

Original Contribution

Full survival of paraquat-exposed rats after treatment with sodium salicylate[☆]

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Abstract

Over the past decades, there have been numerous fatalities resulting from accidental or voluntary ingestion of the widely used herbicide paraquat dichloride (methyl viologen; PQ). Considering that the main target organ for PQ toxicity is the lung and involves the production of reactive oxygen and nitrogen species, inflammation, disseminated intravascular coagulation, and activation of transcriptional regulatory mechanisms, it may be hypothesized that an antidote against PQ poisonings should counteract all these effects. For this purpose, sodium salicylate (NaSAL) may constitute an adequate therapeutic drug, due to its ability to modulate inflammatory signaling systems and to prevent oxidative stress. To test this hypothesis, NaSAL (200 mg/kg ip) was injected in rats 2 h after exposure to a toxic dose of PQ (25 mg/kg, ip). NaSAL treatment caused a significant reduction in PQ-induced oxidative stress, platelet activation, and nuclear factor (NF)- κ B activation in lung. In addition, histopathological lesions induced by PQ in lung were strongly attenuated and the oxidant-induced increases of glutathione peroxidase and catalase expression became absent. These effects were associated with a full survival of the PQ-treated rats (extended for more than 30 days) in comparison with 100% of mortality by Day 6 in animals exposed only to PQ, suggesting that NaSAL constitutes an important and valuable therapeutic drug to be used against PQ-induced toxicity. Indeed, NaSAL constitutes the first compound with such degree of success (100% survival).

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Keywords: Oxidative stress; NF- κ B; Inflammation; Sodium salicylate; Survival

Introduction

Paraquat dichloride (methyl viologen; PQ) is an effective and widely used herbicide, which has a proven safety record when

appropriately applied to eliminate weeds. However, over the past decades, there have been numerous fatalities mainly caused by accidental or voluntary ingestion [1]. The main target organ for PQ toxicity is the lung as a consequence of its accumulation, against a concentration gradient, through the highly developed polyamine uptake system, and due to its capacity to generate redox cycle [2–4] (Fig. 1). Death occurs mostly as a consequence of alveolar epithelial cells (type I and II pneumocytes) and bronchiolar Clara cell disruption, hemorrhage, edema, hypoxemia, infiltration of inflammatory cells into the interstitial and alveolar spaces, proliferation of fibroblasts and excessive collagen deposition [4], and as a consequence of a disseminated intravascular coagulation [5]. Importantly, platelet sequestration has been shown to occur in the lungs of patients with acute respiratory distress syndrome (ARDS) [6] and studies have demonstrated platelet effects on membrane permeability, pulmonary hypertension, activation of neutrophils, endothelial cells, and fibroblasts [7]. Nowadays, no

Abbreviations: ARDS, acute respiratory distress syndrome; AUC, area under curve; CAT, catalase; DNPH, 2,4-dinitrophenylhydrazine; fEMSA, fluorescence electrophoretic mobility shift assay; GPx, glutathione peroxidase; H₂O₂, hydrogen peroxide; HO[•], hydroxyl radical; HOCl, hypochlorous acid; Hyp, hydroxyproline; I κ B, inhibitor κ B; LM, light microscopy; LPO, lipid peroxidation; MDA, malondialdehyde; MPO, myeloperoxidase; NaSAL, sodium salicylate; NF- κ B, nuclear factor kappa-B; PMN, polymorphonuclear leukocytes; PQ, paraquat; ROS, reactive oxygen species; SAL, salicylate; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; TEM, transmission electron microscopy; TMB, 3,3',5,5'-tetramethylbenzidine; TNF- α , tumor necrosis factor alpha.

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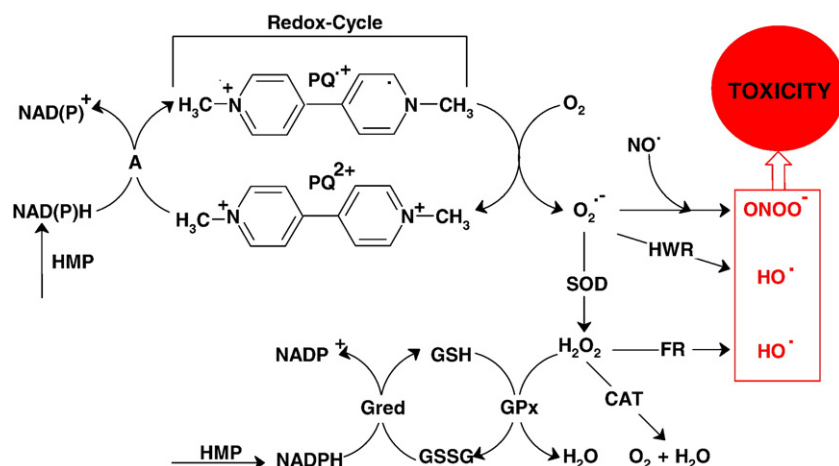


Fig. 1. Schematic representation of the mechanism of paraquat toxicity. A, cellular diaphorases; SOD, superoxide dismutase or spontaneously; CAT, catalase; GPx, glutathione peroxidase; Gred, glutathione reductase; PQ^{2+} , paraquat; $PQ^{\bullet+}$, paraquat cation radical; HMP, hexose monophosphate pathway; FR, Fenton reaction; HWR, Haber-Weiss reaction.

antidote or effective treatment for PQ poisoning has been clinically applied, the survival being mainly dependent on the amount ingested and the time elapsed until the patient is submitted to intensive medical procedures [8].

Despite several studies concerning PQ-induced lung toxicity, few studies focus on the transcriptional regulatory mechanisms responsible for PQ toxicity and the importance of the modulation of these mechanisms in the treatment of PQ poisoning. Nuclear factor (NF)- κ B has been regarded as a key element in the response of cells to inflammatory stimuli. NF- κ B activity is attributed to the Rel/NF- κ B family proteins forming homo- and heterodimers through a combination of the subunits p65 (or RelA), p50, p52, c-Rel, and RelB. In most cells, NF- κ B (the designation for p50-p65, the most frequent heterodimer) is retained in the cytoplasm as an inactive complex bound to inhibitory proteins [I κ B; for revision see [9]]. LPS, IL-1 β , tumor necrosis factor (TNF)- α , UV light, reactive oxygen species (ROS), and double-stranded RNA are classical inducers of NF- κ B. When I κ B α is degraded, NF- κ B migrates to the nucleus, where it binds to the κ B sites in the promoter region of target genes and regulates the transcription of proinflammatory enzymes, cytokines, chemokines, apoptosis inhibitors, cell adhesion molecules, the I κ B α gene, and many others.

Taking into account the above-noted rationale, it may be hypothesized that an antidote against PQ poisonings should have excellent antioxidant, anti-inflammatory (involving inhibition of NF- κ B activation, and antithrombogenic effects. In that sense, salicylate (SAL) and its derivatives seem to be adequate candidates for the task. SAL is a well-known scavenger of ROS [10] and inhibitor of platelet aggregation [11]. Inhibition of the NF- κ B pathway by SAL has also been shown by Kopp and collaborators [12] and several other subsequent studies by impeding I κ B phosphorylation [13,14]. In the present work, the role of oxidative stress, platelet aggregation, NF- κ B activation, and fibrosis in PQ-induced lung toxicity, as well as the remarkable healing effects obtained by the administration of sodium salicylate (NaSAL), is described.

Importantly, taking into account the arrival time of poisoned patients to hospital emergencies, NaSAL was administered 2 h after PQ exposure, conferring more realism to our study. The obtained results exceeded our best expectations since not only the toxicity was reverted but, most significantly, full survival of the PQ-intoxicated rats treated with NaSAL was noted. It may be postulated that NaSAL is the first real PQ antidote described with such degree of success.

Materials and methods

Chemicals and drugs

Paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride), NaSAL (2-hydroxybenzoic acid sodium salt), 3,3',5,5'-tetramethylbenzidine (TMB), 5-sulfosalicylic acid, reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), hydrogen peroxide (H_2O_2), glutathione reductase, 2,4-dinitrophenylhydrazine (DNPH), *trans*-4-hydroxy-L-proline, chloramine-T hydrate, and 4-(dimethylamino)benzaldehyde were all obtained from Sigma (St. Louis, MO). The saline solution (NaCl 0.9%) and sodium thiopental were obtained from B. Braun (Lisbon, Portugal). 2-Thiobarbituric acid (TBA; $C_4H_4N_2O_2S$), trichloroacetic acid (TCA; Cl_3CCOOH), and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). All the reagents used were of analytical grade or from the highest available grade. The following synthetic oligonucleotides, purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), were used: 5'-Cy5-GCC TGG GAA AGT CCC CTC AAC T-3' (NF- κ B-FW-Cy5), 5'-GCC TGG GAA AGT CCC CTC AAC T-3' (NF- κ B-FW), 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (NF- κ B-R), 5'-CGC TTG ATG ACT CAG CCG GAA-3' (AP-1-FW), and 5'-TTC CGG CTG AGT CAT CAA CGC-3' (AP-1-R). Cy5 (indodicarbocyanine) is a fluorescence dye attached at the 5' OH end of the oligonucleotide. Antibodies against p50 and p65 NF- κ B subunits were obtained from Santa Cruz Biotechnology, Inc.

Animals

A total of 84 rats were included in the study; 44 and 16 animals were used for biochemical and histological studies, respectively, and the remaining (24) for survival rate evaluation. Male Wistar rats (aged 8 weeks) were obtained from Charles River S.A. (Barcelona, Spain), with a mean weight of 252 ± 25 g. Animals were kept under standard laboratory conditions (12/12 h light/darkness, $22 \pm 2^\circ\text{C}$ room temperature, 50–60% humidity) for at least 1 week (quarantine) before starting the experiments. Animals were allowed access to tap water and rat chow ad libitum during the quarantine period. Animal experiments were licensed by Portuguese General Directorate of Veterinary Medicine (DGV). Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR 1996). The experiments complied with the current laws of Portugal.

Experimental protocol for biochemical and histological studies

The biochemical and histological studies were carried out with 60 animals randomly, distributed to 10 groups. Each animal was individually housed during the experimental period in a polypropylene cage with a stainless-steel net at the top and wood chips at the screen bottom. Tap water and rat chow were given ad libitum during the entire experiment. Treatments in all groups were always conducted between 8:00 and 10:00 AM.

Each group was treated as follows (for a schematic view, see Fig. 2). (i) Control group, $n = 6$: animals administered with 0.9% NaCl. Animals were administered with one more administration of 0.9% NaCl 2 h later and sacrificed at 24 h after the second injection. (ii) NaSAL group, $n = 18$: animals administered with 0.9% NaCl. Animals were treated with one administration of NaSAL (200 mg/kg) 2 h later and sacrificed at

24 h ($n = 6$, NaSAL 24 h group), 48 h ($n = 6$, NaSAL 48 h group), and 96 h ($n = 6$, NaSAL 96 h group) after the second injection. (iii) PQ group, $n = 18$: animals intoxicated with PQ (25 mg/kg). Animals were administered with one more administration of 0.9% NaCl 2 h later and sacrificed at 24 h ($n = 6$, PQ 24 h group), 48 h ($n = 6$, PQ 48 h group), and 96 h ($n = 6$, PQ 96 h group) after the second injection. (iv) PQ + NaSAL group, $n = 18$: animals intoxicated with PQ (25 mg/kg). Two hours later, animals were treated with NaSAL (200 mg/kg) and sacrificed at 24 h ($n = 6$, PQ + NaSAL 24 h group), 48 h ($n = 6$, PQ + NaSAL 48 h group), and 96 h ($n = 6$, PQ + NaSAL 96 h group) after the second injection.

The administrations of vehicle (0.9% NaCl), PQ, and NaSAL were all made intraperitoneally (ip) in an injection volume of 1 ml. The experimental dose of NaSAL was chosen in such a way that it covers all the desired effects described above, namely that required to inhibit the NF- κ B activation in vivo [13,15]. The PQ administered dose is known to produce severe lung toxicity and death in rats within a few days [16,17].

Surgical procedures

Before sacrifice, anesthesia was induced with sodium thiopental (60 mg/kg, ip). In four rats of each group (biochemical analysis), lungs were perfused in situ through the inferior vena cava with cold 0.9% NaCl for 3 min at a rate of 10 ml/min to remove most trapped blood volume. In the remaining two animals (structural and ultrastructural analysis), lung fixation was initiated in situ by perfusion with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2–7.4) for 3 min at a rate of 10 ml/min. Simultaneous to the perfusion initiation, a cut of the common iliac artery was done to avoid cardiovascular volume overload.

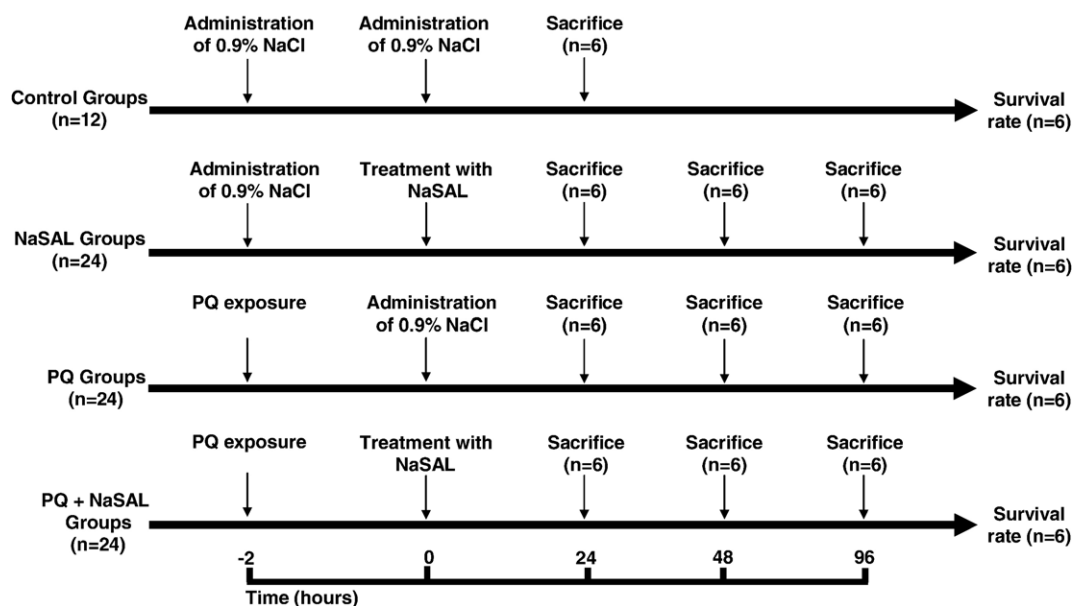


Fig. 2. Schematic representation of the administration protocols for the control, sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ + NaSAL) groups.

Tissue processing for biochemical analysis

Lungs were removed, cleaned of all major cartilaginous tissues of the conducting airways, pat-dried with gauze, weighed [for determination of the relative lung weight (RLW) of each animal], and processed as follows: (i) Right lungs (except the posterior lobe) were homogenized (1:4 m/v, Ultra-Turrax homogenizer) in ice-cold 50 mM phosphate buffer with 0.1% (v/v) Triton X-100, pH 7.4. The homogenate was kept on ice and then centrifuged at 3000g, 4°C, for 10 min. Aliquots of the resulting supernatants were stored (–80°C) for posterior quantification of myeloperoxidase (MPO), catalase (CAT), and glutathione peroxidase (GPx) activity, carbonyl groups, hydroxyproline (Hyp) content, and PQ and protein concentration. The posterior lobe was homogenized (1:4 m/v, Ultra-Turrax homogenizer) in TCA 10% and then centrifuged (13,000g, 4°C, for 10 min). Aliquots of the resulting supernatants were immediately used to measure the degree of lipid peroxidation (LPO). The pellet was used for protein quantification. (ii) Left lungs were used for preparation of nuclear extracts. Briefly, left lungs were homogenized (Ultra-Turrax homogenizer) in a AC buffer [(cell lysis buffer), 1 g of tissue/3 ml] containing 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.2% Igepal, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.25 mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 15 min. After a brief vortexing, the lysates were centrifuged (850g, 4°C for 10 min). The supernatants (cytoplasmic extracts) were discharged and the pellets were resuspended (washing step) in 500 µl of AC buffer and incubated for 15 min on ice and then centrifuged (14,000g, 4°C, for 30 s). The supernatants (cytoplasmic extracts) were discharged and the pellets were resuspended in 500 µl of BC buffer (nuclei lysis buffer) containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 2% Igepal, 0.5 mM EDTA, 20% glycerol, 1 mM DTT, 0.25 mM PMSF, aprotinin (5 µg/ml), pepstatin (5 µg/ml), leupeptin (5 µg/ml) and incubated on ice for 30 min. After a brief vortexing, the lysates were centrifuged (14,000g, 4°C for 10 min). Supernatants (nuclear extracts) were collected, divided into aliquots, and stored at –80°C for future NF-κB semiquantification by fluorescent electrophoretic mobility shift assay (fEMSA). The protein concentration of the extracts was also determined.

Relative lung weight

The RLW of each animal was calculated as a percentage of the absolute body weight at sacrifice.

Quantification of paraquat in rat lung

The lung PQ quantification was performed as previously described [3,16,17].

Tissue processing for structural and ultrastructural analysis

Lung samples were subjected to routine procedures for light microscopy (LM) and transmission electron microscopy (TEM)

analysis as previously described [16,17]. Histopathological evidence of acute lung damage was semiquantified according to a previously described procedure [16,17]. For each group, more than 1000 cells per slide and 100 cells per grid were analyzed in a blind fashion in order to semiquantify the severity and incidence of the following parameters: (i) tissue disorganization, (ii) inflammatory reaction, (iii) necrotic zones, and (iv) interstitial fibrosis. The severity of tissue disorganization was scored according to the percentage of the affected tissue: score 0 = normal structure; score 1 = less than one-third of tissue; score 2 = greater than one-third and less than two-thirds; score 3 = greater than two-thirds of tissue. The severity of inflammatory reaction was scored as follows: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1 to 3 cells by visual field); grade 2 = moderate infiltration (4 to 6 leukocytes by visual field); and grade 3 = heavy infiltration by neutrophils. The severity of necrosis was scored as follows: grade 0 = no necrosis; grade 1 = dispersed necrotic foci; grade 2 = confluence necrotic areas; grade 3 = massive necrosis. The interstitial fibrosis was scored from 0 (normal lung) to 8 (total fibrosis) according to the following criteria: grade 0 = normal lung; grade 1 = minimal fibrous thickening of alveolar or bronchial walls; grades 2–3 = moderate thickening of walls without obvious damage of lung architecture; grades 4–5 = increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass; grades 6–7 = severe distortion of structure and large fibrous areas; “honeycomb lung” was placed in this category; grade 8 = total fibrotic obliteration of the field.

Protein quantification

Protein quantification was performed according to the method of Lowry et al. [18], using bovine serum albumin as standard.

Measurement of toxicity biomarkers

LPO was evaluated by the thiobarbituric acid-reactive substance (TBARS) methodology [19]. Results are expressed as nanomole of malondialdehyde (MDA) equivalents per milligram of protein using an extinction coefficient (ϵ) of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Protein carbonyl groups (ketones and aldehydes) were determined according to Levine et al. [20]. Results are expressed as nanomole of DNPH incorporated per milligram of protein ($\epsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

MPO activity was measured accordingly to the method described before [16,17]. One enzyme unit (U) was defined as the amount of enzyme capable to reduce 1 µl of H₂O₂/min under the assayed conditions. Results are expressed in enzyme unit per gram of protein ($\epsilon = 3.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

CAT activity was measured according to the method of Aebi [21]. Results are expressed in enzyme unit per gram of protein ($\epsilon = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$).

GPx activity was measured according to the method of Flohé and Gunzler [22]. One enzyme unit is equal to

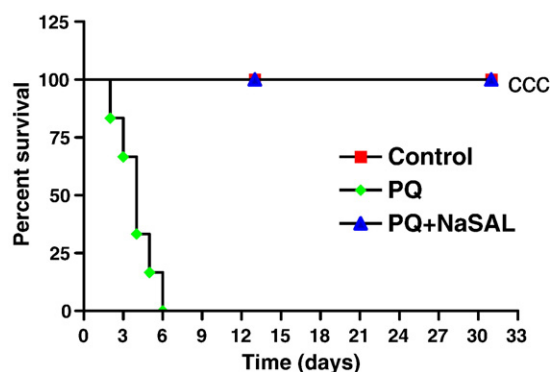


Fig. 3. Percentage of rat surviving in the control, paraquat (PQ), and paraquat plus sodium salicylate (PQ + NaSAL) groups. $^{ccc}P < 0.001$ versus PQ group.

millimole of NADPH oxidized per minute per milligram of protein. Results are expressed in enzyme unit per gram of protein ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

Hydroxyproline quantification was performed accordingly to the method of Reddy and Enwemeka [23], using *trans*-4-hydroxy-L-proline as standard. Total lung collagen content, as an index of the development of fibrosis, was calculated with the assumption that 12.5% of collagen is constituted by hydroxyproline [24]. Results are expressed as milligram of collagen per gram of total protein.

Oligonucleotides and DNA annealing

Oligonucleotides were dissolved in purified water to a final concentration of 0.1 mM prior to use. To generate the double-stranded fluorescent target and the equimolar, amounts of the two complementary single-stranded oligonucleotides (NF- κ B-FW-Cy5 or NF- κ B-FW with NF- κ B-R and AP-1-FW with AP-1-R) were mixed in the annealing buffer (10 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, and 0.5 M NaCl) at final concentrations of 0.05 mM, heated for 2 min at 95°C (denaturing), incubated for 1 h at 37°C (annealing), and then cooled at 4°C.

Determination of transcriptional activation of lung nuclear proteins by fluorescent electrophoretic mobility shift assay

The NF- κ B-binding assay was performed according to a previously reported method [25]. Nuclear extracts (20 μ g of protein) were incubated (1 h at 4°C) in a fresh polypropylene tube with the following mixture: 0.5 pmol of specific double-stranded Cy5-labeled for each transcription factor, DNA-binding buffer [10 mM Hepes (pH 7.9), 0.2 mM EDTA, 50 mM KCl], 2.5 mM DTT, 250 ng of poly(dI-dC) · poly(dI-dC), 1% of Igelal, and 10% glycerol. Nine microliters of the mixture was resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel at 10°C, 800 V, 50 mA, and 30 W for 3 h in 1X TBE (90 mM Tris borate, 2 mM EDTA, pH 8.3) using an ALF-Express DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). The temperature was regulated by an external thermostat ALFexpress II Cooler system (Amersham Pharmacia Biotech).

Specificity of the DNA–protein complexes was confirmed by the addition of a 50-fold excess of either unlabeled specific

competitor (SC, specific probe without the Cy5 label) or unlabeled nonspecific competitor (UC, which was the result of the annealing of AP-1-FW with AP-1-R oligonucleotides). For supershift assays, antibodies against different NF- κ B subunits p50 and p65 were used. Reactions were identical to gel-shift reaction conditions except that for supershift assays the cells extracts were preincubated with the specific antibody (2 μ g) on ice for 15 min before the specific probe was added to the mixtures. Signals were analyzed by an ALFwin 1.03 fragment analyser (Amersham Pharmacia Biotech) and presented as arbitrary units corresponding to area under curve (AUC).

Experimental protocol for the evaluation of survival rate

For the evaluation of survival rate (for a schematic view, see Fig. 2), 24 animals were randomly divided into four groups (control, NaSAL, PQ, and PQ + NaSAL) of six animals each. Animals were kept under the same conditions and treated as described above. Abnormal findings, including weakness and dyspnea, were noted and recorded if present. The lethality was registered every day until Day 30. Rats were weighed daily during the entire study.

Statistical analysis

Results are expressed as mean \pm SE. Statistical comparison between groups was estimated using the Kruskal-Wallis nonparametric method followed by Dunn's test. Comparison of the survival curves was performed using the logrank test. In all cases, *P* values lower than 0.05 were considered as statistically significant.

Results

Survival rate and macroscopic examination

Rats exposed only to PQ (PQ group) displayed approximately 25 and 100% of mortality by the second and sixth day, respectively (Fig. 3). The lethal time (LT)₅₀ for the PQ group was approximately 96 h. Posttreatment of PQ-exposed rats with NaSAL (PQ + NaSAL group) resulted in an enhancement of the

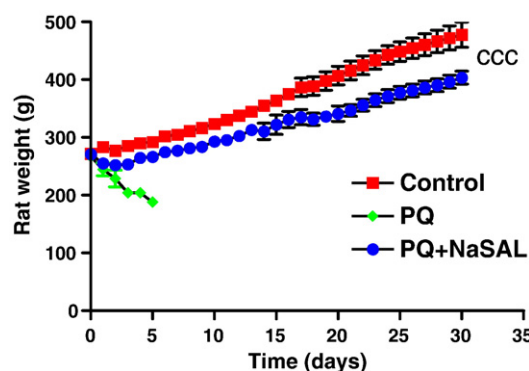


Fig. 4. Rat's body weight for the control, paraquat (PQ), and paraquat plus sodium salicylate (PQ + NaSAL) groups. $^{ccc}P < 0.001$ versus PQ group.

Table 1
Relative lung weight (RLW), PQ levels, and toxicological parameters in the control, sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ + NaSAL) groups

Animal group	Sacrifice time	RLW	TBARS (nmol MDA/mg protein)	Carbonyl groups (nmol/mg protein)	GPx (U/mg protein)	CAT (U/mg protein)	MPO (U/g protein)	Collagen (μg/mg protein)	PQ levels (μg/mg protein)
Control	24 h	0.37 ± 0.01	0.20 ± 0.02	1.81 ± 0.08	69.27 ± 0.89	2.52 ± 0.056	22.76 ± 1.90	9.12 ± 1.54	
NaSAL	24 h	0.36 ± 0.02	0.20 ± 0.01	1.75 ± 0.08	66.34 ± 1.34	2.50 ± 0.10	18.07 ± 2.03	8.96 ± 0.95	
	48 h	0.37 ± 0.02	0.19 ± 0.01	1.76 ± 0.07	68.45 ± 2.43	2.50 ± 0.11	19.95 ± 1.11	8.90 ± 2.06	
	96 h	0.37 ± 0.01	0.21 ± 0.02	1.79 ± 0.05	67.27 ± 2.02	2.46 ± 0.07	19.80 ± 1.86	8.15 ± 1.89	
PQ	24 h	0.44 ± 0.02 ^{a,b}	0.47 ± 0.02 ^{aaa,bbb}	2.28 ± 0.12 ^{a,b}	73.96 ± 2.79 ^{a,bb}	4.50 ± 0.08 ^{aa,bb}	30.20 ± 1.72 ^{a,bb}	9.64 ± 0.54	0.134 ± 0.103
	48 h	0.49 ± 0.03 ^{a,b}	0.40 ± 0.03 ^{aaa,bbb}	2.37 ± 0.10 ^{aa,bb}	75.16 ± 3.45 ^{a,bb}	3.96 ± 0.13 ^{aa,bb}	33.75 ± 2.15 ^{aa,bb}	9.98 ± 1.09	0.061 ± 0.021
	96 h	0.55 ± 0.04 ^{aa,bb}	0.42 ± 0.02 ^{aaa,bbb}	2.50 ± 0.14 ^{aa,bb}	70.65 ± 3.03	3.65 ± 0.08 ^{aa,bb}	25.23 ± 2.50 ^b	11.33 ± 1.12	0.024 ± 0.008
PQ + NaSAL	24 h	0.37 ± 0.03 ^c	0.32 ± 0.02 ^{a,b,c}	1.99 ± 0.09 ^c	68.96 ± 2.79 ^c	2.72 ± 0.17 ^{cc}	24.68 ± 2.50 ^c	9.32 ± 1.04	0.137 ± 0.098
	48 h	0.38 ± 0.02 ^c	0.24 ± 0.03 ^{ccc}	1.91 ± 0.08 ^{cc}	69.32 ± 2.02 ^c	2.53 ± 0.08 ^{cc}	26.45 ± 1.88 ^c	9.45 ± 1.34	0.057 ± 0.013
	96 h	0.36 ± 0.02 ^{cc}	0.22 ± 0.02 ^{ccc}	1.84 ± 0.06 ^{ccc}	70.56 ± 2.88	2.32 ± 0.05 ^{cc}	23.26 ± 1.76	9.60 ± 1.73	0.020 ± 0.003

CAT, catalase; MDA, malondialdehyde; GPx, glutathione peroxidase; MPO, myeloperoxidase; NaSAL, sodium salicylate; PQ, paraquat; TBARS, thiobarbituric acid-reactive substances.

Values are given as mean ± SE ($n = 4$). ^a $P < 0.05$, ^{aa} $P < 0.01$, and ^{aaa} $P < 0.001$ versus control group, ^b $P < 0.05$, ^{bb} $P < 0.01$ and ^{bbb} $P < 0.001$ versus NaSAL group, ^c $P < 0.05$, ^{cc} $P < 0.01$ and ^{ccc} $P < 0.001$ versus PQ group.

survival time to 100%, 30 days post-PQ injection. These animals were sacrificed 30 days post-PQ injection and by that time no signs of toxicity were visible. Concerning body weight (Fig. 4), at the beginning of the study, there were no significant differences among the groups. Significant weight loss occurred in rats of the PQ group and in the PQ + NaSAL group in the first 24 h. Weight stabilized in the PQ + NaSAL group between 24 and 48 h and then increased in a similar rate relative to control and NaSAL groups, during the whole study time. No significant differences were observed in the survival time and body weight between the control and the NaSAL groups (in order to simplify only the control results are presented).

Diarrhea, piloerection, weight loss, anorexia, adipisia, hyperpnea, dyspnea, tachycardia, and a red drainage around the mouth, eyes, and nose were present especially in animals exposed only to PQ during the first 48–96 h. During the same experimental period, it was observed that rats belonging to the PQ group only ingested a few milliliters of water per day (8 ± 6 ml) in comparison with 24 ± 10 ml in the PQ + NaSAL and 36 ± 12 ml in the control group. Of note, it was observed that rats of the PQ + NaSAL 48 h group began to ingest similar or an even higher (in some cases) amounts of water compared to the control group.

Relative lung weight

RLW was assessed as an indication of the edema degree (Table 1). No differences were obtained in RLW values among control, NaSAL, and PQ + NaSAL groups. However, in comparison to these groups, animals from the PQ group showed a significant RLW increase at 24, 48, and 96 h after PQ exposure ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively).

Lung PQ concentrations

The PQ lung concentrations in the PQ and PQ + NaSAL groups are described in Table 1. Animals posttreated with NaSAL did not evidence any significant difference concerning to PQ lung accumulation relatively to the PQ-only exposed group.

Structural and ultrastructural analysis

Major qualitative structural and ultrastructural alterations are depicted in Fig. 5. Results of semiquantitative analysis of the PQ and PQ + NaSAL groups are presented in Table 2.

Animals from the control group presented a normal pulmonary structure at LM, without evidences of alveolar

Fig. 5. Light (A) and electron (B) micrographs from animals of control and NaSAL group, respectively, showing a normal pulmonary structure without evidence of alveolar collapse, vascular congestion, or cellular infiltrations. Light (C) and electron (D) micrographs from animals injected only with paraquat (PQ 24 h group). Suggestive of stasis, it is observed in C an intense vascular congestion with compact and angular erythrocytes (*), and a marked atelectasis (#); in C it is also possible to identify in the alveolar space several phagocytes (pink arrow) and cellular debris (red arrow). In D, beyond endothelial cells with mitochondrial swelling (green arrows), a capillary filled with angular erythrocytes (*) and numerous activated platelets (*), suggestive of an activation of the blood coagulation system, is also observed. Light (E) and electron (F) micrographs from animals sacrificed, respectively, 48 and 96 h after PQ exposure. In E necrotic zones (black arrows) of alveolar walls as well as cellular debris (red arrows) and phagocytes (pink arrow) inside alveolar space are visible. It can also be observed several capillaries filled with angular erythrocytes (*), suggestive of vascular stasis. In F are depicted numerous polymorphonuclear within capillaries and in the interstitial space (yellow arrows); extended interstitial areas filled with collagen fibers and fibroblasts (orange arrows) and evidences of interstitial edema (#) are also shown; cellular debris (red arrows) and phagocyte cells (pink arrow) are also observed in the narrowed alveolar space as well as several angular erythrocytes within capillaries (*). Light (G) and electron (H) micrographs from animals of PQ + NaSAL 96 h group showing in G necrotic areas (black arrows) and debris (red arrows) and phagocytes cells (pink arrow) within alveolar space. Noteworthy, is the preserved pulmonary structure with several cells containing cytoplasmic inclusions and the absence of vascular congestion. In H it is possible to observe cellular debris (red arrows) and a phagocyte within the alveolar space (pink arrow); the presence of edema (#) and collagen fibers (orange arrows) in the interstitial space, and mitochondrial swelling affecting pneumocytes and endothelial cells (green arrows) are also noteworthy.

collapse or cellular infiltrations (Fig. 5A). The TEM evaluation showed an ordinary alveolar wall, without any evidences of edema or cellular infiltration; the pneumocytes and endothelial cells revealed a preserved ultrastructure (score 0). Animals from the NaSAL group also showed normal pulmonary structure at 24, 48, and 96 h after NaSAL administration [score 0 (Fig. 5B)]. On the other hand, PQ

administration induced marked alterations compared to the control pulmonary pattern, mainly characterized by a diffuse alveoli collapse with an increased thickness of its walls. The foremost alterations observed in the animals of the PQ 24 h group include intense vascular congestion with activated platelets and numerous polymorphonuclear cells inside the capillaries, apparently adherent to endothelial cells (Figs. 5C

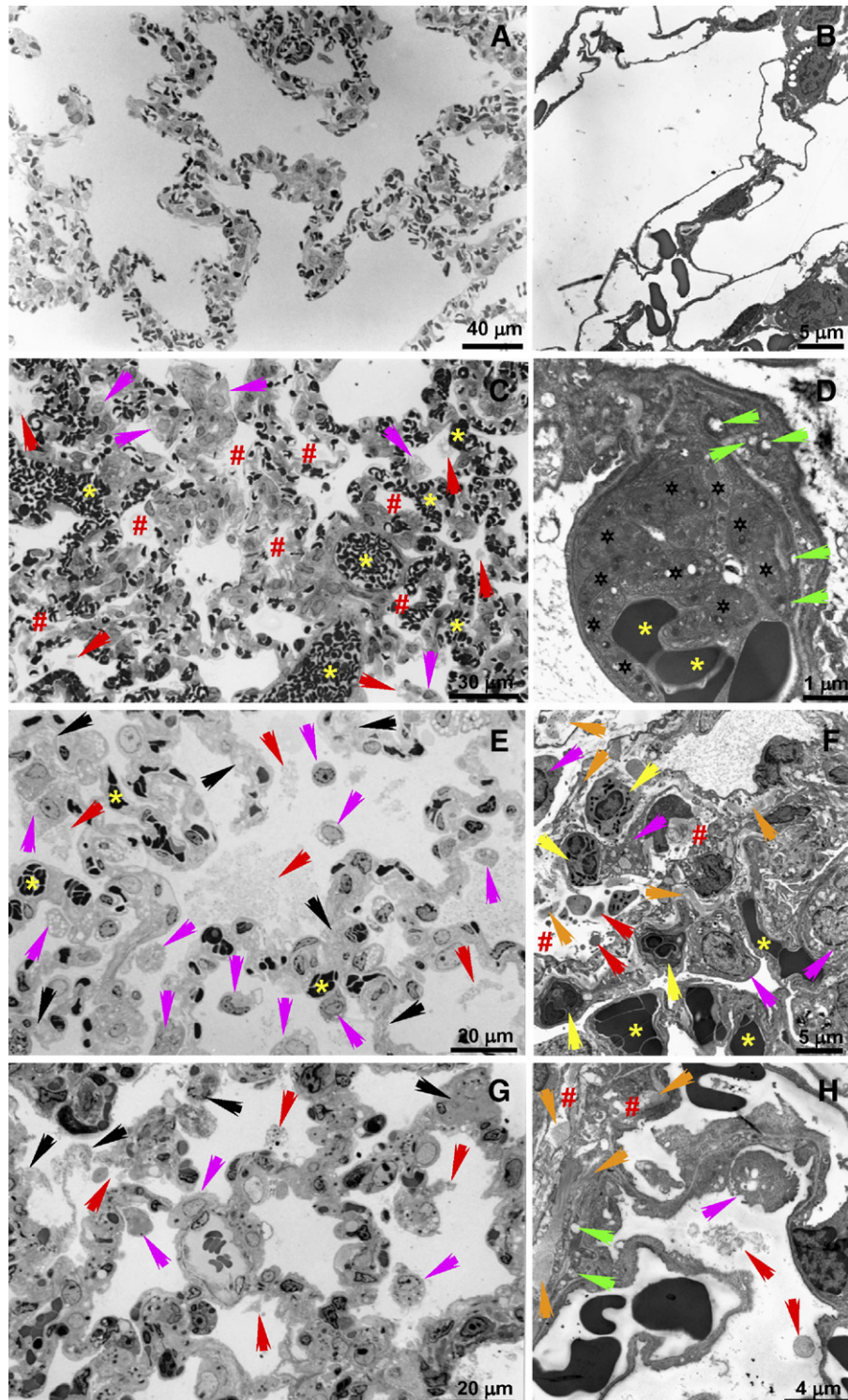


Table 2

Semiquantitative analysis of the morphological injury parameters of the paraquat (PQ) and paraquat plus sodium salicylate (PQ + NaSAL) groups

	PQ			PQ + NaSAL		
	24 h	48 h	96 h	24 h	48 h	96 h
Tissue disorganization	2.1 ± 0.23 ^{a,b}	2.3 ± 0.21 ^{a,b}	2.6 ± 0.16 ^{a,b}	2.0 ± 0.21 ^{a,b}	1.8 ± 0.25 ^{a,b}	1.4 ± 0.16 ^{a,b,c}
Inflammatory reaction	2.0 ± 0.26 ^{a,b}	2.2 ± 0.20 ^{a,b}	2.1 ± 0.18 ^{a,b}	2.1 ± 0.23 ^{a,b}	2.3 ± 0.26 ^{a,b}	1.9 ± 0.20 ^{a,b}
Necrotic zones	1.2 ± 0.20	1.7 ± 0.33 ^{a,b}	2.6 ± 0.16 ^a	1.3 ± 0.21	1.6 ± 0.22 ^{a,b}	1.5 ± 0.17 ^{a,b,c}
Interstitial fibrosis	0.2 ± 0.13	0.7 ± 0.21	2.9 ± 0.3 ^{a,b}	0.5 ± 0.22	1.1 ± 0.23	1.8 ± 0.27 ^{a,b,c}

Values are given as mean ± SE ($n = 2$). ^a $P < 0.05$ versus control group, ^b $P < 0.05$ versus NaSAL group, ^c $P < 0.05$ versus PQ group.

and 5D). Animals also revealed an interstitial edema, indicated by the existence of intercellular vacuolization areas that were characterized by a minor density ultrastructure at TEM. The majority of pneumocytes and endothelial cells showed, at least, one ultrastructural abnormality, with mitochondrial swelling as the most frequent alteration (Fig. 5D). These histopathological alterations became more exuberant at 48 and 96 h after PQ exposure, with the necrotic areas, the fibroblast activation, and extent of interstitial areas occupied by collagen fibers being particularly notorious (Figs. 5E and 5F). Noteworthy, in the PQ + NaSAL 24, 48, and 96 h groups, compared to the PQ-only exposed animals, the occurrence of the above referred alterations was drastically attenuated, particularly the vascular congestion (Figs. 5G and 5H).

NF-κB

EMSA was performed to study the effects of PQ exposure in the activation of rat lung NF-κB. As shown in Fig. 6, PQ induced a significant and time-dependent activation of NF-κB in rat lungs (Lanes 2–4) compared to control (Lane 1) and NaSAL groups (Lanes 8–10). Noteworthy was also the significant reduction of NF-κB lung activation in the NaSAL 24 h group relative to control. Concerning the PQ + NaSAL 24, 48, and 96 groups (Lanes 11, 12, and 13, respectively), NaSAL treatment resulted in a significant reduction of PQ-induced NF-κB activation, the AUC of band 1 being near to that of the control group. The specificity of the DNA–protein complex was confirmed in the PQ 96 h group by maintenance of the bands in the competition experiment with a 50-fold molar excess of the UC (Lane 6) and by its disappearance in the competition experiment with a 50-fold molar excess of the SC (Lane 7). Supershift analysis of the lung samples of rats from the PQ 96 h group, using p50 and p65 antibodies, confirmed the specificity of NF-κB bands (data not shown). The antibody against subunit p65 was able to shift DNA/protein interaction present in band 1. The antibody against subunit p50 shifted band 2 and induced a partial decrease in band 1. Taken together, these results indicated that p50/p65 heterodimers and p50/p50 homodimers corresponded to complexes/bands 1 and 2, respectively. It is interesting to observe that most of the transcriptional activity altered by PQ administration is mediated through the complex p50/p65, which is considered the most frequent active form of NF-κB.

Glutathione peroxidase and catalase activities

PQ produced a significant increase in the GPx and CAT activities in lung tissue when compared with control and NaSAL groups (Table 1). Treatment with NaSAL attenuated the PQ-induced increase of these enzymes activities.

Lipid peroxidation and carbonyl group content

As shown in Table 1, animals from the PQ group exhibited a significant rise of MDA concentration in lung at 24, 48, and 96 h post-PQ exposure ($P < 0.001$), compared with animals from control and NaSAL groups. However, as can be observed in animals from the PQ + NaSAL group, NaSAL led to a significant reduction in lung MDA equivalents compared to animals from the PQ group in every sampled time. Of note, the increase of LPO in the PQ + NaSAL 24 h group was also significantly higher in comparison to control or the NaSAL 24 h group ($P < 0.05$). Analogous results were also obtained for carbonyl group levels (Table 1). In accordance with the results of LPO, NaSAL treatment prevented the increase of protein carbonylation by PQ in all groups.

Myeloperoxidase activity

Aliquots of rat lung samples were assayed for the activity of MPO as an index of lung invasion by neutrophils. As shown in Table 1, lung MPO activities of the PQ 24 and 48 h groups were significantly higher than in rats from control or the NaSAL group. Concerning the PQ 96 h group, a significant difference was only achieved in comparison to the NaSAL 96 h group ($P < 0.05$). Posttreatment with NaSAL prevented the increase of MPO activity as a consequence of PQ exposure in all groups.

Hydroxyproline lung content

Despite the enhanced fibrotic changes observed in the lung histology of PQ-exposed animals relative to control or NaSAL groups, the biochemical measurement of Hyp was not sensitive enough to detect it in these earlier stages. Hyp contents of the lung tissue were comparable among control, PQ, PQ + NaSAL, and NaSAL groups (Table 1).

Discussion

The results obtained in the present study clearly show that NaSAL confers a potent protection against PQ-induced lung

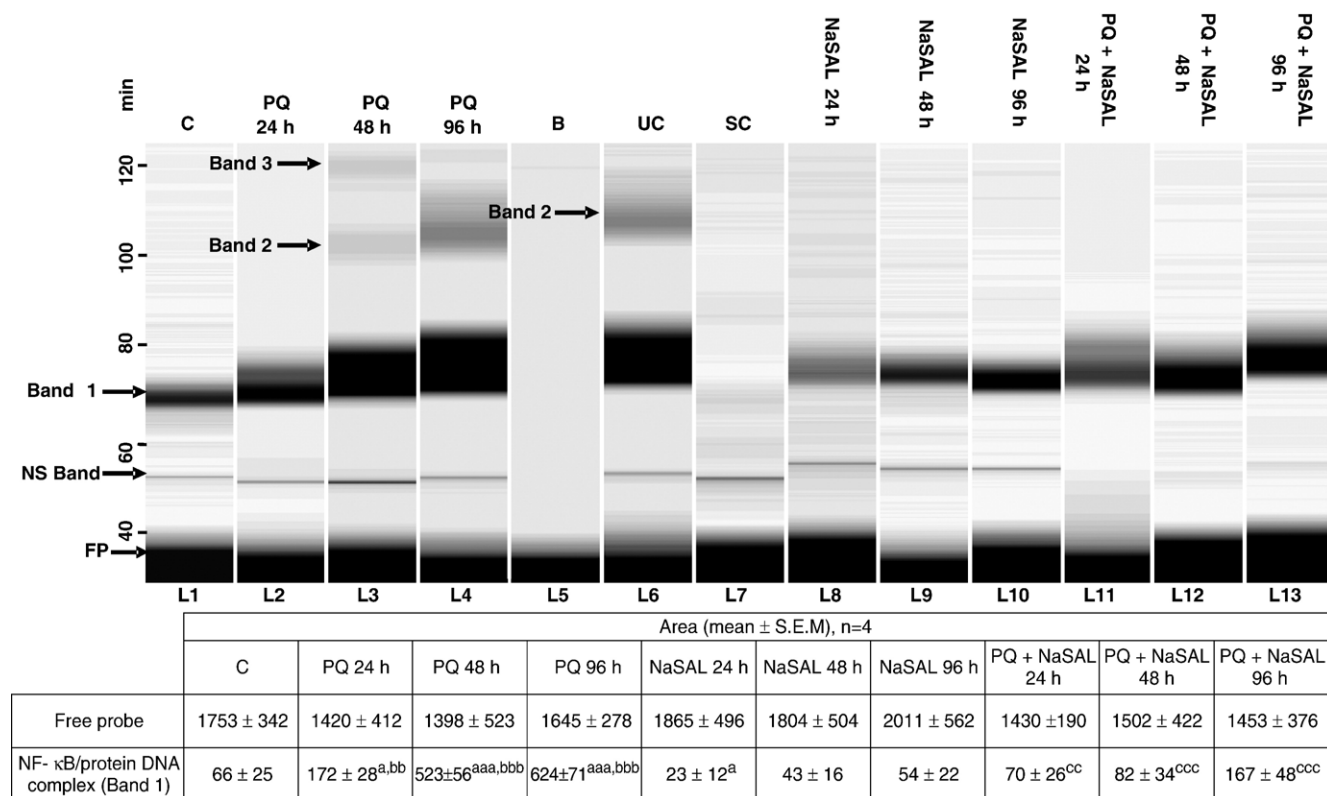


Fig. 6. Time course of NF-κB activation induced by PQ in lungs. fEMSA gel view and binding activities, presented as arbitrary units corresponding to area under curve (AUC), are shown. Nuclear extracts from the different groups were prepared and subjected to fEMSA as described under Materials and methods. Lane 1, control group (C); Lanes 2, 3 and 4, paraquat (PQ) 24, 48, and 96 h groups, respectively; Lane 5, blank (B); Lane 6, competition experiment with a 50-fold molar excess of a nonspecific competitor (UC) compared to specific probe (SP); Lane 7, competition experiment with a 50-fold molar excess of a specific competitor (SC, unlabeled specific probe) compared to SP. Lanes 8, 9, and 10, sodium salicylate (NaSAL) 24, 48, and 96 h groups, respectively; Lanes 11, 12, and 13, PQ + NaSAL 24, 48, and 96 h groups, respectively; The positions of specific NF-κB/DNA-binding complexes (bands 1–3) are indicated. NS Band represents a nonspecific binding. The localization of the free probe (FP) is also indicated. The AUC results are given as mean ± SE ($n = 4$). ^a $P < 0.05$, ^{aa} $P < 0.01$ and ^{aaa} $P < 0.001$ versus control group, ^b $P < 0.05$, ^{bb} $P < 0.01$, and ^{bbb} $P < 0.001$ versus NaSAL group, ^c $P < 0.05$, ^{cc} $P < 0.01$, and ^{ccc} $P < 0.001$ versus PQ group.

toxicity. It is shown, for the first time, that the administration of NaSAL (200 mg/kg ip), 2 h after PQ (25 mg/kg ip) exposure, results in a remarkable decrease of PQ-induced lung toxicity in Wistar rats. The prevention of PQ-induced lung toxicity by NaSAL was evidenced by a significant remission of several biochemical and histopathological biomarkers of toxicity, and resulted in full survival of the intoxicated animals. Importantly, NaSAL was administered 2 h after PQ exposure, conferring more realism to our study since, in the majority of the PQ intoxications, medical assistance is only possible a few hours after PQ intoxication.

As expected, LPO and carbonyl group levels increased significantly in the lung of rats exposed to PQ compared to the control group. These oxidative stress-related alterations, which are in agreement with our previous reports [16,17], were attenuated by NaSAL administration. A plausible justification for this protection conferred by NaSAL is its potent scavenging effect on hydroxyl radical (HO^\bullet). Among ROS, HO^\bullet is thought to be the most damaging species and the mainly responsible for protein oxidation and LPO [26]. Indeed, the major hydroxylation products of HO^\bullet attack on SAL, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), have been used as sensitive markers for the measurement of

HO^\bullet formation in vivo [27]. Thus, the efficacy of NaSAL in preventing PQ-induced lung injury may also be due to its ability to inactivate the HO^\bullet radical. In addition, local biotransformation of NaSAL could play an important role in its ability to markedly attenuate lung oxidative injury due to PQ exposure, since 2,3-DHBA is a potent iron chelator [28] and iron availability is required for HO^\bullet generation via Fenton reaction.

In addition to the increase of oxidative damage markers, it was also observed that NF-κB expression underwent a significant and sustained increase in the lung as a consequence of PQ exposure. The induction of NF-κB expression was time dependent, which seems to point to a continuous inflammatory process that ultimately may cause severe lung damage and death. Indeed, a large oral dose of PQ (>30 mg/kg in humans) rapidly leads to multiorgan failure, with lung damage consisting of disruption of alveolar epithelial cells (type I and II pneumocytes) and bronchiolar Clara cells (CC), hemorrhage, edema, hypoxemia, and infiltration of inflammatory cells into the interstitial and alveolar spaces [29]. To our knowledge this is the first study describing the association of a strong NF-κB activation in the lung along with the aggravation of the health status of rats intoxicated with PQ. NaSAL strongly suppressed the PQ-induced lung NF-κB activation, which probably

contributes to the observed healing effect mediated by this drug. NF- κ B induction by PQ may be correlated with the antioxidant enzyme levels, as previously shown by Zhou and co-workers [30], who exposed skeletal muscle cells to PQ-induced oxidative stress and demonstrated that NF- κ B mediates the induction of GPx and CAT. NaSAL, given 2 h after PQ, attenuated the increase of CAT and GPx activities near to the levels of control and NaSAL groups, which is in accordance with the reported correlation. Accordingly, the same rationale could also be applied to explain the increase of GPx and CAT activities by lung resident cells in PQ-only exposed animals. However, it is important to stress the positive time change correlation between the antioxidant enzymes and the MPO activities, observed in these animals. Despite suggesting an association linking the infiltration of inflammatory cells and the enhancement of antioxidant enzymes, with our experimental design it was not possible to establish a direct cause–effect relationship and thus distinguish the individual contribution of the polymorphonuclear leukocytes (PMN) antioxidant enzymes arsenal from the CAT and GPx overexpression by lung cells as a consequence of PQ exposure. Our biochemical results showed that MPO activity decreased in the lungs of the PQ + NaSAL groups compared to PQ-only exposed groups. Since MPO is located within the primary azurophil granules of PMN, its activity indirectly reflects PMN infiltration through the organs [31] during the inflammatory reaction. It could be argued that the observed NaSAL protective effects against PQ-induced lung toxicity may be the result of less infiltration by inflammatory cells. Histopathological studies confirmed the widespread neutrophil infiltration especially in the lungs of PQ-only exposed animals. Macrophage infiltration and several NK cells were also identified in the interstitial space (Figs. 5C and 5D). It has been shown that the adhesion of circulating PMN to vascular endothelium is crucial to their transendothelial migration [32]. The expression of adhesion molecules on endothelial cells is regulated by NF- κ B [33]. As a consequence of I κ B phosphorylation inhibition, NaSAL has proven to inhibit transendothelial migration of neutrophils [14,33]. However, histopathological results revealed only some amelioration of PMN lung infiltration, in contrast to the drastic decrease of MPO activity observed in rats of PQ + NaSAL groups in comparison to the PQ groups. According to the literature, this apparent discrepancy between the biochemical and the histopathological results could be at least partially explained by a lower generation of the most powerful oxidant produced by human neutrophils, HOCl, as a consequence of MPO inhibition by NaSAL [34]. Thus, it is reasonable to consider that the NaSAL overall protection could also be the consequence of MPO inhibition. In the present study, PQ also caused lung edema, an effect observed as an increase of RLW and by histopathological analysis, which confirms the great contribution of inflammation to the toxic effects mediated by this herbicide. Exuberance of interstitial edema was drastically attenuated in PQ + NaSAL animals.

There are two distinct phases in the development of pulmonary fibrosis (PF) resulting from PQ exposure. The first is a destructive phase in which the alveolar type I and type II and

Clara cells are destroyed within 1–3 days after PQ poisoning, resulting in alveolitis [4]. This stage provides a basis for an extensive PF observed in the second phase, since the cells involved in the alveolitis, e.g., macrophages, lymphocytes, and neutrophils play a key role in producing the factors that regulate the proliferation, chemotactism, and secretory activity of fibroblasts and consequently the extent of the interstitial and intraalveolar fibrosis [35,36]. Alveolar macrophages secrete fibrogenic factors such as transforming growth factor (TGF)- β [37] and gene expression of TGF- β is enhanced in the lungs after PQ exposure [38]. Activated macrophages in inflamed lungs in response to PQ exposure also synthesize increased amounts of several other cytokines, including IL-1 α , IL-1 β , IL-6, platelet-derived growth factor (PDGF)-A, TGF- α , insulin like growth factor, TNF- α , and monocyte chemoattractant protein (MCP)-1 that mediate an enhanced fibroproliferative response [39]. Accordingly, an increase of local TNF- α production was observed in bleomycin-induced PF [40] and Ishida et al. [38] found an up-regulation of TNF- α mRNA expression in lungs of PQ-exposed mice with a concomitant increase in leukocyte infiltration. All these events represent possible causes for unremitting lung fibrosis as a consequence of PQ intoxication as well as potential targets for therapeutic intervention. On its own, NaSAL has been shown to blunt the increase in TNF- α mRNA and to reduce the serum TNF- α protein level of mice [41]. The fact that these events have been observed in the early inflammatory phase suggests that the effect of NaSAL may be related to tissue preservation; that is, a decreased extent of lung lesion at the initial phase in NaSAL-treated animals would imply a reduced fibrotic process at later stages. This hypothesis does not exclude a direct effect of NaSAL on preventing fibrogenesis, since it has been reported that acetylsalicylic acid inhibits collagen synthesis by fibroblast proliferation inhibition *in vitro* [42]. Taking into account the capacity of NF- κ B to activate the transcription of specific genes and classes of genes encoding for various proinflammatory and fibrogenic cytokines, it is reasonable to consider that the inhibition of the NF- κ B activation plays an important role in the NaSAL protective effect against PQ-induced lung toxicity. Our results also confirm earlier studies regarding the fibrogenic effects of PQ. Collagen lung deposition was observed in a time-dependent manner by histology 48 and 96 h after PQ exposure. Despite the enhanced fibrotic changes observed in the lung histology of PQ-exposed animals relative to control or NaSAL groups the biochemical measurement of Hyp was not sensitive enough to detect it in these earlier stages. Hyp contents of the lung tissue were comparable among control, PQ, PQ + NaSAL, and NaSAL groups. Previous studies, where Hyp lung content was determined as a measure of fibrosis, showed that an increase in its levels was observed only after 14 days of PQ exposure [43,44]. The lack of utility for Hyp measurement at the first days after PQ exposure was also confirmed in our study. The histological results showed an attenuation of collagen deposition in the PQ + NaSAL 96 h group, which support a beneficial effect of NaSAL in preventing PF.

Another point of interest resulting from this study comes from the histopathological analysis. Rats from the PQ group

evidenced an intense vascular congestion, the pulmonary capillaries being filled with angular erythrocytes and numerous activated platelets, suggestive of an activation of the blood coagulation system. These findings are in accordance with the disseminated intravascular coagulation described for PQ [5]. One of the main results of NaSAL inclusion in the therapeutic of PQ-poisoned rats was the reduction of vascular congestion without signs of platelet activation, which may be, at least partially, explained by the antithrombogenic effect of NaSAL [11]. It is well known that PQ causes ARDS, which may be the result of blood coagulation and vascular stasis leading to an enhanced pulmonary dead-space fraction [45]. Since blood coagulation evidence in the NaSAL-treated animals was attenuated, it is legitimate to speculate that the ratio of ventilation/perfusion was not severely disturbed as in the PQ group, justifying the survival rate observed in these animals.

We have recently demonstrated that the induction of de novo synthesis of membrane P-glycoprotein (P-gp) by dexamethasone confers a strong protection against PQ-induced lung toxicity by increasing its efflux from the lung [17]. According to the literature, we could not disregard this possibility occurring as well as for NaSAL, since aspirin has proven to enhance expression of P-gp and thus to induce multidrug resistance [46,47]. We could neither ignore the possibility of NaSAL to increase the PQ elimination from pneumocytes, by raising its export by the polyamine export transporter [48]. However, our results did not point to the protection occurring by decreasing PQ lung accumulation, since no statistical difference was observed between the PQ and the PQ + NaSAL groups.

In view of our results, it is plausible to conclude that a therapy with NaSAL, starting as soon as possible after PQ intoxication, may constitute a promising treatment for PQ poisonings. One may consider that the dose of NaSAL used in this study is quite high. According to the literature, the pathophysiologic changes attributable to high doses of NaSAL result in various clinical manifestations depending on the amount ingested; in humans, an oral dose of 200 mg/kg yields approximately a serum concentration of 500–770 mg/L [49–51]. These serum levels originate signals of mild side effects such as nausea, vomiting, tinnitus, hyperventilation, and respiratory alkalosis. In the particular case of life-threatening PQ poisonings, the risk/benefit ratio will most probably flip to the beneficial effects of NaSAL, although this still needs to be carefully confirmed in clinical trials. In addition, NaSAL proved to protect lungs, which was confirmed by a remission of practically all toxicological parameters that were changed in the lung of PQ-challenged rats, through an effective inhibition of proinflammatory factors, scavenging of ROS, inhibition of MPO, and inhibition of platelet aggregation. This protection achieved full survival of the PQ-exposed animals.

In our opinion this therapeutic approach has the potential to be applied in humans, though further preclinical studies are needed, particularly those aimed to explain in more detail the mode of action of this interesting molecule in the protection against PQ-induced lung damage.

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