

Single high dose dexamethasone treatment decreases the pathological score and increases the survival rate of paraquat-intoxicated rats

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Abstract

Dexamethasone (DEX), a synthetic corticosteroid, has been successfully used in clinical practice during paraquat (PQ) poisonings due to its anti-inflammatory activity, although, as recently observed, its effects related to *de novo* synthesis of P-glycoprotein (P-gp), may also strongly contribute for its healing effects. The main purpose of this study was to evaluate the effects of a single high dose DEX administration, which induces *de novo* synthesis of P-gp, in the histological and biochemical parameters in lung, liver, kidney and spleen of acute PQ-intoxicated rats. Four groups of rats were constituted: (i) control group, (ii) DEX group (100 mg/kg i.p.), (iii) PQ group (25 mg/kg i.p.) and (iv) PQ + DEX group (DEX injected 2 h after PQ). The obtained results showed that DEX ameliorated the biochemical and histological lung and liver alterations induced by PQ in Wistar rats at the end of 24 hours. This was evidenced by a significant reduction in lipid peroxidation (LPO) and carbonyl groups content, as well as by normalization of the myeloperoxidase (MPO) activities. Moreover, DEX prevented the increase of relative lung weight. On the other hand, these improvements were not observed in kidney and spleen of DEX treated rats. Conversely, an increase of LPO and carbonyl groups content and aggravation of histological damages were observed in the latter tissues. In addition, MPO activity increased in the spleen of PQ + DEX group and urinary *N*-acetyl- β -D-glucosaminidase activity, a biomarker of renal tubular proximal damage, also augmented in this group. Nevertheless, it is legitimate to hypothesize that the apparent protection of high dosage DEX treatment awards to the lungs of the PQ-intoxicated animals outweighs the increased damage to their spleens and kidneys, because a higher survival rate was observed, indicating that DEX treatment may constitute an important and valuable therapeutic drug to be used against PQ-induced toxicity. © 2006 Elsevier Ireland Ltd. All rights reserved.

Keywords: Paraquat; Dexamethasone; Oxidative damage; Rats; Lung; Kidney; Liver; Spleen

Abbreviations: DEX, dexamethasone; DNPH, 2,4-dinitrophenylhydrazine; H₂O₂, hydrogen peroxide; LM, light microscopy; LPO, lipid peroxidation; MDA, malondialdehyde; MPO, myeloperoxidase; NAG, *N*-acetyl- β -D-glucosaminidase; P-gp, P-glycoprotein; PALS, periarteriolar lymphocyte sheath; PQ, paraquat; RKW, relative kidney weight; RLW, relative lung weight; RLiW, relative liver weight; ROS, reactive oxygen species; ROW, relative organ weight; RSW, relative spleen weight; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TEM, transmission electron microscopy; TMB, 3,3',5,5'-tetramethylbenzidine; TNF- α , tumor necrosis factor-alpha; VER, verapamil

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1. Introduction

Since its introduction in agriculture in 1962, the widespread non-selective contact herbicide paraquat (PQ) used as desiccant and defoliant in a variety of crops has caused thousands of deaths in humans from both accidental and voluntary exposure. It may be considered one of the most toxic poisons involved in suicide attempts. Nevertheless, it is readily available without legal restrictions in several countries where it is registered, due to its herbicide effectiveness and its rapid inactivation in the environment.

Since antidotes for PQ are unknown, over the past 60 years strategies in the management of PQ poisonings have been directed towards modification of its toxicokinetics either by decreasing the absorption or by enhancing its elimination (Dinis-Oliveira et al., 2006a). Besides these approaches, additional protective protocols have also been adopted, particularly those aimed to reduce inflammation (Chen et al., 2002). Indeed, it has been proven that the anti-inflammatory corticosteroid therapy reduces morbidity and mortality if used at an early phase of PQ-induced acute lung injury by ameliorating the respiratory mechanisms, lung histology and the structural remodelling of lung parenchyma in rats (Rocco et al., 2003). Dexamethasone [DEX (a synthetic glucocorticoid)] has been successfully used in the clinical treatment of PQ poisonings (Chen et al., 2002; Dinis-Oliveira et al., 2006a), its positive effects being attributed to the down-regulation of neutrophils recruitment, collagenase activity and proliferation of type II pneumocytes (Meduri et al., 1991). Recently, our group demonstrated that the protection afforded with DEX could also be explained by the overexpression of P-glycoprotein (P-gp) in the cytoplasmic membrane, leading to the elimination of PQ from lung cells and subsequent faecal excretion (Dinis-Oliveira et al., 2006b). Currently, these clinical beneficial effects are mainly supported by the subjacent DEX protective mechanisms described in lungs. However, beyond lung, PQ accumulation has also been observed in other organs such as kidney, liver and spleen (Sharp et al., 1972). In addition, histological and biochemical modifications, suggestive of oxidative stress and damage, have also been described in such organs after acute PQ exposure (Akahori et al., 1987; Burk et al., 1980; Lock and Ishmael, 1979; Melchiorri et al., 1996). In fact, with high ingestion doses of PQ (>30 mg/kg in humans), death occurs within 1 week after intoxication resulting from multiple organ failure (Bismuth et al., 1990; Onyeama and Oehme, 1984).

Considering that the extrapulmonary repercussions of DEX therapy in PQ intoxications, relatively to its

biochemical and histological effects, still remain poorly understood, the aim of this work was to provide comprehensive results about the effect of DEX administration on inflammatory reaction, oxidative stress and related damage, assessed by histological and biochemical parameters in lung, liver, kidney and spleen of acute PQ-intoxicated rats. Moreover, it was also our objective to evaluate the overall healing provided by DEX as well as the hypothetic contribution of P-gp *de novo* synthesis to that protection by presenting the survival rate curves.

2. Materials and methods

2.1. Chemicals and drugs

Paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride), dexamethasone [(11 β ,16 α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], 3,3',5,5'-tetramethylbenzidine (TMB), 4-nitrophenyl *N*-acetyl- β -D-glucosaminide, 2-amino-2-methyl-1-propanol hydrochloride and 2,4-dinitrophenylhydrazine (DNPH) were all obtained from Sigma (St. Louis, MO, U.S.A.). The saline solution (NaCl 0.9%), sodium thiopental were obtained from B. Braun (Lisbon, Portugal). 2-Thiobarbituric acid (C₄H₄N₂O₂S), trichloroacetic acid (TCA; Cl₃CCOOH) and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). All the reagents used were of analytical grade or from the highest available grade.

2.2. Animals

The study was performed in two steps, both using adult male Wistar rats (aged 8 weeks) obtained from Charles River S.A. (Barcelona, Spain), with a mean body weight of 252 \pm 10 g. Animals were kept in standard laboratory conditions (12/12 h light/darkness, 22 \pm 2 °C room temperature, 50–60% humidity) for at least 1 week 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. 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 Portuguese laws.

2.3. Experimental protocol for biochemical and histological studies

The biochemical and histological studies were carried out in 32 animals randomly divided into four groups. Each animal was individually housed in a metabolic cage and kept during the experiment (26 h) for whole urine collection. Animals were fasted during the entire experimental period but water was given *ad libitum*.

The four groups were treated as follows (given doses were kg per body weight): (i) control group, $n=8$: animals treated with 0.9% NaCl. Animals received one more administration of 0.9% NaCl 2 h later. (ii) DEX group, $n=8$: animals treated with DEX (100 mg/kg). Animals received one administration of 0.9% NaCl 2 h later. (iii) PQ group, $n=8$: animals intoxicated with PQ (25 mg/kg). Animals received one administration of 0.9% NaCl 2 h later. (iv) PQ + DEX group, $n=8$: animals intoxicated with PQ (25 mg/kg). Two hours later, animals were treated with DEX (100 mg/kg). The schedule of DEX administration was chosen considering the arrival time of the patient to the hospital, after PQ intoxication. The administrations of vehicle (0.9% NaCl), PQ and DEX were all made intraperitoneally (i.p.) in an injection volume of 0.5 ml. PQ dose was similar to that used in previous studies, conducting to severe lung toxicity (Akahori et al., 1987; Rocco et al., 2003). The reported LD₅₀ for rats in the literature is ~18–28 mg/kg of PQ dichloride (Clark et al., 1966).

Treatments in all groups were always conducted between 8:00 and 10:00 a.m.

2.4. Surgical procedures

Twenty-six hours after the first injection, anesthesia was induced with sodium thiopental (60 mg/kg, i.p.). Animals were placed in the *decubito supino* position and abdomen was opened by two lateral transversal incisions and one central longitudinal incision to expose the portal vein. Five animals of each group (biochemical determinations) were perfused *in situ* with ice-cold 0.9% NaCl for 3 min at a rate of 10 ml/min through the portal vein and completely cleaned of blood. In the remaining three animals (histological analysis), organs' perfusion was done with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2–7.4) in order to pre-fixate tissues for further histological analysis. Simultaneously to the perfusion initiation, a cut at the common iliac arteries was done to avoid overpressure.

2.5. Collection and processing samples for biochemical measurements

Organs were removed, pat-dried with gauze, weighted and processed as following: (i) right lung, right kidney, half-spleen and liver right 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 and at pH 7.4. The homogenate was kept on ice, then centrifuged at 3000 × g , 4 °C, for 10 min. Aliquots of the resulting supernatant's were stored (–80 °C) for posterior quantification of myeloperoxidase activity (MPO), carbonyl groups, PQ and protein content. (ii) The left lung, left kidney, half-spleen and liver left lobe were homogenized (1:4 m/v, Ultra-Turrax[®] Homogenizer) in TCA 10%. The homogenate was kept on ice and then centrifuged at 13,000 × g , 4 °C, for 20 min. Aliquots of the resulting supernatants were immediately used for evaluating the lipid peroxidation (LPO) degree.

The relative organ weight (ROW) of each animal was also calculated as a percentage of the absolute body weight at the sacrifice day.

2.6. Biochemical assays

Protein quantification was performed according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

LPO was evaluated by the thiobarbituric acid reactive substances (TBARS) methodology (Buege and Aust, 1978). Results were expressed as nmol of malondialdehyde (MDA) equivalents/mg protein using an extinction coefficient (ϵ) of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Carbonyl groups (ketones and aldehydes) were determined according to Levine et al. (1994). Results were expressed as nanomole of DNPH incorporated per mg of protein ($\epsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

MPO activity was measured according to the method followed by Suzuki et al. (1983) and Andrews and Krinsky (1982), 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 adding 50 μl of hydrogen peroxide [H_2O_2 (final concentration 1.5 mM)] dissolved in phosphate buffer ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 50 mM, pH 5.4). The rate of MPO/ H_2O_2 -catalyzed oxidation of TMB was followed by recording the absorbance increase at 655 nm at 37 °C during 3 min. One enzyme Unit (U) was defined as the amount of enzyme capable to reduce 1 μl of H_2O_2 /min under assay conditions. Results were expressed in enzyme U/g of protein ($\epsilon = 3.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

Urinary *N*-acetyl- β -D-glucosaminidase (NAG) activity was assayed as previously reported (Carvalho et al., 1999), using the molar extinction coefficient of $18.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. One Unit of NAG was defined as the amount of enzyme that releases one μmol of *p*-nitrophenol in the assay conditions. Results were expressed in $\text{U kg}^{-1} \text{ day}^{-1}$.

2.7. Quantification of paraquat in rat kidney, spleen and liver

Aliquots of the right lung and kidney, half-spleen and liver right lobe supernatants were treated with 5-sulfosalicylic acid (5% in final volume) and then centrifuged (13,000 × g , 4 °C for 10 min). The resulting supernatant fractions were alkalized with NaOH 10N (pH >9) and then gently mixed with 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 (Fuke et al., 1992).

2.8. Tissue processing for histological analysis

After the *in situ* prefixation, lungs, liver, kidneys and spleen were removed, sectioned into ~1 mm³ cubic pieces, and sub-

jected to routine procedures for light microscopy (LM) and transmission electron microscopy (TEM) analysis. Fixation was continued (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 carried out at 4 °C, with exception of the inclusion phase, which was performed at 60 °C. Subsequent to the resin polymerization, semi-thin sections (thickening 1 µm) and ultra-thin sections (500 Å of thickness) were prepared (Ultracut, Leica), respectively, for LM and TEM analysis. The grids, mounted with the ultra-thin specimens sections, were double-contrasted with 0.5% saturated uranyl acetate aqueous solution during 30 min and then with 0.2% lead citrate solution for 15 min. The slides, mounted with semi-thin sections, were stained with toluidine blue. Five slides and three grids from each animal (standing 15 slides and 9 grids per group), were examined in a Zeiss Phomi III photomicroscope and in a transmission electronic microscope (Zeiss EM 10A).

Histopathological evidences of acute tissue damage were semi-quantified according to the methodology described elsewhere (Ascensao et al., 2005; Chatterjee et al., 2000; Chen et al., 2003; Duarte et al., 2005). For each group, at least more than 1000 cells per slide and 100 cells per grid were analyzed in a blind fashion in order to semi-quantify 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 total cell number) and grade 3 = diffuse cell damage (higher than 30% of the total cell number). The severity of inflammatory reaction was scored into: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1–3 cells by visual field); grade 2 = moderate infiltration (4–6 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 and 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 and grade 3 = greater of two-thirds of tissue. For each animal, the highest possible tissue score was 12 and the lowest was 0.

2.9. Experimental protocol for the evaluation of survival rate

For the evaluation of survival rate, 24 animals were randomly divided into four groups of 6 animals each. It was

established a control group, a PQ group, a PQ + DEX group and a PQ + VER + DEX group. In this last group, verapamil (VER, a P-gp inhibitor) was included in attempt to assess the DEX contributory effect by induction P-gp *de novo* synthesis.

Animals were kept in a number of three per 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. The control, PQ and PQ + DEX groups were treated as described for biochemical and histological studies, with a slight modification: 1 h after the first injection the animals received an additional 0.9% NaCl i.p. administration. A fourth group, receiving verapamil (VER) was included for this experimental protocol. We have previously demonstrated (Dinis-Oliveira et al., 2006b) that the induction of the P-gp *de novo* synthesis by DEX decreases PQ lung accumulation and consequently its toxicity and also that VER, a competitive inhibitor of this transporter blocked DEX protective effects, causing instead an increase of PQ lung concentration and an aggravation of toxicity. Therefore, to assess the DEX contributory effect by inducing P-gp *de novo* synthesis and thus study the importance of P-gp in the PQ mortality rate, animals of the fourth group (PQ + DEX + VER) were intoxicated with PQ (25 mg/kg) and treated with VER (10 mg/kg) and DEX (100 mg/kg), 1 and 2 h later (i.p., 0.5 ml of 0.9% NaCl), respectively. The survival rate was registered every day until the 10th day.

2.10. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical comparison between groups was estimated using the non-parametric method of Kruskal–Wallis followed by the 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.

3. Results

The exposure of rats to PQ resulted in histological and biochemical changes in liver, kidneys, spleen and lungs.

3.1. Structural and ultrastructural analysis

Lung—major qualitative structural and ultrastructural alterations are depicted in Fig. 1. The respective semi-quantitative analysis is shown in Table 1. Animals from control and DEX groups presented a normal pulmonary structure at LM and TEM, without evidences of alveolar collapse or cellular infiltrations. PQ administration induced marked alterations compared to the control pattern, mainly characterized by a diffuse alveoli collapse with an increased thickness of its walls. An intense vascular congestion with numerous activated platelets (suggested by changes of discoid shape,

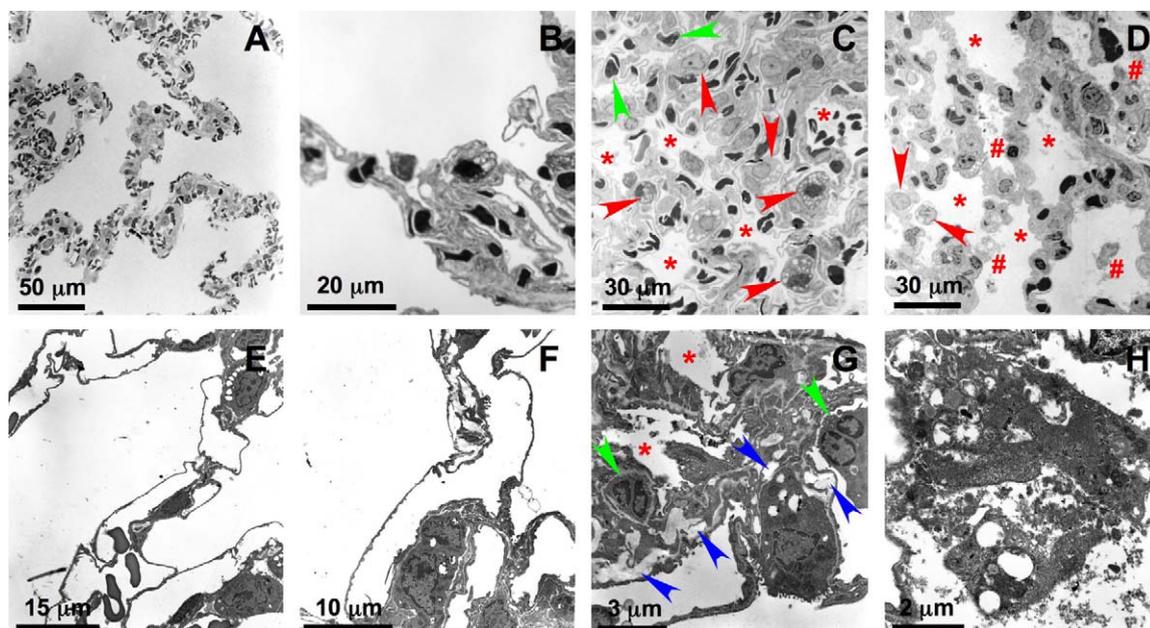


Fig. 1. Optical (above) and electron (below) micrographs from lungs of control (A and E), dexamethasone (B and F), paraquat (C and G) and paraquat plus dexamethasone (D and H) groups. A, B, E and F evidenced a normal structure and ultrastructure with the presence of some pneumocytes type II; C and G depict the alveolar collapse (*) with signs of interstitial edema (blue arrows); several infiltrative macrophages (red arrows) and polymorphonuclear cells (green arrows) adherent to endothelium can also be observed; the alveolar collapse, cellular debris (#) and macrophages in the alveolar space is present in D; In H is depicted a necrotic cell and cellular debris within the alveolar space. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Semi-quantitative analysis of the morphological injury parameters of the control, dexamethasone (DEX), paraquat (PQ) and paraquat plus dexamethasone (PQ+DEX) groups

Organ	Group	Evaluated morphological parameter			
		Cell degeneration	Interstitial inflammatory cell infiltration	Necrotic zones	Tissue disorganization
Lung	Control	0.06 ± 0.06	0.0 ± 0.0	0.0 ± 0.0	0.05 ± 0.05
	DEX	0.09 ± 0.06	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.07
	PQ	2.00 ± 0.15 ^a	2.05 ± 0.18 ^a	1.20 ± 0.09 ^a	2.14 ± 0.20 ^a
	PQ+DEX	1.26 ± 0.10 ^{b,c}	0.11 ± 0.08 ^{b,c}	1.00 ± 0.17 ^b	1.2 ± 0.11 ^{b,c}
Liver	Control	0.17 ± 0.08	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	DEX	0.12 ± 0.06	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	PQ	1.30 ± 0.12 ^a	0.45 ± 0.11 ^a	1.40 ± 0.15 ^a	1.00 ± 0.15 ^a
	PQ+DEX	1.26 ± 0.10 ^b	0.11 ± 0.08 ^{b,c}	1.00 ± 0.17 ^{b,c}	1.2 ± 0.11 ^b
Spleen	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	DEX	0.82 ± 0.18 ^a	0.0 ± 0.0	0.43 ± 0.20 ^a	0.0 ± 0.0
	PQ	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.0 ^a
	PQ+DEX	0.67 ± 0.17 ^c	0.0 ± 0.0	0.44 ± 0.18 ^c	3.0 ± 0.0 ^b
Kidney	Control	0.22 ± 0.15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	DEX	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	PQ	1.00 ± 0.19 ^a	0.57 ± 0.20 ^a	0.88 ± 0.23 ^a	0.50 ± 0.19 ^a
	PQ+DEX	0.91 ± 0.21 ^b	0.40 ± 0.16 ^b	1.22 ± 0.22 ^b	0.56 ± 0.18 ^b

Values are given as mean ± S.E.M. ($n = 3$).

^a $p < 0.05$ vs. control group.

^b $p < 0.05$ vs. DEX group.

^c $p < 0.05$ vs. PQ group.

pseudopodia emissions and degranulation with platelets aggregation, according to Ahnadi et al., 2003) and polymorphonuclear cells inside the capillaries were noticed. The majority of pneumocytes showed, at least, one ultrastructural abnormality, mitochondrial swelling being the most frequent alteration. In the PQ+DEX group, comparatively to PQ group, the occurrence of the above referred alterations were drastically attenuated, particularly the amount of phagocytes observed in interstitial space or within capillaries neighboring endothelial cells. Despite the existence of several pneumocytes with mitochondrial swelling and evidences of interstitial edema, the exuberance of those signals and the ratio of affected cells were drastically attenuated in PQ+DEX animals. Furthermore, comparing to the PQ group, the vascular congestion and the alveolar collapse were not so noticeable in PQ+DEX animals.

Liver—major qualitative structural and ultrastructural alterations are depicted in Fig. 2. The respective semi-quantitative analysis is shown in Table 1. At LM, animals from control and DEX groups exhibited a preserved histological structure. However, a slight cytoplasmic vacuolization identified at TEM as lipid droplets affecting few hepatocytes was observed in both groups. Animals from PQ group evidenced drastic morphological alterations, the closest hepatocytes to arterioles being

the most affected by the cellular degeneration parameters. A wide cytoplasmic vacuolization resulting from intracellular edema and lipid accumulation was observed in these animals. Moreover, extent confluent coagulative necrotic areas and several leukocytes inside sinusoids were also present. The inclusion of DEX in the experimental procedure (PQ+DEX group) attenuated the severity and the incidence of the above referred injuries despite the marked hepatocyte vacuolization adjacent to portal triads.

Spleen—major qualitative structural and ultrastructural alterations are depicted in Fig. 3. The respective semi-quantitative analysis is shown in Table 1. Succinctly, at LM, animals from control group showed normal splenic architecture consisting of areas of white and red pulp in equilibrated proportions. At the periphery of the periarteriolar lymphocyte sheath (PALS) we observed multinuclear macrophages. The treatment with DEX (DEX group) resulted in the disappearance of the white pulp and PALS with a consequent reduction in cellular density. PQ group evidenced an apparent normal histological structure, but with lymphocytes showing clear signs of toxicity namely edema of the endoplasmic reticulum and mitochondrial swelling of the reticular and endothelial cells. Activated platelets were also seen within sinusoids. Histological alterations observed in

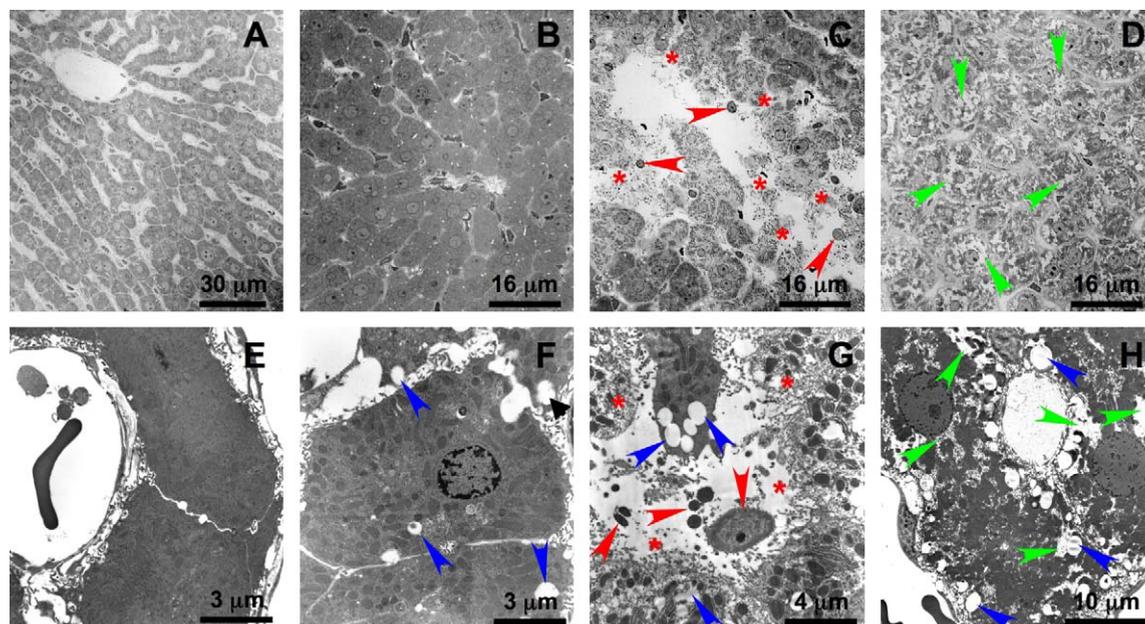


Fig. 2. Optical (above) and electron (below) micrographs from liver of control (A and E), dexamethasone (B and F), paraquat (C and G) and paraquat plus dexamethasone (D and H) groups. A, B, E and F evidenced a normal structure and ultrastructure with some cytoplasmic lipid droplets (blue arrows); C and G depict an extent necrotic area (*), with the presence of organelles (red arrows) in interstitial space and lipid droplets in cytoplasm; D and H shown a diffuse and confluent cytoplasmic vacuolization, suggestive of intracellular edema (green arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

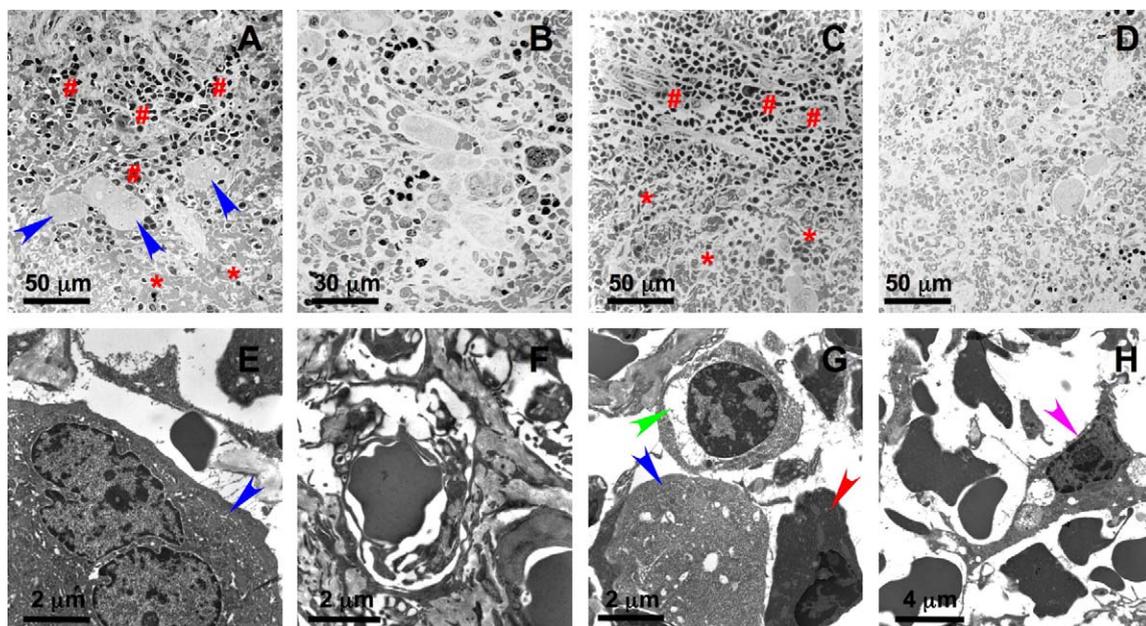


Fig. 3. Optical (above) and electron (below) micrographs from spleen of control (A and E), dexamethasone (B and F), paraquat (C and G) and paraquat plus dexamethasone (D and H) groups. White pulp (#), red pulp (*) and multinuclear macrophages (blue arrows) are observed in A; two nuclei of macrophage are shown in E (blue arrow); B and F evidenced, respectively, the disappearance of the white pulp and the ultrastructure of reticular connective tissue; C depict the a normal white and red pulp and G shows a macrophage, a plasmocytoid cell (red arrow) and a lymphocyte with enlargement of nuclear cisterns (green arrow); D and H show the disappearance of white pulp and a damaged reticular cell (pink arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the PQ + DEX group were similar to those observed in the two previous groups, although with less activated platelets and less endothelial cells exhibiting lesions in comparison to only PQ-exposed group.

Kidney—major qualitative structural and ultrastructural alterations are depicted in Fig. 4. The respective semi-quantitative analysis is shown in Table 1. A regular proximal and distal tubular structure as well as a normal glomerular architecture was registered in the control and DEX groups, although several proximal tubular cells with mitochondrial swelling were observed in DEX group. PQ group evidenced marked tubular lesions, particularly notorious in the proximal tubule with confluent areas of vacuolated cells, apparently resulting from mitochondrial swelling, and coagulative necrosis with a tubular cell loss. Distal tubule was slightly affected. The glomeruli showed moderate alterations affecting endothelial cells. Several infiltrative cells were also observed in interstitial space. In opposition to lung and liver the above referred alterations were not ameliorated by DEX.

3.2. Relative organ weight

Data on the relative weights of lung (RLW), liver (RLiW), spleen (RSW) and kidney (RKW) are present in

Table 2. In comparison to the control group, animals from PQ group showed a significant RLW increase ($p < 0.05$), whereas RLW of DEX-post-treated animals (PQ + DEX group) were near to the control. RLiW in rats from DEX, PQ and PQ + DEX groups were comparable to controls. Animals from the DEX group showed a significant RSW decrease ($p < 0.01$) relatively to control group. RSW in PQ-exposed animals was similar to that of control group. The inclusion of DEX in the PQ treatment (PQ + DEX group) caused a decrease of RSW ($p < 0.01$ versus control group), comparable to the decrease observed in the DEX group. Rats exposed to PQ exhibited an increase of the RKW ($p < 0.05$) that was not reverted by DEX administration ($p < 0.05$ versus control group).

3.3. LPO and carbonyl groups content

As shown in Table 2, animals from PQ group exhibited a significant increase of the LPO in lungs comparing to control group ($p < 0.001$). On the other hand, DEX administration reverted this parameter down to near control levels. Analogous results were obtained for carbonyl groups content (Table 2). Similar profiles were observed for carbonyl groups in the hepatic tissue. LPO and carbonyl groups increased in the kidney of PQ-exposed rats relatively to control group ($p < 0.05$, respectively). In the

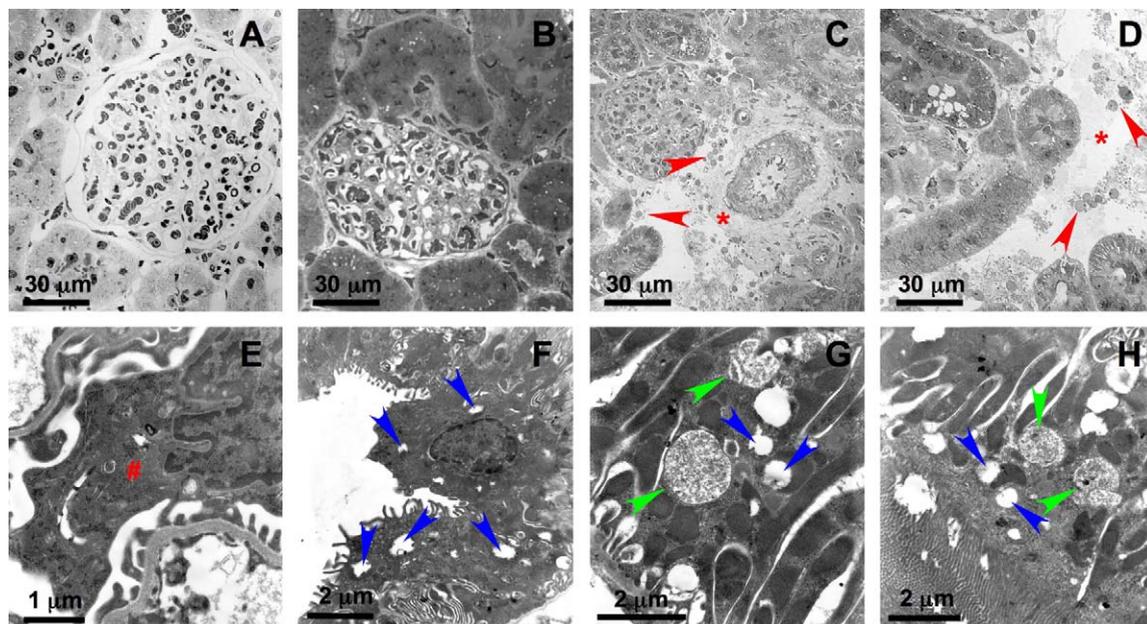


Fig. 4. Optical (above) and electron (below) micrographs from kidney of control (A and E), dexamethasone (B and F), paraquat (C and G) and paraquat plus dexamethasone (D and H) groups. A and B evidenced a glomerular and tubular normal structure while E and F depict, respectively, a podocyte attached to basement membrane (#) and several proximal tubular cells with mitochondrial swelling (blue arrows); in C and D it could be observed a necrotic area (*) with the presence of cell organelles in interstitial space (red arrows); G and H show a cytoplasmic vacuolization of proximal tubular cells resulting from mitochondrial swelling and from the presence of large cytoplasmic vacuoles (green arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spleen, LPO increased in the PQ group in comparison to the control group ($p < 0.05$). Noteworthy are the increase of LPO and carbonyl groups observed in the spleen of DEX group relatively to control group ($p < 0.01$ and 0.05 , respectively), as well as the lack of protective effect of DEX in liver LPO and kidney LPO and carbonyl groups.

3.4. MPO activity

Aliquots of rat organ samples were assayed for the activity of MPO, which is an index of neutrophils sequestration, 26 h after exposure to PQ. As shown in Table 2, lung MPO activity of the PQ-exposed animals was significantly higher (with a $p < 0.05$) than in rats from control group. The post-treatment with DEX, completely prevented the increase of MPO activity. Liver MPO activity revealed similar results to those observed in the lung. In the kidney, an increase of MPO activity in the PQ group in relation to control group was observed ($p < 0.05$), but DEX did not provide any protective effect. MPO expression was also not modified in spleen after PQ exposure, although an increase of its activity was observed in the PQ+DEX relatively to control and PQ group ($p < 0.01$, respectively). The results also showed that DEX led to an increase of MPO activity in the spleen, comparatively to the control group ($p < 0.01$).

3.5. Quantification of paraquat in the rat lung, kidney, spleen and liver

The PQ lung concentration of the PQ group was 0.127 ± 0.010 [(mean \pm S.E.M.), $\mu\text{g}/\text{mg}$ protein]. Animals post-treated with DEX evidenced a significant decrease in PQ lung concentration, down to 0.062 ± 0.008 ($p < 0.05$) (Table 3). The PQ concentration in kidney, spleen and liver of the PQ group did not evidence any significant difference comparatively to PQ+DEX group (Table 3).

3.6. Urinary NAG

NAG urinary excretion was significantly increased 26 h (Fig. 5) after exposure of rats to PQ comparatively to the control group. The administration of DEX (PQ+DEX group) resulted in a further increase of NAG urinary excretion.

3.7. Effect of dexamethasone and verapamil on the survival of paraquat-exposed rats and other observations

Diarrhoea, piloerection, weight loss, anorexia, adipisia, hyperpnea, dyspnea, tachycardia and a red drainage

Table 2

Relative organs weight (ROW) and toxicological parameters of the control, dexamethasone (DEX), paraquat (PQ) and paraquat + dexamethasone (PQ + DEX)

Organ	Group	Evaluated parameter			
		ROW	TBARS (nmol MDA/mg protein)	Carbonyl groups (nmol/mg protein)	MPO (U/g protein)
Lung	Control	0.37 ± 0.01	0.192 ± 0.015	1.860 ± 0.098	21.569 ± 2.232
	DEX	0.36 ± 0.02	0.200 ± 0.020	1.789 ± 0.064	20.067 ± 1.989
	PQ	0.43 ± 0.02 ^{a,b}	0.485 ± 0.033 ^{aaa,bbb}	2.254 ± 0.135 ^{a,b}	29.143 ± 1.915 ^{a,b}
	PQ+DEX	0.36 ± 0.02	0.274 ± 0.039 ^{cc}	2.013 ± 0.193	20.621 ± 2.565 ^c
Liver	Control	4.14 ± 0.11	0.135 ± 0.037	2.020 ± 0.301	11.854 ± 0.549
	DEX	4.01 ± 0.10	0.130 ± 0.040	1.969 ± 0.298	11.278 ± 0.860
	PQ	3.90 ± 0.21	0.200 ± 0.055 ^{a,b}	2.921 ± 0.183 ^{a,b}	15.875 ± 0.975 ^{a,b}
	PQ+DEX	4.08 ± 0.15	0.182 ± 0.082	2.342 ± 0.202	12.984 ± 1.034 ^c
Spleen	Control	0.25 ± 0.01	0.271 ± 0.026	0.582 ± 0.041	75.777 ± 1.298
	DEX	0.18 ± 0.02 ^{aa}	0.441 ± 0.031 ^{aa}	0.652 ± 0.053 ^a	80.201 ± 2.890 ^a
	PQ	0.27 ± 0.03	0.322 ± 0.039 ^{a,b}	0.594 ± 0.072 ^b	73.532 ± 1.927 ^b
	PQ+DEX	0.18 ± 0.01 ^{aa,cc}	0.537 ± 0.105 ^{aa,cc}	0.663 ± 0.091 ^{a,c}	82.939 ± 1.282 ^{aa,cc}
Kidney	Control	0.51 ± 0.01	0.712 ± 0.064	3.110 ± 0.191	6.447 ± 0.204
	DEX	0.52 ± 0.01	0.734 ± 0.039	3.087 ± 0.143	6.767 ± 0.239
	PQ	0.56 ± 0.02 ^{a,b}	0.925 ± 0.169 ^{a,b}	3.932 ± 0.129 ^{a,b}	8.855 ± 0.586 ^a
	PQ+DEX	0.57 ± 0.01 ^{a,b}	1.056 ± 0.223 ^{aa,bb}	3.987 ± 0.152 ^{a,b}	8.448 ± 0.347 ^a

Values are given as mean ± S.E.M. (*n* = 5).^a *p* < 0.05 vs. control group.^{aa} *p* < 0.01 vs. control group.^{aaa} *p* < 0.001 vs. control group.^b *p* < 0.05 vs. DEX group.^{bb} *p* < 0.01 vs. DEX group.^{bbb} *p* < 0.001 vs. DEX group.^c *p* < 0.05 vs. PQ group.^{cc} *p* < 0.01 vs. PQ group.

around the mouth, eyes and nose were present especially in animals exposed to PQ and PQ + VER + DEX during the first 48 h. During the same experimental period, rats belonging to PQ + VER + DEX group did not ingest any amount of water and only a few milliliters were ingested by rats of PQ group. Deep breathing was observed and the thorax was sunken in the animals from PQ and PQ + VER + DEX groups in contrast to those belonging to control, DEX or PQ + DEX-

Table 3

PQ lung, kidney, spleen and liver concentration in the paraquat (PQ) and paraquat plus dexamethasone (PQ + DEX) groups

Organ	PQ levels (µg/mg protein)	
	PQ	PQ + DEX
Lung	0.129 ± 0.062	0.062 ± 0.008 ^a
Kidney	0.029 ± 0.005	0.033 ± 0.011
Spleen	0.015 ± 0.005	0.016 ± 0.003
Liver	0.008 ± 0.001	0.008 ± 0.001

Values are given as mean ± S.E.M. (*n* = 5).^a *p* < 0.05 vs. PQ group.

treated groups. Rats exposed only to PQ (PQ group) displayed approximately, 25 and 100% of mortality by the 2nd and 6th day, respectively (Fig. 6). Hundred percent of mortality was observed by the 4th day in the group PQ + VER + DEX. Post-treatment of PQ-

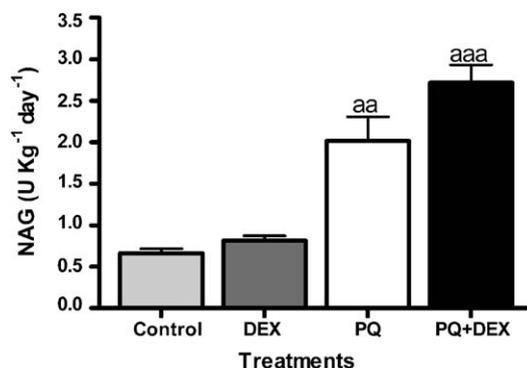


Fig. 5. Urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity in the control, paraquat (PQ) and paraquat plus dexamethasone (PQ + DEX) groups. Values are given as mean ± S.E.M. (*n* = 8). ^{aa}*p* < 0.01 and ^{aaa}*p* < 0.001 vs. control.

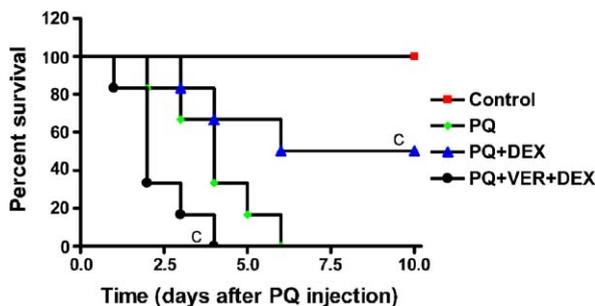


Fig. 6. Percentage of rat survival in the control, paraquat (PQ), paraquat plus dexamethasone (PQ+DEX) groups and paraquat plus verapamil and dexamethasone (PQ+VER+DEX) groups. ^c $p < 0.05$ vs. PQ group.

exposed rats with DEX (PQ+DEX group) resulted in a significant enhancement of the survival time (50% of survival at 10th day, $p < 0.05$). Logrank test showed significant differences between the survival curves of PQ versus PQ+DEX and PQ+VER+DEX ($p < 0.05$, respectively).

4. Discussion

The main objective of the present study was to assess the effect of DEX in PQ-exposed rats, in the lung as well as in other organs and systems, but also to support our hypothesis that the protective effect of DEX against PQ toxicity might, at least, be partially mediated by P-gp functionality. Lung was undoubtedly the most affected organ, which is in accordance to the accumulation of PQ in this organ through a highly developed polyamine uptake system (Dinis-Oliveira et al., 2006c; Nemery et al., 1987; Rannels et al., 1989). As we showed previously (Dinis-Oliveira et al., 2006b) the high dose of DEX used in the present study leads to a stunning increase of lung P-gp expression. The involvement of P-gp in decreasing PQ-lung concentration is evidenced by the lowering effect on PQ levels mediated by DEX and the respective inhibition by VER, a competitive inhibitor of this transporter. In this study, we observed that this therapeutic approach conducts to an increase in the survival rate of animals belonging to PQ+DEX group in comparison to animals only exposed to PQ.

The increase of the RLW (Table 2) and the presence of interstitial edema evidenced by histopathological analysis (Fig. 1) confirmed that PQ induced lung edema, an effect that was drastically attenuated in PQ+DEX treated animals. The cellular damage mediated by PQ is essentially due to its redox-cycle leading to continuous superoxide radicals ($O_2^{\bullet-}$) production (Bus et al., 1974). This then sets in the well-known cascade leading

to generation of the hydroxyl radical (HO^{\bullet}) (Youngman and Elstner, 1981), which has been implicated in the initiation of membrane injury by lipid peroxidation (LPO) during the exposure to PQ *in vitro* (Bus et al., 1975) as well as *in vivo* (Chen and Lua, 2000). Besides, researchers have been suggesting the hypothesis of cytotoxicity via mitochondrial dysfunction caused by PQ (Dinis-Oliveira et al., 2006d; Fukushima et al., 1994). We have previously demonstrated that the increase of LPO induced by PQ-exposure was significantly reduced by DEX (Dinis-Oliveira et al., 2006b). The present study corroborates those results. Besides lipids, ROS are also known to oxidatively modify DNA, carbohydrates and proteins. 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 protein oxidative reactions (Dean et al., 1997). Accordingly, it was also shown that PQ administration increased the carbonyl groups content in lung and in accordance to previous results (Dinis-Oliveira et al., 2006b), DEX protected against PQ-induced increase of carbonyl groups content. In the present study, the histopathological findings confirmed that PQ induced marked alterations to the normal pattern of lung, with majority of pneumocytes showing, at least, one ultrastructural abnormality, mitochondrial swelling being the most frequent alteration. These morphological evidences of cellular aggression were again attenuated by DEX-treatment, results evidenced by qualitative and quantitative analysis of the morphological injury (Table 1 and Fig. 1). In addition, the reduced amount of activated platelets within the capillaries observed in DEX-treated animals might be interpreted as a consequence of endothelial cells protection against PQ-toxicity.

Considering the liver, RLW measurements did not reveal any difference between the experimental groups, although a wide cytoplasmic vacuolization was observed in the PQ group. Besides the low PQ concentrations quantified in this organ, necrotic zones and tissue disorganization were notorious in PQ-exposed animals, particularly surrounding centrilobular region. The more susceptibility of this region can be explained by its higher concentration in NADPH-cytochrome *P*-450 reductase (Jungermann and Kietzmann, 1996), essential to PQ redox-cycle (Clejan and Cederbaum, 1989) and consequent oxidative stress propagation. Moreover, although the PQ elimination occurs mainly through kidneys, the biliar excretion (Dinis-Oliveira et al., 2006b; Hughes et al., 1973) may also have contributed to the discrepancy observed between PQ concentrations and the extent of the lesions in this organ. Similar histopathological

results were also previously described in animals and humans (Burk et al., 1980; Parkinson, 1980). However, our results showed, for the first time, that DEX significantly reduced signs of cell degeneration, interstitial inflammatory cell infiltration, necrotic zones and tissue disorganization in the liver of PQ-exposed rats. Additionally, and as it was previously observed in lung, hepatic alterations in the LPO and protein carbonylation, were correlated with the extent of histological damage.

Regarding the spleen, no significant changes were observed for RSW between control and PQ group. Taking into account that one of the major functions of the spleen is to remove damaged erythrocytes, and since PQ proved to damage erythrocytes by altering its antioxidant status (Hernandez et al., 2005), it is expected that injured erythrocytes will be ultimately scavenged by the spleen, generating ROS and subsequent tissue injury. In the present study, LPO increased in the spleen of PQ-exposed rats in relation to control group. According to that, qualitative and quantitative analysis of the morphological injury revealed tissue disorganization of PQ-exposed rats, mitochondrial swelling of the reticular and endothelial cells being the most significant alteration observed in this group. Interestingly, a decrease of the RSW was observed in the PQ + DEX group, which may be due to a consequence of spleen atrophy caused by DEX (Orzechowski et al., 2002). Our results also showed that DEX, by itself, reduced RSW and caused the disappearance of the white pulp (DEX group). Since white pulp reflects T-cell mass, such effect probably corresponds to the immunosuppressive effectiveness of DEX, that it is in accordance with current therapeutic guidelines to prevent pulmonary fibrosis (Mason et al., 1999).

The lysosomal enzyme NAG is generally regarded as an indicator of renal tubular dysfunction and disease (Price, 1982). In this work, the increase of NAG urinary excretion induced by PQ was accompanied by an increase in LPO, protein carbonylation, proximal tubular damage, coagulative necrosis and tubular cell loss. Similar results were also documented by Murray and Gibson (1972). Urine is the main excretion via of PQ and this toxicity seems to result from intracellular redox-cycle generated by PQ in proximal tubules (Lock and Ishmael, 1979). Noteworthy were the results observed in the PQ + DEX exposed rats. Unexpectedly, DEX aggravated PQ-induced kidney toxicity, leading to an increase of LPO and protein carbonylation. In accordance, NAG urinary excretion steeply increased and RKW did not ameliorate in the PQ + DEX group relatively to PQ group. This lack of kidney protection caused by DEX in PQ-exposed rats might be the consequence of the organ specificity regarding the P-gp expression as consequence

of DEX treatment. Indeed, while DEX increases P-gp expression in liver and lung, it has an opposite effect in the kidney (Demeule et al., 1999). In this way, less P-gp expression by DEX in the kidneys will cause an extended presence of PQ in the proximal tubules and consequently, more damage and urinary NAG release will take place. Nevertheless, although a tendency for higher PQ levels (~11%) was observed in the kidney of PQ + DEX group, comparatively to the PQ group, the non-significance of this result indicates that other mechanisms are probably involved.

It should be considered that the observed DEX protective effects against PQ-induced lung and liver toxicity may also result from its anti-inflammatory effects. Indeed, according to Hybertson et al. (1995), the toxicity provoked by PQ is assumed to be associated with the activation of neutrophils. Furthermore, various inflammatory mediators have been found to be increased in the alveolar space during the early phase of ARDS, including tumor necrosis factor- α (TNF- α), interleukin-1 β , interleukin-6 and chemokines (Pugin et al., 1999), which stimulate the infiltration of polymorphonuclear leukocytes (PMN) into the lungs. DEX has been shown to decrease TNF- α concentrations in the bronchoalveolar lavage fluid of PQ treated rats (Chen et al., 2001). DEX presents also an inhibitory effect on ROS production by macrophages and neutrophils (Maridonneau-Parini et al., 1989). Since MPO is located within the primary azurophil granules of PMN, its activity indirectly reflects PMN infiltration through the organs (Schultz and Kaminker, 1962) during the inflammatory reaction. As expected, our results showed that MPO activity is markedly elevated in lung, liver and kidney of PQ-exposed animals. Our histopathological results confirmed the widespread neutrophils infiltration in these organs. DEX administration caused a significant decrease of the interstitial inflammatory cell infiltration score, in lung and liver, of animals exposed to PQ.

Despite the beneficial effects observed in the lungs and liver of PQ-intoxicated rats treated with DEX, this protection appears not be achieved in the kidney and spleen. In fact, this study confirmed once more that PQ poisoning is an extreme frustrating condition to manage. In attempt to verify the contribution of these apparent contradictory results to the final outcome we assessed the survival rate of this approach. If still some doubts existed, DEX proved to increase the survival rate by shifting the time course of deaths (Fig. 6). Giving credit to this protection, VER showed the tremendous contribution of P-gp functionality to the final outcome. Indeed rats that received VER prior to DEX (PQ + VER + DEX group) died within 48 h, faster than rats that were only PQ-

exposed (PQ group). On the other hand, only PQ + DEX group had animals that survived beyond the 5th day, with 50% rats remaining alive by the 10th day. It is important to focus that this improvement in the survival rate was obtained with only a single dose of DEX. It might be supposed that repetitive DEX therapy could extend survival time and allow a lung transplant to be performed in the PQ-poisoned patients. Following these encouraging results, further studies are needed to clarify these protective effects.

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