tissue samples of lung were divided into four parts. One of them was fixed in 10% formaldehyde and the other three parts were taken for biochemical assays. Lung specimens were kept frozen at −70°C until analysis.

### Measurement of lung tissue myeloperoxidase activity

One part of the tissue was homogenized in 10 parts HETAAB buffer for myeloperoxidase activity assay. The determination of tissue MPO activity depends on the fact that it reduces dimethoxibenzidine. Lung tissues were homogenized in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-(N-morpholino) propanesulfonic acid and centrifuged at 15,000 g for 40 min. An aliquot of supernatant was mixed with a solution of 1% (w/v) dimethoxybenzidine and 1 mM hydrogen peroxide. After a 30-min incubation, the reaction was stopped by adding 3 M HCl. Activity was measured spectrophotometrically as the change in absorbance at 410 nm at 37°C by using a spectrophotometer (13). Results are expressed as units MPO activity per g tissue.

### Measurement of lung tissue nitrite and nitrate levels

One part of the tissue was homogenized in 10 parts 100 mM sodium phosphate buffer for tissue nitrite/nitrate assay. The levels of nitrite and nitrate were determined by using a photometric endpoint determination (Roche Diagnostic GmbH, Mannheim, Germany catalog no: 1 756 281). Principle of nitrate is reduced to nitrite by reduced nicotinamide dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase. The formed nitrite reacts with sulphanilamide and N-(1-naphtyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range (550 nm). Values were expressed as $\mu$mol per gram wet tissue weight.

### Detection of lipid peroxidation

One part of the tissue was homogenized in 10 parts 15 mmol/L KCL for malondialdehyde (MDA) assay. MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Measurement of MDA content by TBA reactivity is the most widely used method for assessing lipid peroxidation (14). The principle of the method is based on measurement of the absorbance of the pink color produced by interaction of TBA with MDA at 530 nm. Values were expressed as nmol per gram wet tissue weight.

### Histopathological examination

The specimens were fixed in 10% formalin for 24 h, and standard dehydration and paraffin-wax embedding procedures were used. Hematoxylin and eosin-stained slides were prepared by using standard methods. Light microscopic analysis of lungs was performed by blinded observation to evaluate pulmonary architecture, tissue edema formation, and infiltration of the inflammatory cells. The results were classified into four grades, where grade 1 represented normal histopathology; grade 2 indicated only few neutrophil leukocyte infiltration; grade 3 represented moderate neutrophil leukocyte infiltration perivascular edema formation, and partial destruction of pulmonary architecture; and finally grade 4 included dense neutrophil leukocyte infiltration, abscess formation, and complete destruction of pulmonary architecture.

### Evaluation of apoptosis by immunohistochemical study

In this study, we used immunohistochemical staining for cytokeratin 18 with M30 cytodeath and caspase 3. M30 is a monoclonal antibody that recognizes a neoepitope of cytokeratin 18 (CK18) in paraffin-embedded tissue. This neoepitope is produced by caspase cleavage of CK18 during apoptosis and is not present in nonapoptotic cells (15). Caspase 3 is a cysteine protease protein (CPP 32) that exists as inactive zymogen in nearly all cells and is involved in the development of apoptotic cell death in cell turnover. Immunohistochemistry for M30 CytoDeath and caspase 3 were performed using a combination streptavidin-biotin-peroxidase method and microwave antigen retrieval on formalin-fixed paraffin-embedded tissues. Paraffin sections of 5 micron were cut and deparaffinized in xylene and rehydrated with graded ethanol, water, and Tris-buffered saline. The slides were treated with 10% hydrogen peroxidase in distilled water to block the endogenous peroxidase activity. Antigen retrieval was performed by microwave pretreatment. Citric acid buffer (pH 6) was preheated by incubation in a microwave oven at 750 W for 15 min according to the manufacturers' protocols with modification. After cooling, nonspecific binding of antibody was blocked by Ultra V block (Laboratory Vision/ Fremont, CA 94539) for 20 min, both primary M30 CytoDeath antibody (monoclonal antibody, dilution 1:50, Roche, Mannheim, Germany) and caspase 3 antibody (CPP32, Ab-4 dilution 1:100, Neomarkers Labvision, Fremont, CA 94539) were incubated in a moist chamber for 1 h at room temperature, followed sequentially with biotinylated goat anti-polyvalent (Laboratory Vision) for 30 min and streptavidin peroxidase complex (Laboratory Vision) for 30 min. 3-Amino-9 Ethylcarbazole (AEC; Laboratory Vision) was used as the Chromagen and hematoxylin was used for nuclear counterstain. Omitting the primary antibody performed the negative control and a case of breast carcinoma was included as positive control for M30 and caspase 3. Sections were examined by light microscopy (Olympus BX50). The numbers of positively stained cells in five high-power fields (X 400) were counted in the most intensely stained areas and their mean was calculated. In the contrary to the evaluation of caspase 3 staining, during the evaluation of M 30 staining, positively stained macrophages have also been counted because they ingest apoptotic cells (16) and these ingested cells may be stained in the cytoplasms of the macrophages as mentioned in the manufacturer's prospectus for the M30 CytoDeath kit, therefore the method reflects an indirect measurement of apoptotic epithelial cells.

### Statistical analysis

Biochemical values are given as mean ± SD values. Statistical differences for myeloperoxidase, MDA and nitrite/nitrate values were evaluated using one-way analysis of variance followed by Student Newman-Keuls test. Comparison of hematoxylin and eosin, M30 and caspase 3 staining scores was analyzed using Kruskall-Wallis variance analysis followed by Dunn test. $P$ values less than 0.05 were considered significant.

## RESULTS

All animals in the sham-operated, sham-operated + NAC, and CLP + NAC groups survived the experimental period. Two rats in the CLP group died on the second and third days of the experiment.

### Tissue MPO activity

The activity of MPO in lung tissue is demonstrated in Figure 1. In the CLP group, lung tissue MPO activity was found to be significantly increased (3.74 ± 0.48 vs. 7.06 ± 0.75) whereas NAC caused a decrease in MPO activity (4.63 ± 0.60; $P < 0.05$). The decrease of MPO activity was also paralleled with the decrease in the accumulation of polymorphonuclear neutrophil. Although MPO activity levels were still higher in NAC

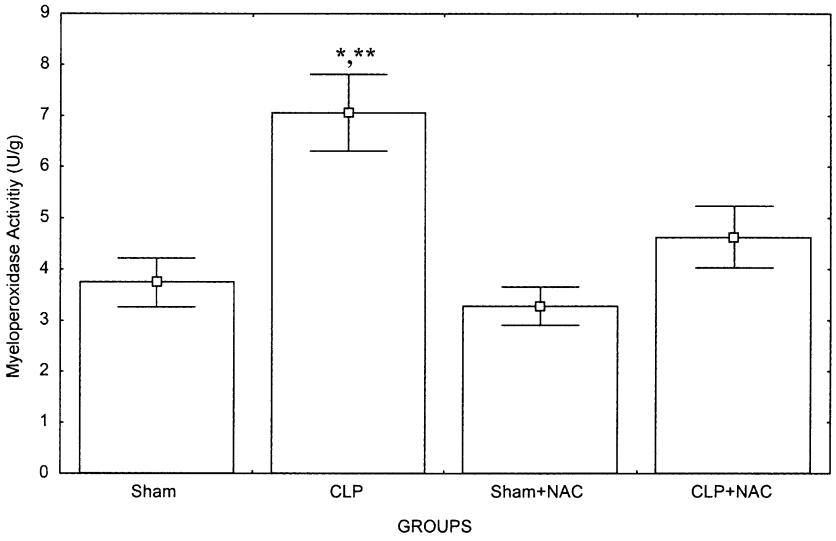


FIG. 1. **Lung MPO.** CLP resulted in increased lung MPO activity compared with sham-operated animals. The CLP-induced increase was reduced by NAC treatment. *$P < 0.05$ compared with sham, **$P < 0.05$ compared with CLP + NAC.

group when compared with the sham, this difference was not statistically significant.

### *Tissue MDA levels*

In all groups, lung tissue MDA levels correlated with MPO activity. This CLP model significantly increased tissue lipid peroxidation so that MDA levels were found to be increased in the CLP group (7.03 ± 1.83 vs. 39.75 ± 11.48) while CLP + NAC group presented lower lung tissue MDA levels (9.27 ± 1.84; $P < 0.05$; Fig. 2). Nearly six times higher MDA levels were obtained in CLP model in comparison with sham. However, NAC treatment prevented the formation of lipid peroxidation.

### *Tissue nitrite/nitrate levels*

No significant difference was observed in lung tissue nitrite/nitrate levels among the study groups (Fig. 3).

### *Light microscopy findings*

There were no significant light microscopic differences between lungs of sham and sham + NAC group. In the CLP group, interstitial edema with massive infiltration of the inflammatory cells was observed and the pulmonary architecture was severely damaged. In the CLP + NAC group pulmonary architecture was preserved and infiltration of inflammatory cells and edema decreased (Figs. 4 and 5).

### *Immunohistochemical findings*

In the sham and sham + NAC groups, the immunohistochemical study performed with M30 and caspase3 stainings resulted in 0–1 positive cell in five high-power fields. In the other groups, CLP and CLP + NAC, the number of M30 and caspase 3-positive cells was found to be significantly increased especially in the areas where dense neutrophil infiltration was present.

Because the macrophages also became M30 positive as the result of phagocytized apoptotic epithelial cells, the total number of M30-positive cells represent an indirect measurement of epithelial cell apoptosis. The mean number of M30-positive cells in the CLP group was higher than the sham-operated group (33.33 ± 3.72 vs. 0.57 ± 0.78; $P < 0.05$). In CLP + NAC group, however, the number of the M30 stained cells was reduced significantly indicating a lower rate of apoptosis (14.12 ± 1.96).

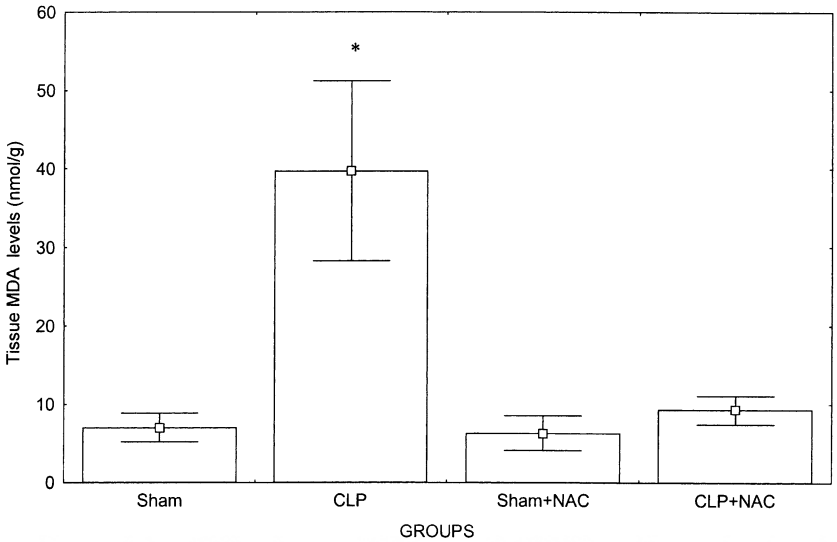


FIG. 2. **Lung MDA levels.** CLP resulted in increased lung MDA levels compared with sham-operated animals. The CLP-induced increase was reduced by NAC treatment. *$P < 0.05$ compared with other groups.

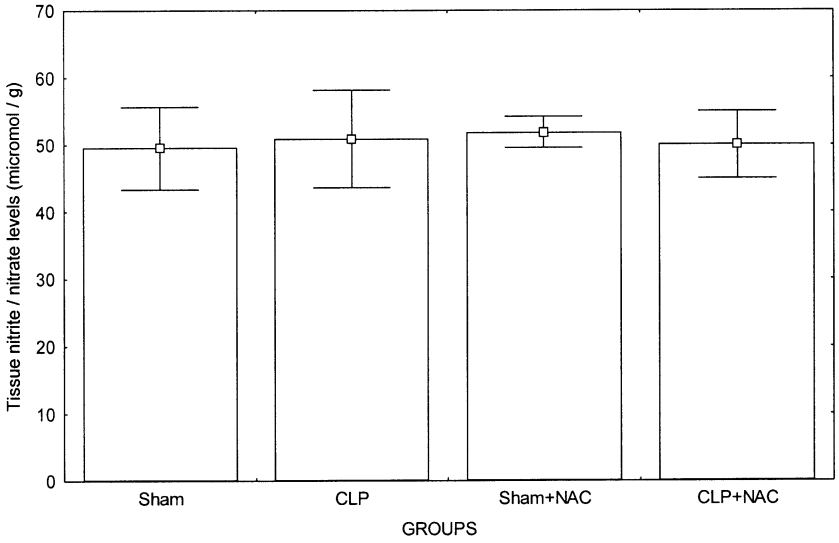


FIG. 3. **Lung nitrite/nitrate levels.** No differences have been observed between the groups. Data were expressed as mean ± SD.

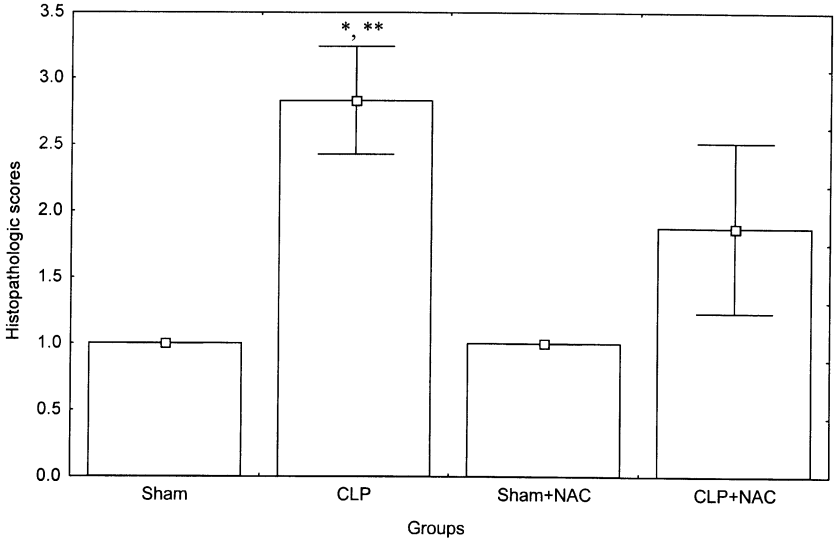


FIG. 4. **Histopathological scores of the lung tissue.** CLP resulted in increased lung histopathologic scores compared with sham-operated animals. The CLP-induced increase was reduced by NAC treatment. $^{*}P < 0.05$ compared with sham, $^{**}P < 0.05$ compared with CLP + NAC.

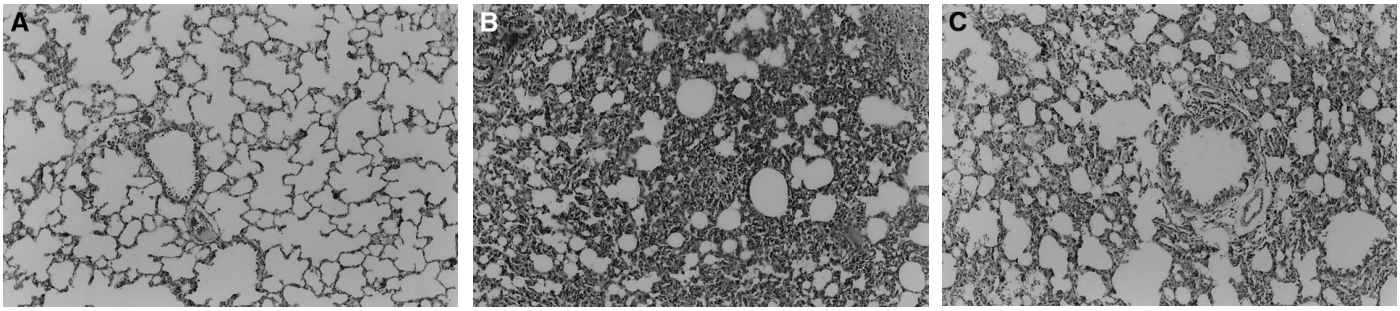


FIG. 5. **Photomicrographs of lung tissues (Hematoxylin-eosin ×200).** Normal pulmonary histology in sham group (A), severe interstitial infiltration of neutrophils and destroyed pulmonary architecture in the CLP group (B), and the protection of the normal pulmonary histology in CLP + NAC group (C).

Similar results were obtained regarding the caspase 3 staining. Although positive cells were higher in number in CLP group than sham group (19.63 ± 3.16 vs. 0.28 ± 0.48), they were found to be significantly reduced in CLP + NAC group (6.130 ± 1.64). The significant differences between the groups concerning the number of M30 and caspase 3 positive apoptotic cells are also clearly reflected by the bar graphics in Figures 6 and 7, respectively. The photomicrographic appearance of this difference is represented in Figure 8 for M30 and in Figure 9 for caspase 3 immunohistochemical stainings.

## DISCUSSION

In the present study, we demonstrated NAC to reduce the number of apoptotic cells in lung and to alleviate lung injury in a CLP-induced sepsis model. Exposure of rats to CLP

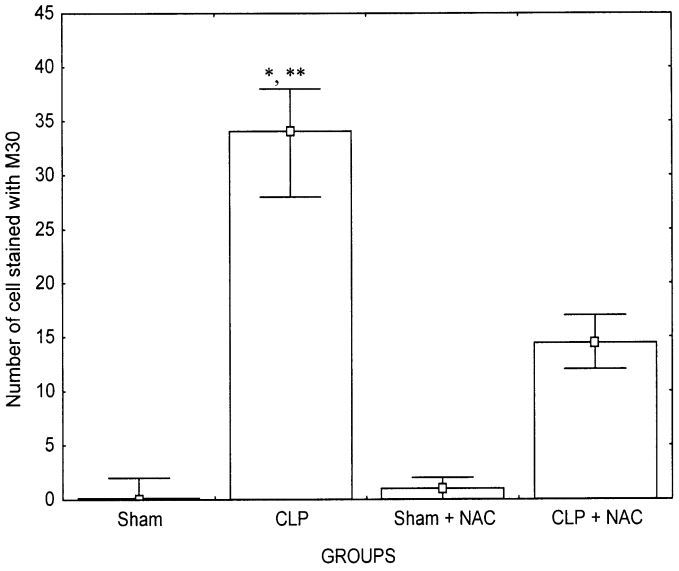


FIG. 6. **Number of positively stained cells with M30 cytodeath**. CLP resulted in increased number of positively stained cells with M30 compared with sham-operated animals. The CLP-induced increase was reduced by NAC treatment. *$P < 0.05$ compared with sham, **$P < 0.05$ compared with CLP + NAC.

increased MPO activity and MDA levels in lung tissue, indicating the infiltration of polymorphonuclear neutrophils and the development of oxidative lung injury. Histopathological and immunohistochemical assessment also confirmed that the model of CLP used here substantially injured lung tissue, which was associated not only with necrosis but with apoptosis as well.

NAC is a well-known precursor of glutathione (GSH) and was found to increase GSH levels in bronchoalveolar fluid (17). Clinically, NAC supplementation has been shown to reduce oxidative stress by improving the thiol redox status (18). Recently, Ortolani et al. documented an increase in GSH levels and prevention of lipid peroxidative damage in patients with septic shock (19). In concordance with previous findings, we found that chronic usage of NAC inhibited lipid peroxidation in CLP-induced sepsis model. The preservation of lung histology, which was documented with hematoxylin-eosin staining, also supports this finding. On the other hand, lipid peroxidation products and ROS/reactive nitrogen species (RNS) have been shown to elicit apoptosis (20, 21). They are known to be toxic to cells and are capable of inducing all of the morphologic and chromatin changes leading to apoptosis in lungs (22). This process may result in a decrease of functional lung units as seen in ARDS, which can contribute to severity of acute lung injury. In the present study, chronic NAC treatment significantly attenuated CLP-induced apoptosis, which was documented by M30 staining immunohistochemically.

We found MPO activity, one of the markers of neutrophil accumulation in lung tissue, elevated in CLP group. In some recent studies MPO was found to be increased in lungs in CLP-induced sepsis (23, 24). Another observation in this study was that NAC treatment attenuated this elevation and thus limiting the severity of lung injury. As a matter of fact, Cuzzocrea et al. showed the MPO activity to decrease with NAC treatment in the lung and ileum tissues, supporting our findings (23, 25). These results suggest that the inhibition of the increase of MPO activity may result in lesser generation of ROS/RNS so that lipid peroxidation is prevented and consequently the apoptotic pathway cannot be triggered. Thus, the lung barrier integrity may be protected distinctly by these two different mechanisms. The capability of NAC to inhibit the adhesion molecules may also have a role at the very initial step of both mechanisms (11).

We did not observe any significant differences among the groups regarding lung tissue nitrite/nitrate levels in this study. This may be the result of initial elevation of nitric oxide synthase activity during sepsis (26) and of production of nitric oxide in organs other than the lungs (27), especially the intestines (28). As we continued the experiment for a longer period after CLP, similar levels of nitrite/nitrate levels can be considered as a normal finding.

ROS/RNS, generated by neutrophils, activates nuclear factor (NF)-$\kappa$B, resulting in the excessive production of inflammatory cytokines, which may be the primary triggering agents for apoptosis in septic states. It was demonstrated experimentally

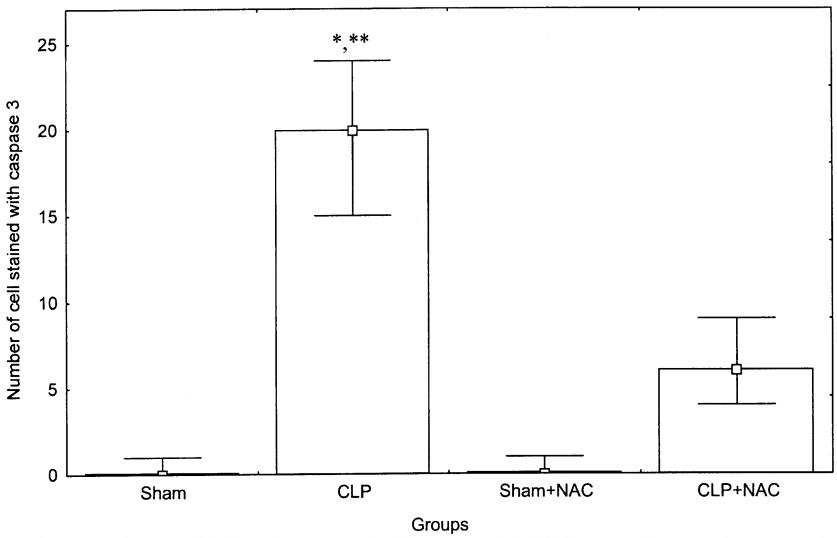


FIG. 7. **Number of positively stained cells with caspase 3.** CLP resulted in increased number of positively stained cells with caspase 3 compared with sham-operated animals. The CLP-induced increase was reduced by NAC treatment. *$P < 0.05$ compared with sham, **$P < 0.05$ compared with CLP + NAC.

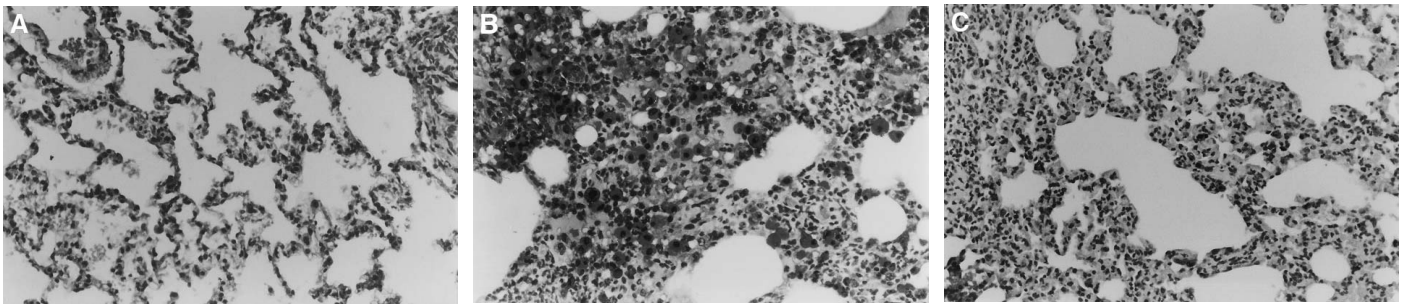


FIG. 8. **Representative immunohistochemical staining for apoptosis in lung tissues by M30 cytodeath (×200).** No staining is observed in sham group (A); clusters of M30-stained apoptotic cells and apoptotic bodies in the macrophages are seen in CLP group (B); there is a significant decrease in the number of apoptotic cells in CLP + NAC group (C).

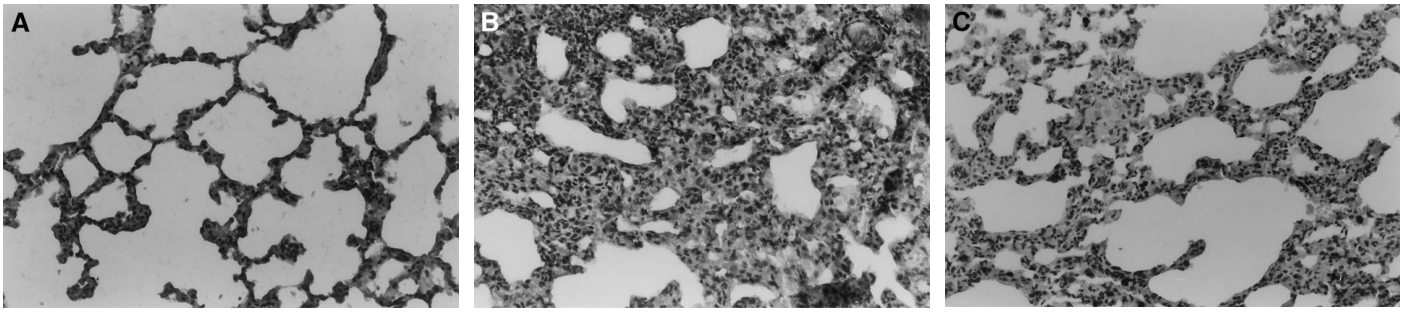


FIG. 9. **Representative immunohistochemical staining for apoptosis in lung tissues by caspase 3 (×200).** No staining is observed in sham group (A); caspase 3-stained apoptotic cells and apoptotic bodies are seen in CLP group (B); a relatively smaller number of apoptotic cells are observed in CLP + NAC group (C).

that endotoxemia caused the activation of NF-κB in lungs through ROS-dependent mechanisms (29). Clinically, NF-κB activation was also determined in patients with acute lung injury (30). Regarding the data related to inhibition of cytokine production and inhibition of NF-κB activation by NAC, our finding of attenuated lung injury after chronic use of NAC in CLP model may also be associated with prevention of NF-κB activation. In fact, the role of NF-κB in the regulation of cell viability is multidimensional because it has also been shown to be able to simultaneously trigger antiapoptotic mechanisms as well as upregulation of mitochondrial Bcl-2 (31).

Growing evidence suggests that cellular oxidative processes have a major role in inflammation also through the activation of stress kinases (c-Jun n-terminal kinase [JNK], mitogen-activated protein kinase, and p38), which are components of the intracellular signaling pathway (29). As a matter of fact, the increase in JNK activity has been documented to participate in the onset of apoptosis and thus inhibition of tyrosine kinase signaling may represent a novel therapeutic approach for improving organ functions and decreasing the death rate from subsequent sepsis (32, 33). It has been shown that NAC blocked this intracellular signaling pathway by the inhibition of the increase in JNK activity, caspase-3 cleavage, and subsequent apoptosis (34). However, NAC has been documented to block lipopolysaccharide-activated mitogen-activated protein kinases even in environments where NAC behaves as an antioxidant. This may be interpreted as a direct effect of NAC, which is independent from its antioxidant properties (35). In concordance with previous studies NAC inhibited apoptosis in lung in our study as well. This prevention of apoptosis by NAC may be not only caused by the regulation of NF-κB expression but also by blockage of intracellular stress-activated kinases.

As conclusion, chronic use of NAC inhibited MPO activity and lipid peroxidation, which resulted in the reduction of apoptosis in lung in this CLP model. We believe that chronic use of NAC deserves additional investigation for its possible antiapoptotic potential in septic states besides its documented antioxidant and antiinflammatory effects.

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