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Original Contribution

P-glycoprotein induction: an antidotal pathway for paraquat-induced lung toxicity☆

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Abstract

The widespread use of the nonselective contact herbicide paraquat (PQ) has been the cause of thousands of deaths from both accidental and voluntary ingestion. The main target organ for PQ toxicity is the lung. No antidote or effective treatment to decrease PQ accumulation in the lung or to disrupt its toxicity has yet been developed. The present study describes a procedure that leads to a remarkable decrease in PQ accumulation in the lung, together with an increase in its fecal excretion and a subsequent decrease in several biochemical and histopathological biomarkers of toxicity. The administration of dexamethasone (100 mg/kg ip) to Wistar rats, 2 h after PQ intoxication (25 mg/kg ip), decreased the lung PQ accumulation to about 40% of the group exposed to only PQ and led to an improvement in tissue healing in just 24 h as a result of the induction of *de novo* synthesis of P-glycoprotein (P-gp). The involvement of P-gp in these effects was confirmed by Western blot analysis and by the use of a competitive inhibitor of this transporter, verapamil (10 mg/kg ip), which, given 1 h before dexamethasone, blocked its protective effects, causing instead an increase in lung PQ concentration and an aggravation of toxicity. In conclusion, the induction of P-gp, leading to a decrease in lung levels of PQ and the consequent prevention of toxicity, seems to be a new and promising treatment for PQ poisonings that should be further clinically tested.

Keywords: Paraquat; Lung toxicity; P-glycoprotein; Dexamethasone; Free radicals

Paraquat dichloride (methyl viologen; PQ) is an effective and widely used herbicide as desiccant and defoliant in a variety of crops. Despite PQ being the third most extensively used herbicide in the world, it can be considered one of the most toxic over the past 60 years. Indeed, PQ has caused thousands of deaths from both accidental and voluntary ingestion, as well as from dermal exposure [1]. Depending on the ingested dose, different clinical patterns and outcomes have been observed in animals and humans [1]. A large oral dose of PQ (>30 mg/kg in humans) rapidly leads to death from multiorgan failure, with lung damage consisting of disruption of alveolar epithelial cells (type I and II pneumocytes) and bronchiolar Clara cells, hemorrhage, edema, hypoxemia, and infiltration of inflammatory cells into the interstitial and alveolar spaces [1]. Smaller doses of PQ (from 16 mg/kg) may also lead to death, but this occurs after several days as a result of a progressive lung fibrosis and consequent respiratory failure, by proliferation of fibroblasts and excessive collagen deposition [1].

In 1974, Rose et al. [2] demonstrated that the accumulation of radioactively labeled [^{14}C]PQ in rat lung slices was energy dependent and obeyed saturation kinetics. Other studies led to the conclusion that PQ accumulated in the lung through a system in which polyamines are the natural substrates and that in comparison to other organs, the lungs, and more specifically the alveolar epithelial and Clara cells, were endowed with a particularly active polyamine uptake system [3]. Although PQ

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proved to be a "poor" substrate for the polyamine uptake system, it undoubtedly accumulates in the lung through this transport pathway. The mechanism of PQ-induced acute lung toxicity is well known. It is essentially due to its redox cycle [4]: PQ is reduced enzymatically, mainly by NADPH-cytochrome P450 reductase [5] and NADH:ubiquinone oxidoreductase (complex I) [6–8], to form a PQ monocation free radical. The PQ monocation free radical is then rapidly reoxidized in the presence of oxygen (which has a high partial pressure in lungs) with the subsequent generation of the superoxide radical ($O_2^{\bullet -}$) [9,10]. This then begins the well-known cascade leading to the production of other reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$).

Currently, no antidote or effective treatment for PQ poisoning has been identified, survival being mainly dependent on the amount ingested and the time elapsed until the patient is submitted to intensive medical measures to inactivate or to eliminate PQ, before its cellular uptake. These approaches include procedures such as induction of emesis or intestinal transit, gastric lavage, administration of oral adsorbents, hemodialysis, and hemoperfusion [11–13]. In addition to these treatments, protective measures have also been adopted: (i) to prevent the generation of ROS, namely the effective iron chelation by desferrioxamine [14]; (ii) to scavenge ROS, including the maintenance of effective levels of antioxidants [15]; and (iii) to reduce the inflammation [16,17]. However, such treatments have a general low efficacy and the fatality rate remains very high. It was precisely this lacuna in the treatment of PQ intoxication that impelled our study.

In this work we propose a new approach for PQ poisonings by induction of *de novo* synthesis of a plasma membrane phosphoglycoprotein, P-glycoprotein (P-gp). P-gp, a member of the ATP-binding cassette superfamily, was initially identified in tumor cells as an ATP-dependent transporter, which can export a wide variety of unmodified substrates out of the cell, namely *Vinca* alkaloids, colchicine, antibiotics, anthracyclines, cardiac glycosides, organic cations, and pesticides [18–20]. This drug transport occurs against the concentration gradient and is independent of an electrochemical transmembrane potential or proton gradient [21].

A number of reports exist noting that dexamethasone (DEX) induces P-gp levels in liver, brain, and intestinal tissue and also in lung tissue [22], an effect that seems to be glucocorticoid concentration-dependent. This induction phenomenon is rapid, because a maximum effect may be observed 24 h after a single administration [22]. On the other hand, P-gp-mediated efflux can be pharmacologically inhibited using several drugs, namely verapamil (VER), cyclosporin A, and amiodarone [23]. Thus, in the present study, we investigated, for the first time, the process of *de novo* synthesis of P-gp, by DEX, in the concentration of PQ in rat lung and its urinary and fecal excretion. The preventive effect of this pharmacological approach against PQ-induced lung toxicity was also evaluated using both biochemical and histopathological biomarkers of toxicity. VER, as competitive inhibitor of P-gp, was used to confirm the importance of this transporter in PQ excretion.

Materials and methods

Chemicals and drugs

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride), dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], (±)-verapamil hydrochloride (5-[(3,4-dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride), 3,3′,5,5′-tetramethylbenzidine (TMB), 5-sulfosalicylic acid, NADPH (nicotinamide adenine dinucleotide phosphate reduced), GSH (reduced glutathione), GSSG (oxidized glutathione), 2-vinylpiridine, and 2,4-dinitrophenylhydrazine (DNPH) were all obtained from Sigma (St. Louis, MO, USA). Saline solution (NaCl 0.9%) and sodium thiopental were obtained from B. Braun (Lisbon, Portugal). Sodium hydroxide (NaOH), sodium dithionite ($Na_2S_2O_4$), 2-thiobarbituric acid ($C_4H_4N_2O_2S$), and trichloroacetic acid (Cl_3CCOOH) were obtained from Merck (Darmstadt, Germany). All the reagents used were of analytical grade or of the highest available grade.

Animals and experimental design

The study was performed using adult male Wistar rats obtained from Charles River S.A. (Barcelona, Spain), with a mean weight of 252±8 g. Animals were kept under standard laboratory conditions (12/12 h light/darkness, 22±2°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 the Portuguese General Directorate of Veterinary Medicine. 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.

After the quarantine period, 52 animals were randomly divided into four groups of 13 animals each. Each animal was individually housed in a metabolic cage where it was kept during the whole time of experiment (26 h). Animals were fasted during the entire experimental period but water was given *ad libitum*. Urine and feces were collected over ice during the 26-h period, for quantification of PQ.

The administrations of vehicle (0.9% NaCl), PQ, DEX, and VER were all done intraperitoneally (ip) in an injection volume of 0.5 ml. The four groups were treated as follows: (i) The control group ($n=13$) animals were treated with 0.9% NaCl. Animals were treated with two more administrations of 0.9% NaCl, 1 and 2 h later, respectively. (ii) The PQ group ($n=13$) animals were intoxicated with PQ (25 mg/kg). Animals were treated with two administrations of 0.9% NaCl, 1 and 2 h later, respectively. (iii) The PQ+DEX group ($n=13$) animals were intoxicated with PQ (25 mg/kg). Animals were treated with 0.9% NaCl and DEX (100 mg/kg), 1 and 2 h later, respectively. The schedule of DEX

administration was chosen considering the lag time necessary for the arrival of the patient at the hospital after PQ intoxication. (iv) The PQ+DEX+VER group ($n=13$) animals were intoxicated with PQ (25 mg/kg). Animals were treated with VER (10 mg/kg) and DEX (100 mg/kg), 1 and 2 h later, respectively.

The experimental dose of DEX has been applied in numerous studies for inducing *de novo* synthesis of P-gp [22]. The VER dose was selected according to some reported studies referring to P-gp competitive inhibition *in vivo* [24]. The PQ dose was similar to that used in previous studies resulting in severe lung toxicity [25,26].

The treatments for all groups were always conducted between 8:00 and 10:00 AM.

Collection and processing of lung samples

Twenty-six hours after PQ administration, anesthesia was induced with sodium thiopental (60 mg/kg, ip). Animals were placed in the *decubito supino* position and tracheotomy and tracheal cannulation was done, followed by the immediate connection of the cannula to a mechanical ventilation system that supplied a tidal volume of 2 ml at a respiratory frequency of 60 breaths/min. The thorax was opened by two lateral transversal incisions and one central longitudinal incision to expose the pulmonary artery. In 10 rats of each group, lungs were perfused in situ through the pulmonary artery with cold 0.9% NaCl for 3 min at a rate of 10 ml/min to be completely cleaned of blood. At the same time that this perfusion was initiated, a cut at the left wall ventricle was done to avoid overpressure. Lungs were removed, cleaned of all major cartilaginous tissues of the conducting airways, pat-dried with gauze, weighed, and processed as follows: (i) The right lung (except the posterior lobe) was homogenized (Ultra-Turrax homogenizer) in a cold mixture of phosphate buffer [(KH_2PO_4 + $Na_2HPO_4 \cdot H_2O$) 50 mM, pH 7.4] and 0.1% (v/v) Triton X-100, 1 g of tissue/4 ml of mixture, and centrifuged (3000*g*, 4°C, for 10 min). Aliquots of the resulting supernatants were stored (−80°C) for posterior quantification of the pulmonary remaining PQ, myeloperoxidase activity (MPO), superoxide dismutase activity (SOD), carbonyl groups, and protein levels. Aliquots of the resulting supernatants were then centrifuged at 33,000*g*, 4°C, for 30 min. The pellet containing the crude membrane fractions was resuspended in 50 mM mannitol, 20 mM Hepes–Tris, pH 7.5, and stored at −80°C for posterior confirmation of P-gp induction. The posterior lobe was homogenized in perchloric acid (5% final concentration) and then centrifuged (13,000*g*, 4°C, for 10 min). Supernatants were stored (−80°C) for posterior quantification of GSH and GSSG. The pellet was used for protein quantification. (ii) The left lung was homogenized (Ultra-Turrax homogenizer) in trichloroacetic acid 10% (1/4 m/v) and then centrifuged (13,000*g*, 4°C, for 10 min). Aliquots of the resulting supernatants were immediately used for evaluating the degree of lipid peroxidation (LPO). The pellet was used for protein quantification.

The relative lung weight (RLW) of each animal was calculated as a percentage of the absolute body weight on the sacrifice day.

Quantification of PQ in rat lung, urine, and feces

Aliquots of right lung supernatants were treated with 5-sulfosalicylic acid (5% in final volume) and then centrifuged (13,000*g*, 4°C, for 10 min).

Feces were treated with 5-sulfosalicylic acid (5% in final volume) and then centrifuged (13,000*g*, 4°C, for 20 min). Urine samples were centrifuged (13,000*g*, 4°C, for 20 min).

The resulting supernatant fractions from lung, urine, and feces were alkalinized with 10 N NaOH (pH >9) and then gently mixed with a few crystals of a reductant (sodium dithionite) to give the blue color characteristic of the PQ cation radical. PQ quantification was carried out by a previously reported method based on second-derivative spectrophotometry [27].

Evaluation of P-gp induction

Proteins (40 μg) from lung tissue homogenates of the four groups were separated by SDS–PAGE on a 6.25% acrylamide gel according to the method of Laemmli [28]. P-gp was detected by Western blot analysis using the polyclonal antibody sc-1517 (Santa Cruz Biotechnology, Inc.). A horseradish peroxidase-conjugated anti-goat IgG was used as secondary antibody. The bands were visualized by treating the immunoblots with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier's instructions, followed by exposure to X-ray films (Kodak Biomax Light Film; Sigma). The films were analyzed with QuantityOne Software (Bio-Rad). Optical density results were expressed as percentage variation of control values.

Tissue processing for structural and ultrastructural qualitative and semiquantitative analysis

Three animals of each group were assigned to histological analysis. Lung samples were subjected to routine procedures for light microscopy (LM) and transmission electron microscopy (TEM) analysis. With the animals under anesthesia, lung fixation was initiated *in situ* by perfusion through the pulmonary artery, with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2–7.4) for 3 min. Subsequently, lungs were excised, sectioned into ~1-mm^3 pieces and fixed (by diffusion) in the same fixative for 2 h. After two washing steps, of 30 min each with buffer solution, the specimens were dehydrated in graded alcohol for 2 h and then embedded in Epon. Propylene oxide was the compound used in the dehydration–impregnation transition. The inclusion phase lasted 2 days. All the procedures were done at 4°C, with the exception of the inclusion phase, which was performed at 60°C. Subsequent to the resin polymerization, semithin sections (1 μm thick) and ultrathin sections (500 Å thick) were prepared (Ultracut, Leica), respectively for LM and TEM analysis. The grids, mounted with the ultrathin specimen sections, were double-contrasted with 0.5% saturated uranyl acetate aqueous solution for 30 min and then with 0.2% lead citrate solution for

15 min. The slides, mounted with semithin sections, were stained with toluidine blue. Five slides and three grids from each animal (totaling 10 slides and six grids per group), were examined in a Zeiss Phomi III photomicroscope and in a transmission electronic microscope (Zeiss EM 10A).

Histopathological evidence of acute tissue damage was semiquantified according to the methodology described elsewhere [29–32]. 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 in every slide or grid: (i) cellular degeneration, (ii) interstitial inflammatory cell infiltration, (iii) necrotic zones, and (iv) tissue disorganization. Considering the cellular degeneration, its severity was scored according to the number of cells showing any alterations (dilatation, vacuolization, pyknotic nuclei, and cellular density) in the LM visual field: grade 0, no change from normal; grade 1, a limited number of isolated cells (until 5% of the total cell number); grade 2, groups of cells (5–30% of the cell total number); and grade 3, diffuse cell damage (higher than 30% of the total cell number). The severity of inflammatory reaction was scored as 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, confluent necrotic areas; grade 3, massive necrosis. The severity of tissue disorganization was scored according to the percentage of the affected tissue: grade 0, normal structure; grade 1, less than one-third of tissue; grade 2, greater than one-third and less than two-thirds; grade 3, greater of two-thirds of tissue. For each animal, the highest possible total tissue score was 12 and the lowest was 0.

An examiner blinded to each tissue sample analyzed all grids and slides independently.

Protein quantification

Protein quantification was performed according to the method of Lowry et al. [33] using bovine serum albumin as standard.

Measurement of toxicological biomarkers

LPO was evaluated by the thiobarbituric acid-reactive substances methodology [34]. Results are expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram protein using an extinction coefficient (ε) of 1.56×10^5 M^{-1} cm^{-1}.

Protein carbonyl groups (ketones and aldehydes) were determined according to Levine et al. [35]. Results are expressed as nanomoles of DNPH incorporated per milligram of protein ($\varepsilon=2.2\times10^4$ M^{-1} cm^{-1}).

MPO activity was measured according to the method followed by Suzuki et al. and Andrews et al. [36,37], with slight modifications. Briefly, the supernatants were initially submitted to three cycles of snap freezing. The assay mixture consisted of 50 μl of supernatant and 50 μl of TMB (final concentration 7.5 mM) dissolved in dimethyl sulfoxide. The enzymatic activity was initiated by addition of 50 μl of H_2O_2 (final concentration 1.5 mM) dissolved in phosphate buffer ($Na_2HPO_4\cdot 2H_2O$ 50 mM, pH 5.4). The rate of MPO/H_2O_2 system-catalyzed oxidation of TMB was followed by recording the absorbance increase at 655 nm at 37°C for 3 min. One enzyme unit (U) was defined as the amount of enzyme capable of reducing 1 μl of H_2O_2/min under assay conditions. Results are expressed in enzyme U/g of protein ($\varepsilon=3.9\times10^4$ M^{-1} cm^{-1}).

GSH and GSSG concentrations were determined by the 5,5′-dithiobis-2-nitrobenzoic acid–GSSG reductase recycling assay as described before [38]. Results are expressed in nanomoles of GSH or GSSG per milligram of protein.

Copper/zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) were assayed using the method of Flohé and Otting [39] with modifications. A xanthine–xanthine oxidase system was used to generate $O_2^{\bullet-}$. The subsequent reduction of nitroblue tetrazolium (NBT) by $O_2^{\bullet-}$ was monitored at 560 nm. Potassium cyanide (2 mM) was used to allow the measurement of MnSOD. Enzyme activity was expressed in U/mg of protein 1 U of SOD is defined as the amount of enzyme required to inhibit the rate of NBT reduction by 50%).

Statistical analysis

Results are expressed as means ± SEM (standard error of the mean). Statistical comparison between groups was estimated using the nonparametric method of Kruskal–Wallis followed by Dunn's test. In all cases, p values lower than 0.05 were considered statistically significant.

Results

Macroscopic observations

Diarrhea, piloerection, weight loss, anorexia, adipsia, hyperpnea, dyspnea, tachycardia, and a red drainage around the mouth, eyes, and nose were present especially in animals subjected to only PQ or to PQ + VER + DEX. During the experimental period rats belonging to groups PQ and PQ + VER + DEX did not ingest any amount of water. Deep breathing was observed and the thorax was sunken in the animals from these groups, in contrast to those treated with DEX.

Lung PQ concentrations

The concentration of PQ in lungs of the PQ-treated group was 0.127 ± 0.010 (mean ± SEM; μg/mg protein). Animals post-treated with DEX evidenced a significant decrease in PQ lung concentration, down to 0.051 ± 0.012 ($p<0.05$) (Fig. 1). VER significantly reverted ($p<0.001$) the DEX effect and even contributed to a higher lung concentration of PQ (up to 0.309 ± 0.038).

Urinary and fecal excretion of PQ

Quantification of urinary PQ levels showed that almost all the PQ administered was eliminated by urine within 26 h (nearly 90%). The inclusion of DEX did not result in any increment of urinary excretion of PQ. The same result was obtained in rats exposed to DEX and VER (Fig. 1). On the other hand, and as shown in the Fig. 1, rats that received DEX in addition to PQ (PQ+DEX group) had a significant increase in PQ fecal excretion, up to 0.651±0.088 (mean± SEM; mg kg $BW^{-1} \cdot 26\ h^{-1}$) ($p<0.05$), compared to rats treated only with PQ (0.466±0.053), this increase being abolished and even reverted by the previous administration of VER (down to 0.366±0.064).

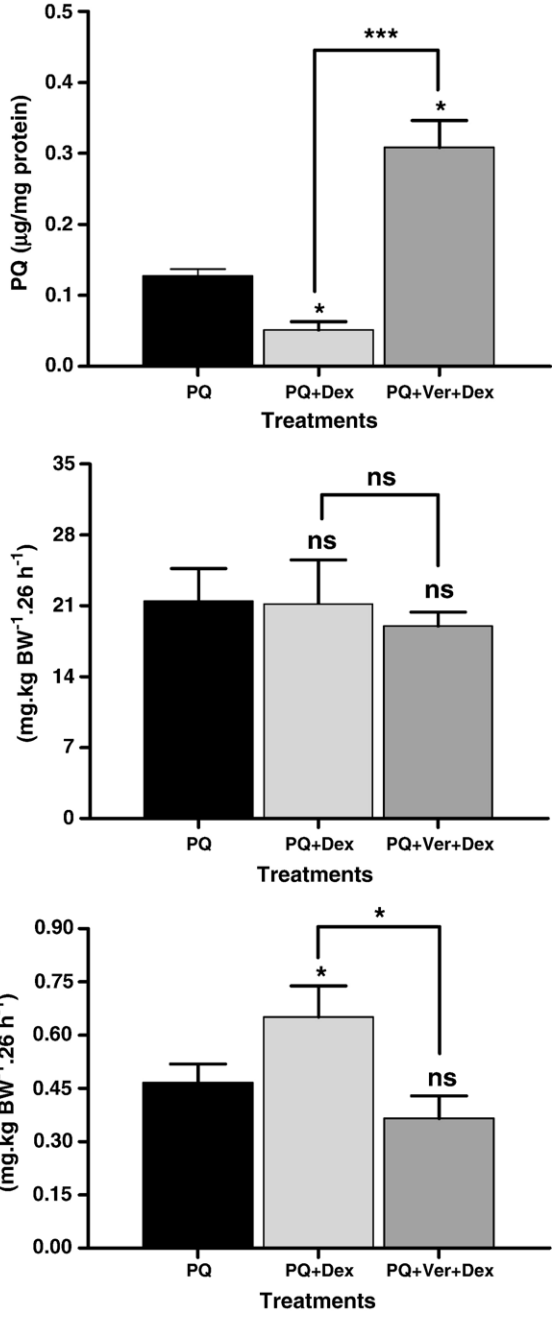

Fig. 1. Levels of PQ in the lung, urine, and feces of the paraquat (PQ), paraquat+dexamethasone (PQ+Dex), and paraquat+verapamil+dexamethasone (PQ+Ver+Dex) groups. Values are given as means±SEM ($n=10$). $^{ns}p>0.05$, $*p<0.05$, $***p<0.001$; BW, body weight.

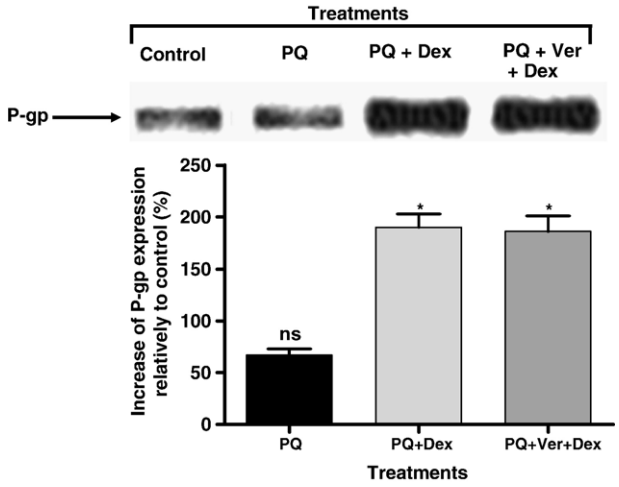

Fig. 2. Immunodetection of the P-glycoprotein (P-gp) in the lung of PQ, PQ+Dex, and PQ+Ver+Dex groups. Values are given as means±SEM ($n=3$). $*p<0.05$.

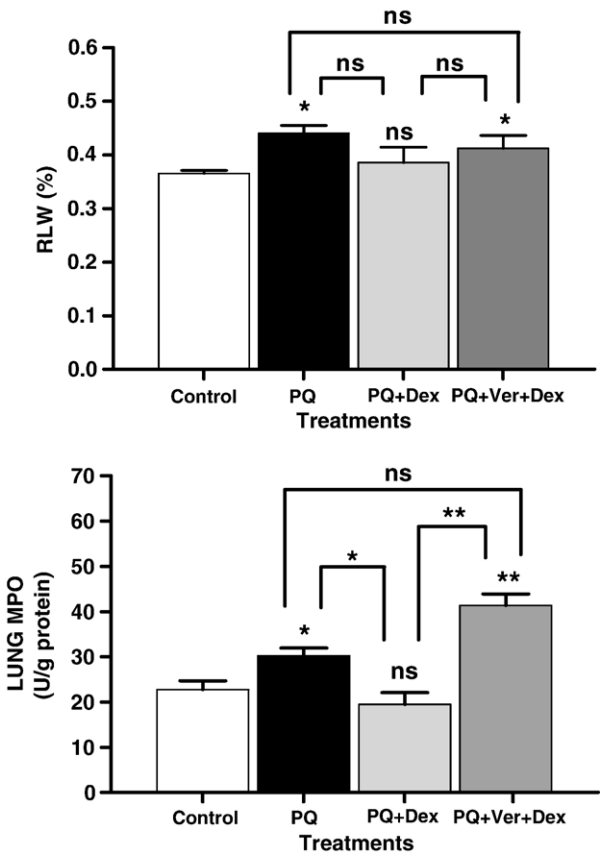

Fig. 3. Biomarkers of lung injury. RLW (%) and MPO lung activity (µmol/min/g of protein) of the control, PQ, PQ+Dex, and PQ+Ver+Dex groups. Values are given as means±SEM ($n=10$). $^{ns}p>0.05$, $*p<0.05$, $**p<0.01$; RLW, relative lung weight; MPO, myeloperoxidase.

DEX-induced P-gp expression

In lung, P-gp expression increased about 1.9-fold ($p<0.05$) (Fig. 2) in the DEX-post-treated groups compared to control rats.

RLW

RLW was assessed as an indication of the degree of edema. In comparison with the control group, animals from the PQ group showed a significant RLW increase ($p<0.05$), whereas RLWs of the PQ+DEX group were near to the control (nonsignificant difference) (Fig. 3).

MPO activity

Aliquots of rat lung samples were assayed for the activity of MPO as an index of lung invasion by neutrophils, 26 h after exposure to PQ. As depicted in Fig. 3, lung MPO activity of the PQ-exposed animals was significantly higher ($p<0.05$) than in rats from the control group. The post treatment with DEX completely prevented the increase in MPO activity. In opposition, lung MPO increased significantly ($p<0.01$) in the VER-treated group, compared to rats exposed to PQ alone or PQ+DEX.

Qualitative and semiquantitative histological analysis

Animals from the control group presented a normal pulmonary structure at LM, without evidence of alveolar collapse or cellular infiltrations (Fig. 4A). The TEM evaluation showed an ordinary alveolar wall, without any evidence of edema or cellular infiltration; the pneumocytes and endothelial cells revealed a preserved ultrastructure (Fig. 4B). PQ administration, however, 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 PQ group animals are illustrated in Figs. 4C and 4D. The LM and TEM analyses evidenced an intense vascular congestion with activated platelets and numerous polymorphonuclear cells inside the capillaries, apparently adherent to endothelial cells. Despite the few polymorphonuclear cells detected in the interstitial space, a diffuse macrophage infiltration, suggested by their cellular shape by LM and TEM, was observed in this space (Fig. 4C); several natural killer (NK) cells, suspected by their characteristic

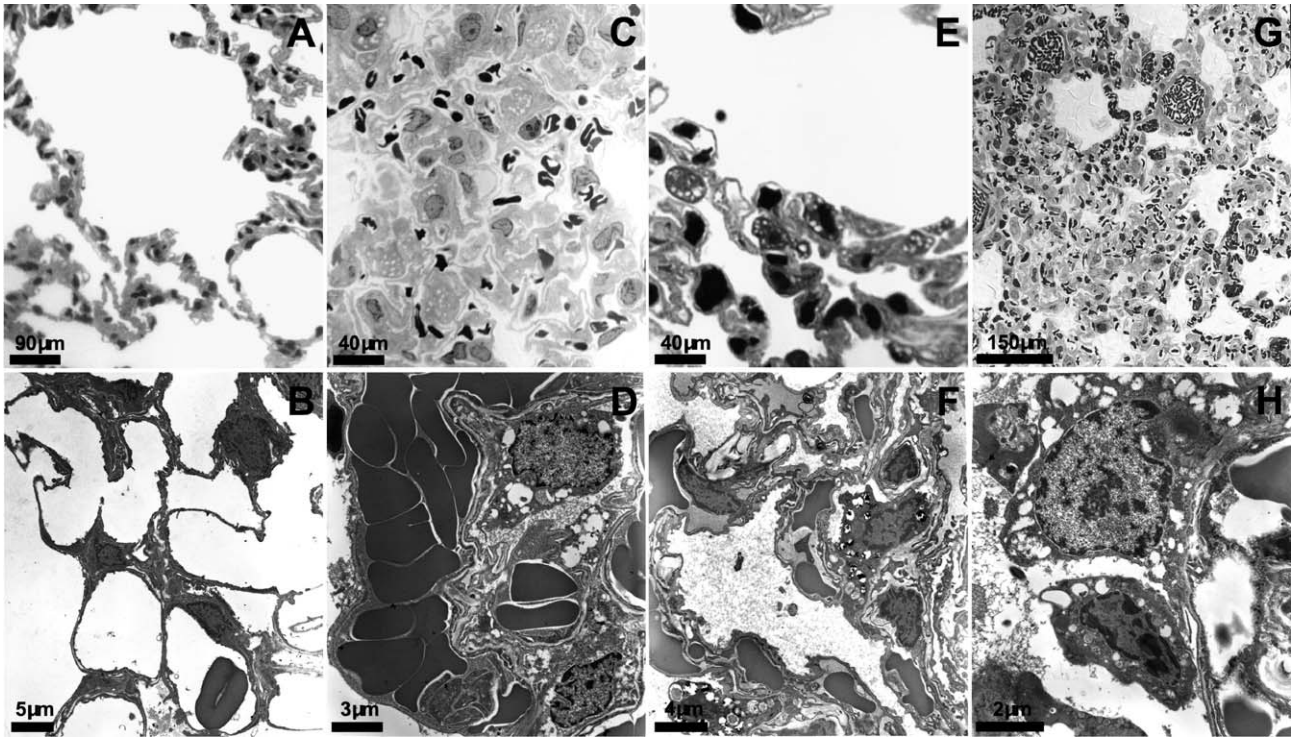

Fig. 4. Structural and ultrastructural qualitative histological analysis of the lung. (A) Light and (B) electron micrographs from control animals (control group) showing a normal pulmonary structure without evidence of alveolar collapse, vascular congestion, or cellular infiltrations. (C) Light and (D) electron micrographs from animals injected only with paraquat (PQ group). In panel C, a marked alveolar collapse and atelectasis with an interstitial infiltration of vacuolated colorless cells are visible and also one polymorphonuclear cell within a capillary at the center of the picture; in panel D an intense vascular congestion with numerous activated platelets can be seen, as well as a vacuolated infiltrative cell, these vacuoles being similar to those described for natural killer cells, and type II pneumocytes with mitochondrial swelling. (E) Light and (F) electron micrographs from animals injected with paraquat plus dexamethasone (PQ+DEX group). In E, a slight decrease in the alveolar space can be observed as well as the existence of several infiltrative vacuolated cells in the interstitial and in the alveolar space; slight signals of vascular congestion and interstitial edema are depicted in panel F. (G) Light and (H) electron micrographs from animals treated with paraquat plus dexamethasone and verapamil (PQ+VER+DEX group) showing, in panel G, a marked vascular congestion with alveolar collapse and numerous vacuolated cells in the interstitial space. In H can be observed a type II pneumocyte with mitochondrial swelling as well as phagocytes (looking like natural killer cells) inside the alveolar space.

cytoplasmic vacuoles, were also identified in the interstitial space (Fig. 4D). Animals from the PQ group also revealed an interstitial edema, indicated by the existence of intercellular vacuolization areas that were characterized by a minor density ultrastructure by TEM. The majority of pneumocytes showed, at least, one ultrastructural abnormality, mitochondrial swelling being the mostly frequent alteration (Fig. 4D). In the PQ group, the TEM analysis evidenced a few endothelial cells with chromatin condensation in the nuclear periphery, suggestive of the occurrence of apoptosis, and the LM analysis revealed the presence of several pyknotic nuclei. In the PQ+DEX group, compared to PQ animals, the occurrence of the above-mentioned alterations was drastically attenuated, particularly the amount of phagocytes observed in interstitial space or within capillaries neighboring endothelial cells. Moreover, despite the existence of several pneumocytes with mitochondrial swelling and evidence of interstitial edema, the exuberance of those signals and the ratio of affected cells were drastically attenuated in PQ+DEX animals (Figs. 4E and 4F). Furthermore, compared to the PQ group, the vascular congestion and the alveolar collapse were not as evident in the PQ+DEX animals (Fig. 4F). Some pyknotic nuclei were also observed in this group but with an apparently lower occurrence compared to the PQ group. Figs. 4G and 4H illustrate the main structural and ultrastructural alterations observed in animals injected with PQ plus VER and DEX (PQ+VER+DEX group). In general, the majority of histological changes detected by LM and TEM had been qualitatively and quantitatively identical to those observed in the PQ group. However, the occurrence of interstitial phagocyte infiltration and the signals of extracellular edema were apparently more severe in the PQ+VER+DEX group.

Concerning the semiquantitative analysis, the total score obtained was 0.03±0.02, 1.82±0.09, 0.96±0.08, and 1.90±0.10 in the control, PQ, PQ+DEX, and PQ+VER+DEX groups, respectively. Significant differences were observed between all groups exposed to PQ and the control group ($p<0.05$). However, compared to PQ or PQ+VER+DEX, the animals of the PQ+DEX group showed a significant reduction of evidence of acute tissue damage ($p<0.05$).

LPO and carbonyl groups

As shown in Fig. 5, animals from the PQ group exhibited a significant increase in the MDA concentration in lung 26 h post-PQ exposure ($p<0.001$), compared with animals from the control group. On the other hand, animals from the PQ+DEX group showed a significant reduction in lung concentration of MDA equivalents (down to near control levels) compared to animals from the PQ group ($p<0.01$). Pretreatment with VER completely prevented the protective effect of DEX. Analogous results were obtained for carbonyl group levels, as depicted in Fig. 5. In accordance with the results of LPO, DEX post treatment prevented the increase in protein carbonylation by PQ. Also similar to LPO, VER reverted the protection conferred by DEX, this group showing a dramatic increase in the lung carbonyl content ($p<0.001$) compared to the control group.

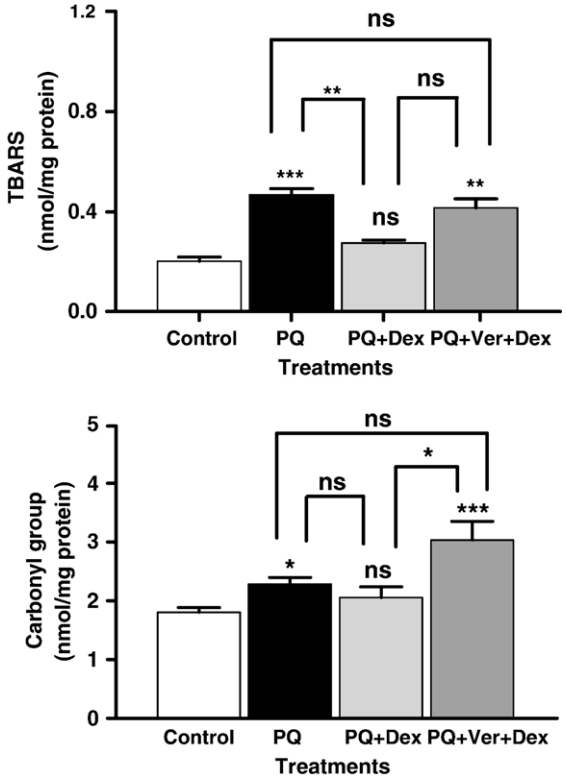

Fig. 5. Lung lipid and protein oxidation. Concentrations of TBARS (nmol MDA/mg of protein) and carbonyl groups (nmol/mg protein) of the control, PQ, PQ+Dex, and PQ+Ver+Dex groups. Values are given as means±SEM ($n=10$). $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; TBARS, thiobarbituric acid-reactive substances.

GSH and GSSG levels

Treatment with PQ induced a pronounced depletion of lung GSH levels ($p<0.05$) (Fig. 6). Nevertheless, no significant difference in the GSH levels between animals from groups PQ, PQ+DEX, and PQ+VER+DEX was observed. In parallel, GSSG (Fig. 6) increased in rats exposed to PQ compared with controls ($p<0.01$). Again, nonsignificant difference in the GSSG levels between animals from groups PQ, PQ+DEX, and PQ+VER+DEX was observed.

SOD

As depicted in Fig. 6, a significant induction in MnSOD and CuZnSOD activity in the group exposed to PQ was observed ($p<0.05$). DEX post treatment prevented the increase in MnSOD activity but not that of CuZnSOD. MnSOD and CuZnSOD activities of the VER-treated group were again significantly increased compared to control ($p<0.05$ vs $p<0.01$).

Discussion

The results obtained in the present study clearly show that DEX confers a potent protection against PQ-induced lung toxicity. Indeed, it is shown, for the first time, that the induction

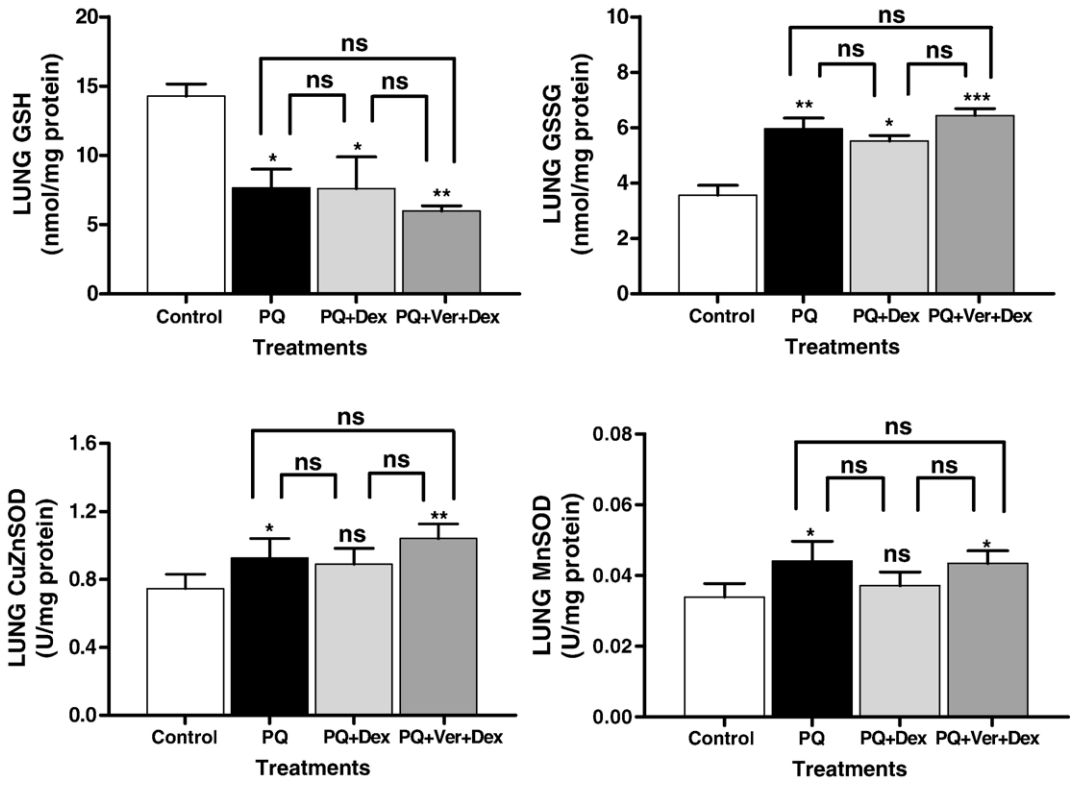

Fig. 6. Characterization of the lung antioxidant defenses. GSH (nmol GSH/mg of protein) and GSSG (nmol GSSG/mg of protein) levels and MnSOD and CuZnSOD activity (U/mg of protein) in the control, PQ, PQ+Dex, and PQ+Ver+Dex groups. Values are given as means±SEM ($n=10$). $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.01$.

of *de novo* synthesis of P-gp by DEX (100 mg/kg ip), 2 h after PQ exposure (25 mg/kg ip), results in a remarkable decrease in PQ lung accumulation (to about 40% of the PQ-only-exposed group in just 24 h) and an increase in its fecal excretion, in Wistar rats. As expected, the decrease in lung PQ levels resulted in the prevention of PQ-induced lung toxicity, which was evidenced by a significant decrease in several biochemical and histopathological biomarkers of toxicity. VER (10 mg/kg ip), a competitive inhibitor of P-gp, given 1 h before DEX, blocked its protective effects and led to an increase in PQ lung concentration (up to about twice that of the PQ-only-exposed group in just 24 h) and toxicity, indicating the important role of this transporter in PQ excretion. As a calcium channel (L-type) modulator, VER is widely used in the treatment of hypertension and certain cardiovascular disorders. Although it might be hypothetically expected that strenuous variations in blood pressure could modify PQ toxicokinetics, it was previously shown that VER (10 mg/kg ip) does not seem to modify the blood pressure in normotensive conscious Wistar rats [40].

Our proposed antidotal mechanism is depicted in Fig. 7. Succinctly, after diffusion through the plasma membrane, DEX binds to the glucocorticoid receptor (GR) in the cytoplasm and causes its release from interactions with heat shock proteins (hsp90s) [41]. Then the receptor undergoes a conformational change and becomes activated. Subsequently the GR/DEX complex translocates to the cell nucleus where it binds as a dimer to DNA at its glucocorticoid response elements (GRE) [41,42]. The interaction of GR–glucocorticoid dimers with the DNA double helix in these GRE regions will lead to an induction of *de novo* synthesis of P-gp [43]. In humans, P-gp involved in multixenobiotic resistance is encoded by *MDR1* [44]. Rodents have two homologues of *MDR1*, *mdr1a* (*mdr3*) and *mdr1b* (*mdr1*). Both *mdr1* gene products are functional xenobiotic transporters but have different substrate preferences and physiological localization [45]. The newly synthesized P-gp will ultimately be inserted into the phospholipid membrane where it can act as an efflux pump of PQ and hence contribute to the protection of lung cells against its toxicity. Although several P-gp inducers have been reported in the literature [46,47], DEX (a synthetic glucocorticoid) was chosen because it is already used in clinical practice to prevent the inflammation in PQ poisonings, although in these cases it has been administered about 4 days after the occurrence of intoxication, at doses that do not induce P-pg expression [48]. According to our results we strongly recommend a pulse therapy with DEX starting as soon as possible after PQ intoxication. In view of literature reports of DEX human pulse therapy, 200 mg perfused intravenously over 1 h seems to be the most approximate dose regimen [49]. DEX is cleared from the circulation within 24 h, so repetitive pulses on subsequent days are not expected to lead to DEX accumulation in the blood [49].

One of the main results of this study was the increase in PQ excretion in feces in rats treated with DEX. It is highly probable that the observed increase in PQ fecal excretion is due to its biliary excretion, because a strong expression of P-gp at the

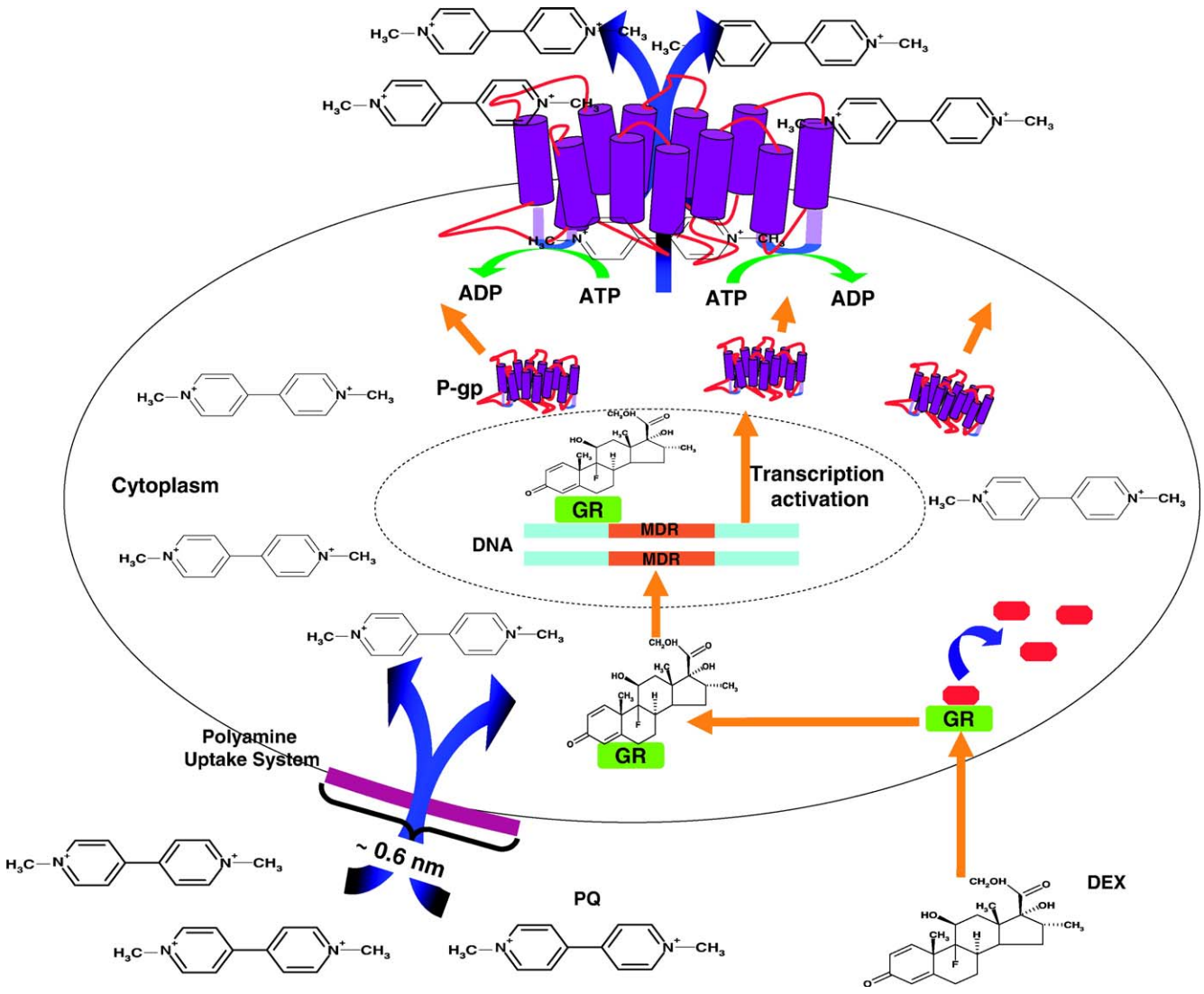

Fig. 7. Proposed scheme for the PQ efflux mediated by P-gp. *Abbreviations used*: GR, glucocorticoid receptor; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; MDR, multidrug resistance gene.

canalicular membrane of hepatocytes [50,51] and PQ excretion in the bile [52] have already reported. Interestingly, P-pg is also expressed at the luminal part of the intestinal mucosa [20,53]. Its physiological function is mainly to avoid the absorption of toxic xenobiotics and/or metabolites. Therefore, the induction of enterocyte P-pg may also gain clinical importance to prevent further PQ absorption after oral intake, although this has still to be tested.

It is well known that injury to the air–blood barrier and impairment of surfactant production in the lung can cause pulmonary edema and collapse of the fine airways. In the present study PQ caused lung edema, observed by the increase of RLW, an effect that was attenuated by DEX. Histopathological analysis confirmed that animals from the PQ group revealed an interstitial edema, indicated by the existence of intercellular vacuolization areas that were characterized by a minor density ultrastructure at TEM. Exuberance of interstitial edema was drastically attenuated in PQ+DEX animals (Figs. 4E and 4F). In a previous study [54], the pretreatment of animals with α-tocopherol liposomes or liposomes containing both α-tocopherol and GSH did not alter significantly the PQ-induced changes in RLW.

In this study, it is highly probable that the anti-inflammatory effect of DEX contributed to its protective effect against PQ-induced lung toxicity. Indeed, according to Hybertson et al. [55], the pulmonary toxicity caused by PQ is assumed to have a connection with the activation of neutrophils. It was previously shown that the treatment of rats with DEX significantly reduced Sephadex-induced recruitment of inflammatory cells to bronchoalveolar fluid [56]. Furthermore, various inflammatory mediators have been found to be increased in the alveolar space during the early phase of acute respiratory distress syndrome, including tumor necrosis factor-α (TNF-α), interleukin-1β, interleukin-6, and chemokines [57]. TNF-α, a potent inflammatory mediator, triggers the synthesis of leukotrienes and prostaglandin E2, which then stimulate the infiltration of polymorphonuclear leukocytes into the lungs. DEX has also been shown to decrease TNF-α concentrations in the

bronchoalveolar lavage fluid of PQ-treated rats to about half those of control animals [58]. Of note, DEX improves gas exchange in PQ-treated animals by alleviation of lung damage after PQ-induced lung injury [58]. DEX presents also an inhibitory effect on ROS production by macrophages and neutrophils [59]. Because neutrophils are recruited to the lungs during the inflammatory reaction generated by PQ exposure, MPO activities were assessed. As expected, our results showed that MPO activity is markedly elevated in the lungs of animals exposed to PQ. Histopathological studies confirmed the widespread neutrophil infiltration into the lungs of these animals. Macrophage infiltration and several NK cells were also identified in the interstitial space (Fig. 4D). DEX clearly reduced the lung infiltration by neutrophils observed in the interstitial space or within capillaries neighboring endothelial cells (Figs. 4E and 4F), an effect that could be attributed both to the anti-inflammatory and to its P-gp-inducing properties. However, taking into account that animals exposed to VER (in addition to DEX) showed a significant increase ($p<0.01$) in MPO activity compared to animals from group PQ+DEX, it is more likely that the observed effect is mainly due to PQ efflux from lungs. In addition, the majority of histological changes detected by LM and TEM from group PQ+VER+DEX were qualitatively and quantitatively identical to those registered in the PQ group.

As reviewed elsewhere [1], PQ toxicity is mainly mediated by oxidative stress as a result of ROS production during its redox cycle. SOD was assessed due to its significance in cellular protection against PQ toxicity by dismutation of $O_2^{\bullet -}$, produced in the reoxidation of the PQ free radical in the presence of oxygen. PQ induced a significant increase in both MnSOD and CuZnSOD activities. Noteworthy, DEX was preventive of MnSOD induction only after PQ exposure, which is in accordance with a cellular adaptation to higher concentrations of PQ and therefore of $O_2^{\bullet -}$ derived from the PQ redox cycle. Histologically, the majority of pneumocytes showed at least one ultrastructural abnormality, mitochondrial swelling being the most frequent alteration (Fig. 4D), which gives credit to the involvement of this organelle in PQ cytotoxicity [60] and provides some rationale for the observed effectiveness of DEX in preventing MnSOD (a mitochondrial enzyme) induction by PQ.

It was also shown that LPO increased significantly in the lungs of rats exposed to PQ compared to the control group. These observations are in agreement with other reports showing that LPO is a biomarker of PQ toxicity *in vitro* [61] and *in vivo* [10,62].

In addition to lipids, ROS are also known to oxidatively modify DNA, carbohydrates, and proteins. One such modification is the formation of carbonyl groups in protein amino acid residues. Free radical damage to proteins has been implicated in the oxidative inactivation of several key metabolic enzymes. Fragmentation of polypeptide chains, increased sensitivity to denaturation, formation of protein–protein cross-linkages, as well as modification of amino acid side chains to hydroxyl or carbonyl derivatives, are possible outcomes of oxidation reactions [63]. Once more, as expected, PQ administration increased the carbonyl group content. In accordance with what would be anticipated from a vital release of PQ from lungs, DEX significantly reduced the lung LPO and protein carbonyl formation.

In this study a severe depletion of lung GSH in PQ-exposed rats compared to the controls was observed. In parallel, the levels of GSSG also increased significantly. Surprisingly, DEX post-treatment or VER+DEX was unable to change PQ-induced oxidation of GSH. A plausible justification for this comes from studies showing that DEX causes a decrease in the activity of γ-glutamylcysteine synthetase, which is the rate-limiting enzyme in GSH synthesis, and glutathione synthetase [64]. GSH, present in high concentrations in lung epithelial lining fluid [65], has an important role in maintaining the integrity of the epithelial air space, both in type II alveolar cells *in vitro* and in lungs *in vivo* [66]. Its depletion has been shown to intensify LPO and predispose cells to oxidative damage [67]. Thus, these effects suggest that in addition to the induction of P-gp it would be wise to continue with the antioxidant therapeutic approaches followed so far [48] in order to maximize the treatment of PQ-intoxicated patients.

Despite the tendency of the lung toxicity to increase in the PQ+VER+DEX group compared to the PQ-only-exposed group, confirmed by the aggravation of carbonyl group content, MPO activity, more pronounced decrease in GSH levels and increase in GSSG levels relative to controls, and, apparently, more severe histopathological damage observed, these results do not fully correlate with the twofold increase in lung PQ concentration observed in the PQ+VER+DEX group, compared to the PQ group. This may be due to the anti-inflammatory effect of DEX, which might be important in preventing the VER-induced aggravation of toxicity. It might be also hypothesized that the lung PQ concentration achieved in the PQ group was enough to cause a depletion of the cellular NADPH pool, required for the redox cycle [68], and thus further increases in PQ accumulation may not be directly correlated with PQ-induced changes in toxicity. Furthermore, the PQ-induced deleterious effects in the first hours post-intoxication would be only mildly affected by VER because it depends on P-gp expression, which is maximal only 24 h after DEX administration [22].

In conclusion, it was demonstrated that the DEX induction of *de novo* synthesis of P-gp is an important antidotal pathway against PQ lung toxicity, mainly mediated by an effective reduction in lung PQ accumulation. On the opposite side, VER caused an increase in lung PQ concentration, probably resulting from the impairment of P-gp-mediated efflux hindrance. The protection here conferred by the P-gp *de novo* synthesis induction was confirmed by an amelioration of practically all toxicological parameters that were changed in the lungs of PQ-challenged rats. Because the DEX dose required to induce P-gp is quite high, this therapeutic approach should be applied in humans only after a careful assessment of its safety in clinical trials. Nevertheless, PQ poisoning is an extremely frustrating condition to manage clinically, due to the elevated morbidity and mortality observed so far, despite therapeutic efforts, which may endorse this type of drastic treatment. Also, further preclinical studies are needed, particularly those aimed to

develop new, specific, and more potent inducers of *de novo* synthesis of P-gp. These inducers may be useful tools in the reduction of systemic exposure and specific tissue access of potential harmful xenobiotics, like PQ.

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