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Severe acidosis affects the anti-inflammatory properties of N-acetylcysteine on lipopolysaccharide-activated macrophages

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Abstract

Critical illness is exemplified by a state of profound disruption in physiological homeostatic mechanisms. Tissue acidosis is an hallmark of inflammation/ischemia and tumor processes and although it remains uncertain whether there is a true cause-effect relation between acidosis and adverse clinical outcomes it remains a powerful marker of poor prognosis in critically ill patients. Patients with severe sepsis and septic shock exhibit a complex metabolic pattern of acidosis at intensive care unit admission, caused predominantly by hyperchloremic acidosis, which was more pronounced in non survivors. Abnormalities in systemic acid-base balance may also induce significant alterations in the immune response. In this study we evaluated the effect of N-acetylcysteine (NAC), an important cellular antioxidant, during severe hyperchloremic acidosis, *in vitro*, in Lipopolysaccharide from *E.coli* (LPS)-treated J774A.1 murine/macrophage cell line. Our results show that NAC, in hyperchloremic acidosis conditions, reduces nitric oxide (NO) and reactive oxygen species (ROS) release and iNOS expression. Furthermore these effects are associated to alterations in J774A.1 macrophage cell cycle distribution but not to impairments of cell viability and apoptosis induction. Our data report a reduced inflammatory response exerted by NAC in condition of hyperchloremic acidosis indicating that the use of NAC during inflammation further impairs immune response associated to acidosis associated disease, as septic shock.

KEY WORDS: ACIDOSIS, MACROPHAGES, N-ACETYLCYSTEINE, LPS

Introduction

Systemic acidosis appears to be common in critically ill and injured patients. Although it remains uncertain whether there is a true cause-effect relation between acidosis and adverse clinical outcomes, acidosis is a powerful marker of poor prognosis in critically ill patients. This condition may be a result of increases in arterial PCO_2 (respiratory acidosis) or from a variety of organic or inorganic fixed acids (metabolic acidosis). Metabolic acidosis results from a variety of common etiologies [1], including lactic acidosis [2, 3], hyperchloremic acidosis [4, 5], renal failure [6], and ketones [7]. Although the underlying disease process associated with each of these subtypes carries its own clinical consequences, acidosis itself might potentially contribute to the adverse effects of these conditions. A potentially important consequence of acidosis is its effect on the immune response. Several studies have documented the effects of decreased extracellular pH on the synthesis and release of inflammatory mediators, especially as nitric oxide (NO) and TNF-α [8].

Patients with severe sepsis and septic shock exhibit a complex metabolic acidosis at intensive care units admission, caused predominantly by hyperchloremic acidosis, which was more pronounced in nonsurvivors patients [9]. Inflammatory response resulted associated to oxidative stress, commonly considered as an excessive reactive oxygen species (ROS) production due to an imbalance between pro- and anti-oxidant cellular mechanisms, also associated to various diseases. Because of the involvement of ROS in the ethiology of human diseases such as atherosclerosis, neurodegeneration, inflammation, diabetes, and aging, there is a growing interest in the use of antioxidant, as a protective strategy against these diseases by blocking or removing oxidative stresses. Thus, a large number of chemicals (naturally occurring as well as synthetic), foreign to mammalian cells have been accredited as agents aimed to reduce ROS release and inflammation [10, 11, 12]. Sepsis occurs as a result of complex host-pathogen interactions, leading to release of inflammatory mediators, as well as ROS and reactive nitrogen intermediates (RNS), as NO. Monocyte/macrophages and neutrophils are the sentinel phagocytic cells primarily responsible for engulfment and destruction of pathogens organisms during infection of the host. ROS and RNS are antimicrobial agents produced by these cells that can directly destroy microbial pathogens. During sepsis, excessive production of ROS and RNS can be detrimental, inducing significant cytotoxicity to organs and contributing to the sequelae of unresolved sepsis, multiorgan system failure [13].

Moreover ROS and RNS are known to directly induce cytotoxicity to organs and can also alter cell signaling pathways [14]. Lipopolysaccharide (LPS), a component of the membrane of Gram-negative bacteria, is one of the most potent activators of macrophages [15]. LPS activation of macrophages results in a wide range of responses, including secretion of growth factors and proinflammatory mediators, expression of adhesion molecules and coagulation factors, phagocytosis, and cytoskeletal rearrangement [16]. Antioxidants can protect both extracellularly by scavenging toxic ROS and intracellularly by interrupting lipid peroxidation within the membrane. Antioxidant agents can also interfere early in inflammatory responses by blocking or modifying the signal transduction of inflammatory cytokines [17, 18]. N-Acetylcysteine (NAC) is an important cellular antioxidant that decreases inflammation in various diseases [19, 20].

The aim of this study is to evaluate the anti-inflammatory activity of NAC in vitro on LPS-stimulated J774A.1 murine/macrophages in condition of hyperchloremic acidosis.

Methods

Reagents

Unless stated otherwise, all reagents and compounds were purchased from Sigma Chemicals Company (Sigma, Milan, Italy). The N-acetyl-L-cysteine powder, stored at 2 ° -8 ° C, soluble in

water, was dissolved just before use in cell culture medium and used at concentrations of 5-10 - 15 mM. The Lipopolisaccharide from *Escherichia coli* (LPS), in lyophilized powder was diluted in the medium of treatment to obtain the concentration of 1 mg / ml. The sterile hydrochloric acid in 1.0 N solution was diluted to obtain the concentrations of 17.1 mM and 2.5 mM corresponding to values of pH 6.5 and 7.4 as previously reported [8].

Cell culture

J774A.1 murine monocyte macrophage cell line (American Type Culture Collection, Rockville, MD), was grown in adherent to Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mM HEPES, 2 mM glutamine, 100 u/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

Nitrite determination

Macrophages J774A.1 were seeded in 24-well $3x10^5$/well and allowed to adhere for 24h. After they were pretreated with medium at two different pH (pH 6.5 e pH 7.4) for 1h. After the medium was replaced with fresh medium and cells were stimulated with NAC (15-10-5 mM) alone or in combination with LPS (1µg/ml) for further 24 h.

NO production was measured as nitrite (NO_2^-, µM), index of NO released by cells, in the culture medium 24 h after LPS stimulation, as previously reported [21]. NO_2^- amounts were measured by Griess reaction. Briefly, 100 µL of cell culture medium were mixed with 100 µL of Griess reagent – equal volumes of 1% (w:v) sulphanilamide in 5% (v:v) phosphoric acid and 0.1% (w:v) naphtylethylenediamine-HCl and incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm in a microplate reader Titertek (Dasit, Cornaredo, Milan, Italy). The amount of NO_2^-, as µM concentration, in the samples was calculated using a sodium nitrite standard curve.

Western blot analysis for iNOS

iNOS protein expression was assessed by Western blot analysis as previously reported [21]. Macrophages J774A.1 were seeded in p60 plated (1.8×10^6/P60) and allowed to adhere for 24h. After pretreatment with medium at two different pH (pH 6.5 e pH 7.4). After the medium was replaced with fresh medium and cells were stimulated with NAC (5-10-15 mM) alone or in combination with LPS (1µg/ml) for further 24 h.

After nitrite determination cells were scraped off, washed with ice-cold phosphate-buffered saline (PBS), and centrifuged at 5.000 g for 10 min at 4°C. The cell pellet was lysed in a buffer containing 20 mM Tris hydrogen chloride (HCl; pH 7.5), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 mM sodium fluoride, 150 mM sodium chloride, 10 mg/ml trypsin inhibitor, and 1% Tween-20.

Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (50 µg) were dissolved in Laemmli's sample buffer, boiled, and run on a SDS polyacrylamide gel electrophoresis (SDS-PAGE) minigel (8% polyacrylamide) and then transferred to hybond polyvinylidene difluoride membrane for 40 min. at 5 mA cm2 into 0.45 µm.

Membranes were blocked for 40 min in PBS and 5% (w/v) nonfat milk and subsequently probed overnight at 4°C with mouse monoclonal anti-iNOS (BD Laboratories), or anti-tubulin (Santa Cruz Biotechnologies) in PBS, 5% w/v non fat milk, and 0.1% Tween-20.

Blots were then incubated with horseradish peroxidase conjugated goat antimouse immunoglobulin (Ig)G (1:5.000) for 1h at room temperature. Immunoreactive bands were visualized using electro-chemiluminescence assay (ECL) detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS on XOmat films were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD U.S.A.). Data are normalized with tubulin expression, used as reference protein, and expressed as arbitrary densitometric units as previously reported [22].

Measurement of intracellular reactive oxygen species (ROS)

ROS formation was evaluated by means of the probe 2',7'-dichlorofluorescin-diacetate (H_2DCF-DA) by modifying a previously reported method [23]. H_2DCF-DA is a non-fluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby traps it within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, J774A.1 cells were plated at a density of $7x10^5$cells/well into 24-well plates. Cells were allowed to grow for 24h; thereafter, the medium was replaced with fresh medium and cells were exposed to the medium at the different pH (pH 6.5 e pH 7.4) for 1 h. After the medium was replaced with fresh medium and cells were stimulated with NAC (15-10-5 mM) alone or in combination with LPS (1µg/ml) for further 24 h.

Cells were then collected, washed twice with phosphate buffer saline (PBS) and then incubated in PBS containing H_2DCF-DA (10µM) at 37°C. After 45 minutes, cells fluorescence was evaluated using a fluorescence-activated cell sorting (FACSscan; Becton Dickinson) and elaborated with Cell Quest software. Data are expressed as mean fluorescence intensity.

Antiproliferative activity

J774A.1 cells ($5x10^4$/well) were plated on 96-well plates and allowed to adhere for 24 h. Thereafter, the medium was replaced with of fresh medium and of serial dilutions of NAC (15-10-5 mM) was added for 24 h at various pH. Cell viability was assessed through MTT assay as previously reported [24, 25]. Briefly, 25 µL of MTT (5 mg/mL) were added and cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 µL of a solution containing 50% (v:v) N,Ndimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. Macrophage viability in response to treatment with NAC, was calculated as:

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% dead cells= 100 x [(OD treated/OD control)
                    x100]
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Cell viability analysis using trypan blue

Because of the probable interference with the colorimetric MTT assay [26], we also used the method of direct count by exclusion with Trypan Blue for the calculation of the cell viability of the macrophages J774A.1, with NAC (5-10-15 mM) alone or in combination with LPS (1µg/ml) for 24h at two different pH, 6.5 and 7.4.

The Trypan blue exclusion test of cell viability is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not.

A cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

J774A.1 macrophages were detached and an aliquot of cell suspension was centrifuged for testing the vitality 5 min at 100 xg and the supernatant was discarded. The aliquot should contain a convenient number of cells to count in a hemacytometer suspended in 1 ml PBS and then diluted again by mixing with 0.4% trypan blue (e.g., 5 × 105 cells/ml). Cell pellet was resuspend in 1 ml PBS. One part of 0.4% trypan blue was mixed with 1 part cell suspension (dilution of cells) and mixture was incubated at room temperature. Apply a drop of the trypan blue/cell mixture was applyed to a hemacytometer. The hemacytometer was placed on the stage of a binocular microscope and focus on the cells. The unstained (viable) and stained (nonviable) cells was counted separately in the hemacytometer. Cell viability was calculated as:

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% Of viability = (number of live cells / total
          number of cells) x 100.
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Analysis of apoptosis

Hypodiploid DNA was analyzed using PI staining by flow cytometry as previously reported [27, 28]. Briefly, J774A.1 (7 x 10^5) cells were grown in 24-well dishes and allowed to adhere. Thereafter the medium was replaced with fresh medium and cells were exposed to medium at two different pH (pH 6.5 e pH 7.4) for 1 h. After the medium was replaced with fresh medium and cells were incubated with NAC (5-10-15 mM) alone or in combination with LPS (1µg/ml) for further 24 h. Following treatment, culture medium was removed, cells washed once with PBS and then resuspended in 500 µL of a solution containing 0.1% (w/v) sodium citrate, 0.1% Triton X-100 and 50 µg/mL propidium iodide (PI). Culture medium and PBS were centrifuged and cell pellets were pooled with cell suspension to retain all dead and living cells for analysis. After incubation at 4° C for 30 min in the dark, cell nuclei were analyzed with Becton Dickinson FACScan flow cytometer using CellQuest program and the DNA content of the nuclei was registered on a logarithmic scale. Cellular debris was excluded from the analysis by raising the forward scatter threshold and the percentage of cells in the hypodiploid region (sub G_o/G_1) was calculated.

Analysis of cell cycle

The distribution of cells in different phases of the cell cycle was assessed by flow cytometry as previously reported [29]. The assay involves the permeabilization and the treatment of the cells with a fluorochrome, propidium iodide (PI) and the subsequent analysis via software in the percentage of cells in the various phases of the cell cycle. In order to determine the effect on cell cycle we tested graded concentration of NAC (5-10-15 mM). J774A.1 cells were seeded in a 12-well plastic plate at 1.5x10^6cells/well and allowed to adhere. Thereafter cells were exposed to medium at two different pH (pH 6.5 e pH 7.4) for 1 h and then were treated with NAC (5-10-15 mM) for 24h. After incubation, J774A.1 were harvested and fixed in cold 70% ethanol at –20°C. Cell cycle profiles were evaluated by DNA staining with PI (2.5 mg/ml) in phosphate-buffered saline (PBS) supplemented with 100 U/mL ribonucleases A, for 30 min at room temperature. Samples were analysed with a FACScan flow cytometer (Becton Dickinson, CA) using Mod FitLT program.

Data analysis

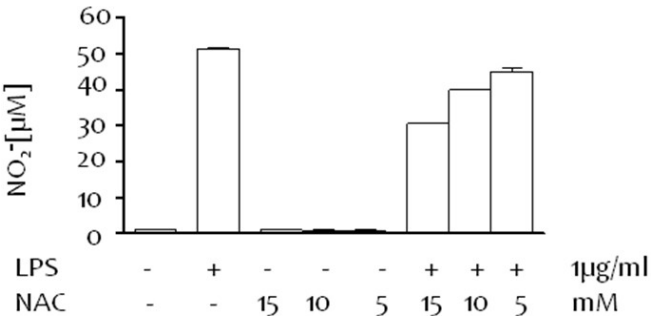
Data are reported as mean±standard error mean (s.e.m.); values of at least three independent experiments. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test. A ***P***-value less than 0.05 was considered significant.

Results

In conditions of hyperchloremic acidosis, NAC reduces NO release, iNOS expression from LPS-stimulated macrophage

To asses if NAC influences NO production we measured nitrite release, a stable end-product of NO, in cellular medium of the murine macrophages cell line J774A.1 incubated with NAC (5-10-15 mM) alone or in combination with LPS (1ìg/ml) for 24h. In macrophages incubated with the two different pH medium was observed an increase of nitrites production after stimulation with LPS for 24 h. NAC alone at different concentrations tested (05/10/15 mM) did not induced any change on the nitrite production, (Fig. 1, Panel A and B). In cells incubated with culture medium at pH 7.4 (Fig. 1, Panel A) NAC induced a significant inhibition in NO release, which was further inhibited in acidosis conditions (P <0.001 vs LPS; P <0.001 vs pH 7.4).

A



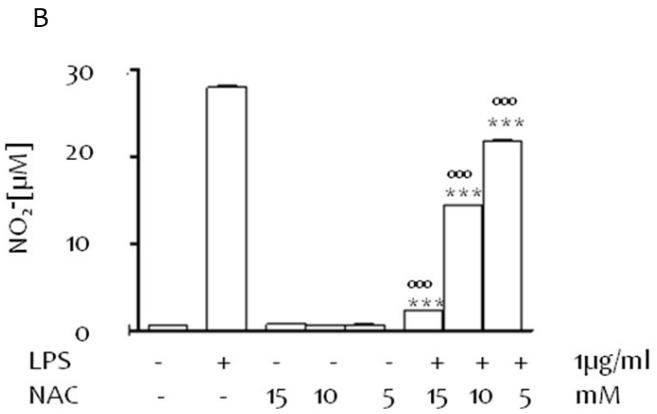
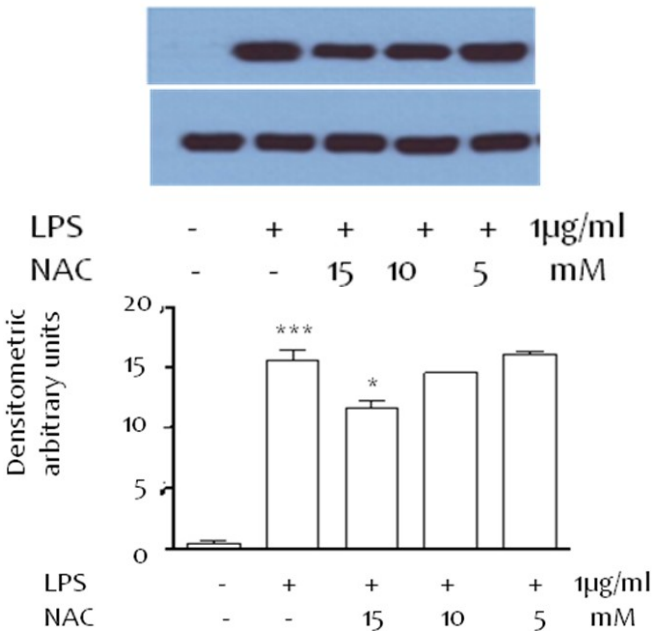


Fig. 1 Effect of NAC (5-15 mM) on nitrite production of in the medium of J774A.1 macrophages co-incubated for 24 h with LPS (1 μg / mL), in the two conditions of pH tested, pH 7.4 (Panel A) and pH 6.5 (Panel B). The results obtained are expressed as mean ± SEM of 3 independent experiments. *** P<0.001 vs LPS; °°° P<0.001 vs pH 7.4.

Under the same experimental conditions we also observed a significant induction in iNOS expression in macrophage treated with LPS alone (P<0.001 vs control; Fig. 2, Panel A). When NAC (5-10-15 mM) was added to J774A.1 macrophages simultaneously with LPS, at pH 7.4 and 6.5, NAC exerted inhibitory effect iNOS expression only at the highest concentration, but in hyperchloremic conditions, NAC (15 mM) exerted a stronger inhibition an INOS respect to pH 7.4 (P<0.05 a pH 7.4; P<0.001 a pH 6.5; Fig. 2, Panel B).



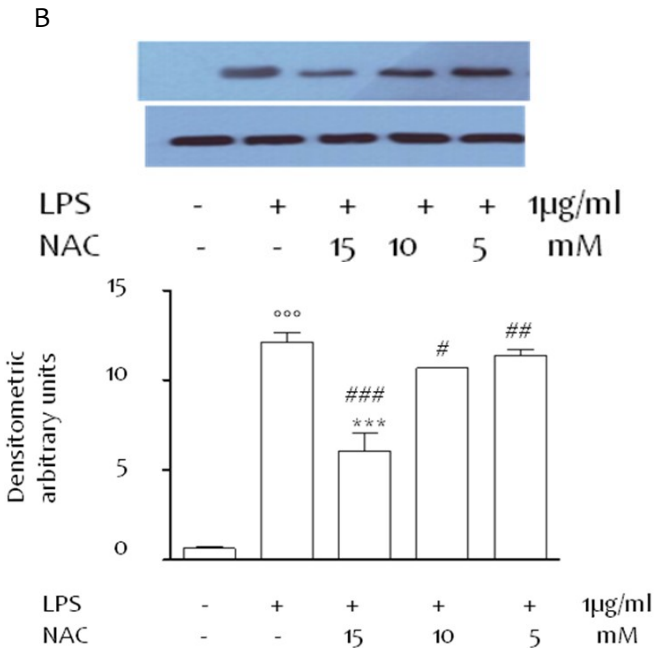
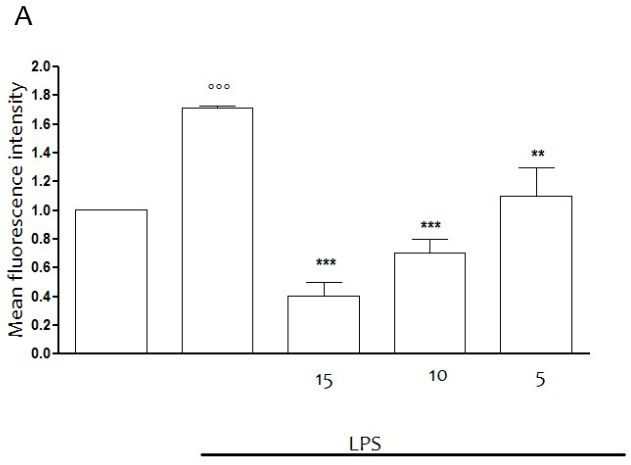


Fig. 2 *Effect of NAC (5-15 mM) on the expression of iNOS in macrophages J774A.1 co-incubated with LPS (1 μg / mL) for 24 h in the two conditions of pH tested, pH 7.4 (Panel A) and pH 6.5 (Panel B). The results obtained are expressed as mean ± SEM of 3 independent experiments.* °°° *P <0.001 vs CON, * P <0.05, *** P <0.001 vs LPS;# P<0.05, ## P<0.01, ### P<0.001 vs pH 7.4.*

NAC enhances ROS release from macrophages

To investigate whether NAC mediates oxidative stress in J774A.1 macrophages at different pH, we evaluated its effect on intracellular ROS by flow cytometry. Kinetic studies showed an increased production of ROS in the presence of LPS alone in both conditions of pH of medium. The co-incubation with NAC and LPS reduced the intracellular ROS levels only at pH 7.4 and the effect appeared to be concentration-dependent.



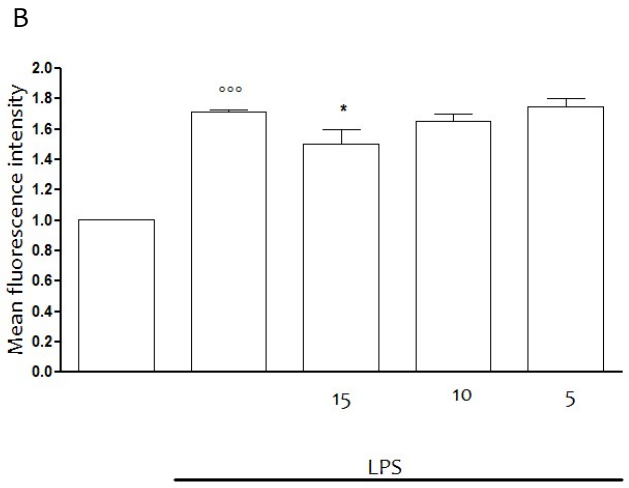


Fig.3 Effect dell'NAC (5, 10 and 15 mM) on intracellular ROS production in conditions of co-incubation with LPS (1 µg / mL) for 24 h in the two conditions of pH tested, pH 7.4 (Panel A) and pH 6.5 (Panel B) shown by the histograms flow cytometry of the fluorescence of DCFH-DA.

NAC doesn't affect macrophage viability

To elucidate the influence of NAC, in our experimental conditions, on viability in J774A.1 macrophages, cells were treated with NAC (5-10-15mM) for 24h. Our data, both by MTT assay and trypan blue counting, indicated that J774A.1 macrophage viability was not affected by NAC treatment (data not shown).

NAC did not induce apoptosis in J774A.1 cells

In order to confirm the absence of antiproliferative effects on the part of NAC and exclude the possible involvement of apoptotic pathways, cytofluorimetric analysis was assessed incubating J774A.1 with graded concentration of NAC (15-10-5 mM), alone alone and associated with LPS (1 µg/mL) for 24h. Apoptosis was measured by cytofluorimetric analysis of PI staining of hypodiploid nuclei. Our results indicate that the treatment of macrophages J774A.1 with the NAC at concentrations of 5-10-15 mM, alone and associated with LPS at a concentration of 1 g / mL, for 24 h did not result in any significant variation in the percentage of hypodiploid nuclei detected regardless the pH of the incubation medium. (pH 6.5 pH 7.4; Figure 4, Panel A and *B).*

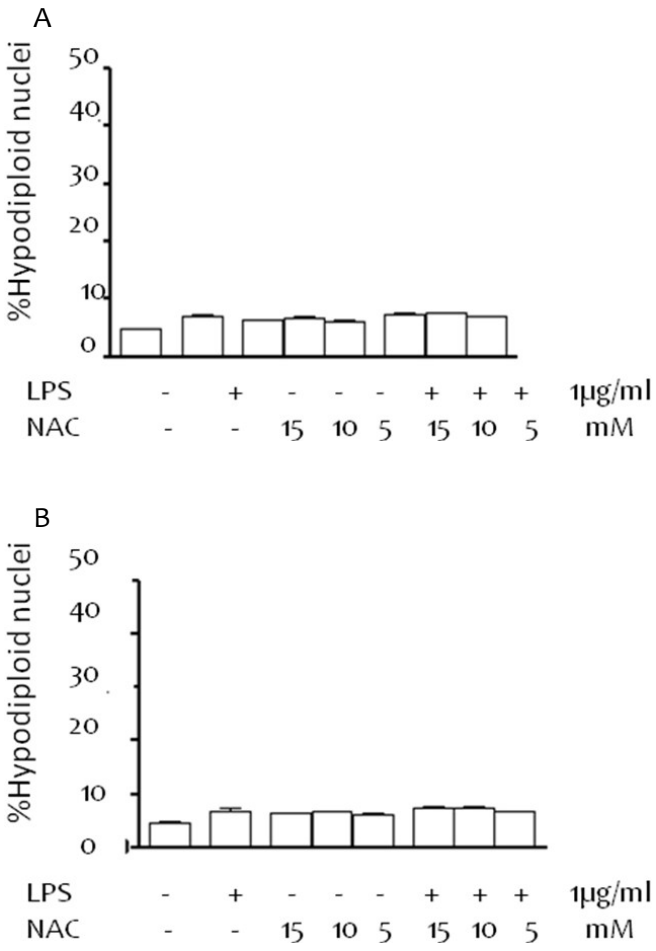


Fig.4 *Proapoptotic activity of NAC (15-10-5 mM) alone and combined with LPS (1µg/mL), in the two conditions of pH tested, pH 7.4 (Panel A) and pH 6.5 (Panel B) on J774.A1 macrophages. The results obtained are expressed as mean ± SEM of 3 independent experiments and are expressed as a percentage of hypodiploid nuclei.*

NAC impairs cell cycle distribution

In response to DNA damage, resulting from apoptotic pathway activation, the cell cycle checkpoints (G_1, S, G_2/M) are activated and cell cycle progression is arrested to allow time for repair.

In order to determine if NAC could affect cell cycle cells distribution we carried out a cell cycle analysis. Incubation of macrophages with NAC at concentrations of 5-10-15 mM for 24 h in medium at pH 7.4 did not alter the distribution percentage of cells in different phases of the cell cycle (Fig. 5, Panel A). On the contrary NAC treatment in medium at pH 6.5 induced a reduction in the percentage of cells in the G0/G1 phase that appeared significant

only at the highest concentration tested (P <0.01 vs CON) and an increase of cells in S-phase that was significant at the concentrations of 10 mM (P <0.05 vs. CON) and 15 mM (P<0.01 vs CON; Fig. 5, Panel B) .

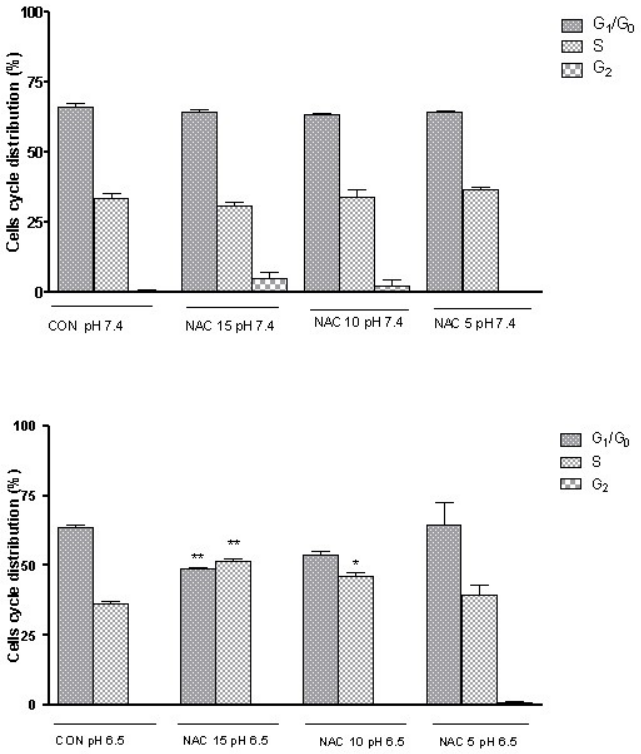


Fig.5 Effect of NAC (15-10-5 mM) on cell cycle distribution at the two tested pH, pH 7.4 (upper panel) and pH 6.5 (lower panel) on J774.A1 macrophages. Results are expressed as mean ± SEM of 3 independent experiments.

Discussion

Sepsis is the most common cause of mortality in intensive care units and its incidence is increasing despite advances in healthcare and science [30]. Marked oxidative stress is a result of the inflammatory responses associated with sepsis and may result in organ damage. Normally, a complex system of interacting antioxidant defences is able to balance oxidative stress and prevents damage. Despite the accepted role that oxidative stress-mediated injury plays in the development of organ failure, there is still little conclusive evidence of any beneficial effect of systemic antioxidant supplementation in patients with sepsis and organ dysfunction [31]. LPS activates macrophages and modifies the oxidative intracellular redox state. Depletion of the important intracellular antioxidant GSH exerts an inflammatory effect. NAC increases cellular GSH levels and may have an anti-inflammatory effect. Song et al. [32] showed an increase of GSH levels and an inhibition of LPS-induced lipid peroxidation by the action of NAC. In the present study we investigated NAC anti-inflammatory properties in LPS- stimulated J774A.1 macrophages, in condition of hyperchloraemic acidosis, which also occurs during sepsis.

LPS enhances NO formation, following the induction of iNOS, that has been implicated in the pathogenesis of shock and inflammation. Therefore, LPS has been extensively used to reproduce oxidative and inflammatory conditions in *in vitro* and *in vivo* models [33-36]. Our results indicate that the antioxidant compound NAC in condition of hyperchloraemic acidosis downregulates inflammatory response reducing NO release and iNOS expression respect to ph 7.4. Furthermore NAC, in condition of hyperchloraemic acidosis didn't inhibits ROS release but impairs macrophages cell cycle distribution, without inducing apoptosis or affecting cell viability.

NO is a pleiotropic mediator that acts in a variety of physiological and pathophysiological processes. This molecule is produced from the oxidation of L-arginine by NOS enzyme, which occurs in two major classes: one is constitutive (including endothelial and neuronal isoforms), and another is the inducible including macrophagic isoform [37-39]. The iNOS may be expressed in different cell types (e.g. macrophages, smooth muscle cells, epithelia) by various proinflammatory agents such as LPS. NO can be considered an immune modulator owing to its complex activity during host cellular defence. When macrophages are activated by LPS or by IFN-γ, iNOS is significantly expressed, and massive amounts of NO are produced to exert a nonspecific immune response. Induced NO, in addition to being a final common mediator of inflammation, is essential for the up-regulation of the inflammatory response. Furthermore, NO contributes to tissue damage both directly via the formation of peroxyni-

trite, with its associated toxicity, and indirectly through the amplification of the inflammatory response [38, 39]. In our experiments, LPS induced in J774A.1 macrophages a marked increase in NO release associated to an increased iNOS expression; NAC significantly reduced NO release and iNOS expression mostly in acidosis condition respect to physiological pH. Our results regarding the reduced iNOS activity and expression are in according with previous studies reporting the effect of hyperchloremic acidosis in RAW264.7 cells stimulated with LPS [8, 41].

ROS, as well as NO, generation in the inflammatory site is typically induced as part of defensive reaction intended to clear infectious and environmental threats, including microbial agents and particulate material. Alternatively, ROS activation could acts as a significant and adverse participant in abnormal inflammatory disease. In our experimental model observed that LPS-induced ROS release in macrophages was the same at both tested pH. NAC reduced ROS release only at ph 7.4 resulting unable to affect ROS release in hyperchloremic condition. Our results are in according with previous studies demonstrating the effect of NAC in decreasing macrophage ROS release in physiological conditions, this may be explained by the ability of NAC to restore GSH [42]. In our experiments both at pH 6.5 and at pH 7.4, with or without LPS, NAC induced no significant change in macrophages cell viability and not triggers apoptotic pathway, as demonstrated by the evaluation of hypodiploid nuclei, indicative of apoptosis. However, cell cycle analysis showed that the treatment of macrophages with NAC the medium at pH 6.5, corresponding to the condition of hyperchloremic acidosis, change the percentage distribution of cells in the various phases of the cell cycle. Infact, we observed a significant reduction in the percentage of cells in the G0/G1 phase to the highest concentration tested and a cell number increase in the S phase. The G1 phase is the point of the interval between the M phase, which occurs during the mitosis and cytokinesis, and during the S phase occurs DNA replication, the phase Go is a state of "quiescence" temporary or permanent brings out the cells from the cell cycle. The variations described are not evident incubating cells with NAC with a pH 7.4 cellular medium, thus in physiological conditions. Thus NAC at high concentrations and in states of hyperchloremic acidosis promotes in macrophages the processes of DNA synthesis and cell division. The modulation of the macrophage functions during inflammation by NAC it has been previously reported [41, 42]. NAC ability in reducing inflammatory response can be considered beneficial in presence of an excessive macrophage response, not proportionate to the nature of the stimulus, but on the other side in some conditions can assumes a negative value as indicated by our results. Considering that in disorders associated to hyperchloraemic acidosis, as sepsis, there is a reduction of macrophages immune response the use of NAC could be useful to further reduce macrophage function and thus worsen the patient's clinical conditions.

Acknowledgments

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