Establishing the Use of Melatonin as an Adjuvant Therapeutic Against Paraquat-Induced Lung Toxicity in Rats

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It is well known that the intake of paraquat (PQ) causes severe tissue injury leading to numerous fatalities. Considering that the main target for PQ toxicity is the lung and involves the production of reactive oxygen and nitrogen species, transcription factors and inflammatory cytokines, it may be hypothesized that the combination of a potent antiinflammatory and antioxidant agent may counteract more of PQ's effects than an antiinflammatory agent alone. For this purpose, combination of dexamethasone (Dex) and melatonin (Mel) was compared with Dex alone. A total of 40 male Wistar albino rats were divided into four groups as control, PQ, Dex only, and Dex plus Mel. The animals were given intraperitoneally a toxic dose of 19 mg/kg PQ dissolved in 1 ml saline. Control animals were injected with the same amount of saline only. A dose of 1 mg/kg Dex was administered 2 hrs after PQ administration. In the combination treatment group, 20 mg/kg Mel was given with Dex. All drugs were given every 12 hrs for a total of six doses. Five animals in PQ group and three animals in Dex only group died by the end of the study. No deaths occurred in the Dex+Mel group. Dex exerted improvements in several oxidative and antioxidative parameters. However, combination treatment provided beneficial effects against PQ toxicity far greater than Dex alone. This difference was also apparent when tissues were histologically compared. In conclusion, Mel exhibited strong additive beneficial effects with Dex and can be considered as a safe treatment modality against PQ toxicity. Exp Biol Med 233:1133–1141, 2008

Key words: paraquat; lung damage; nitrosative stress; oxidative stress; dexamethasone; melatonin

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Introduction

Paraquat (PQ), a quaternary nitrogen herbicide, is a highly toxic substance for humans and animals with many cases of acute poisoning and death having been reported. PQ causes extensive damage to lungs, kidneys, and liver (1, 2). Death occurs primarily as a consequence of alveolar epithelial cell (type I and II pneumocytes) and bronchiolar cell disruption, hemorrhage, edema, hypoxemia, infiltration of inflammatory cells into the interstitial and alveolar spaces, proliferation of fibroblasts and excessive collagen deposition, and finally as a result of a disseminated intravascular coagulation (3).

The toxicity of several drugs including PQ, doxorubicin (4), mustards (5–7), and others has been shown to share a similar pathophysiological mechanism. This may be summarized as the generation of the superoxide radical leading to the production of other reactive oxygen species (ROS) (8, 9) and induction of inducible nitric oxide synthase (iNOS) resulting with peroxynitrite formation (10). Reactive oxygen molecules and subsequent oxidative stress are likely to contribute to PQ-induced lung toxicity. The role of superoxide in PQ-mediated cytotoxicity has been confirmed in studies showing protection against the radical by overexpression of superoxide dismutase (SOD) activity or treatment with SOD mimetics (11, 12). Recent results also indicate that nitric oxide (NO) plays a critical role in PQ-caused lung injury (13, 14). Several reports indicate that the toxicity of PQ is partially inhibited by blocking nitric oxide synthase (NOS) activity (15–17). Excess superoxide and NO readily react with each other to form peroxynitrite. Activation of this "devil's triangle" may be the core of the pathophysiological process of PQ toxicity (4, 6).

Furthermore, PQ itself, ROS and peroxynitrite induce intracellular transcription factors such as nuclear factor (NF)-κB and activator protein-1. NF-κB induces many proinflammatory genes including iNOS, several cytokines, and

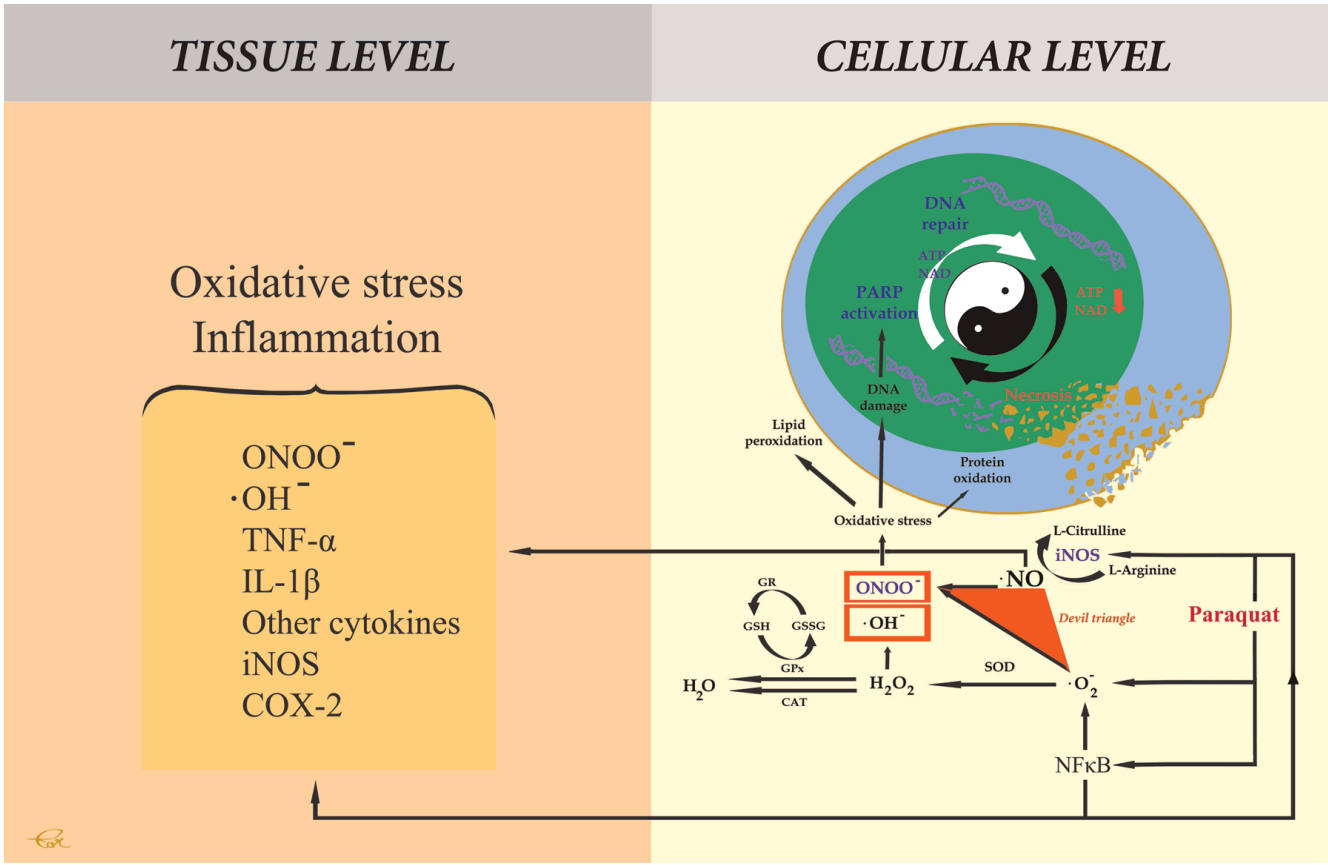

Figure 1. Summary of rationalizing the Dex+Mel combination regarding proposed mechanism. PQ exposure results in severe oxidative and nitrosative stress in cells. This nitro-oxidative stress causes cell necrosis *via* lipid peroxidation, protein oxidation, and DNA damage leading to an energy crisis. NF-κB induction propagates the reaction and spreads out the nitro-oxidative and inflammatory reaction into tissue. Intracellular enzymatic and other antioxidant defense mechanisms protect the cells from nitro-oxidative stress. NF-κB inhibitors have a crucial role to restrict the reaction. Dexamethasone has the ability to act as a multifunctional antiinflammatory agent including NF-κB, iNOS, COX-2, and further cytokine inhibitory effects. Melatonin is a multifunctional antioxidant, including stimulating the mRNA levels of SOD and GPx (28–30), scavenging all oxygen- and nitrogen-based reactive species as well as multifunctional capacity of NF-κB, iNOS, COX-2 inhibition (31, 32). A color figure is available in the online journal.

cyclooxygenase-2 (COX-2) all of which exaggerate the inflammatory process (18). It is documented that antiinflammatory corticosteroid therapy reduces morbidity and mortality when used at an early phase of PQ-induced acute lung injury by ameliorating the respiratory mechanisms, lung histology, and the structural remodeling of lung parenchyma (19). Dexamethasone (Dex) (a synthetic glucocorticoid) has been successfully used in the clinical treatment of PQ poisonings (20, 21); its positive effects are attributed to its multifunctional antiinflammatory properties (22). Another multifunctional agent, melatonin, has shown beneficial effects against PQ and diquat toxicity (23–25). These effects can be attributed to the potent antioxidative and antiinflammatory features of this molecule (26, 27). A proposed mechanism of PQ toxicity and how a combination of Dex and melatonin may exert more beneficial effects is summarized in Figure 1. In the current report, the potential beneficial effects of melatonin in combination with Dex were investigated.

Materials and Methods

Animals. A total of 40 male albino Wistar rats weighing between 200 and 230 g were divided into four groups ($n = 10$ for each) using the "simple random sampling method." They were given food and water *ad libitum*. Our institutional animal care and use committee approved the experimental protocol.

Chemicals. All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), and all organic solvents were from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade, were prepared fresh daily (except the phosphate buffer), and stored in a refrigerator at 4°C. The reagents were equilibrated at room temperature for 0.5 hr before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at 4°C for 1 month.

Experimental Design. The animals were injected intraperitoneally (ip) with a toxic dose of 19 mg/kg PQ dissolved in 1 ml saline. The PQ dose used in this experiment has been previously found to kill 50% of the

animals (LD_{50}) within 7 days. Control animals were injected with saline only. A dose of 1 mg/kg Dex was administered 2 hrs after PQ application. In the combination group, 20 mg/kg melatonin (Mel) was used with Dex. All drugs were given every 12 hrs for a total of six doses. Of importance, taking into account the arrival time of poisoned patients in hospital emergency rooms, both Dex and Mel were started 2 hrs after PQ exposure, consistent with the delay that often occurs after PQ exposure. After Dex and Mel treatments were discontinued, the animals were allowed to survive for an additional 96 hrs. The total experiment duration took 7 days.

Tissue Preparation. After the 7-day experimental period, all animals were anesthetized with ketamine (85 mg/kg) and xylazine (12.5 mg/kg) and their thoracic cavities were opened. The lung parenchyma was flushed with 10 ml of ice-cold physiological saline *via* a right ventricular puncture to the heart to remove blood from tissues and to slow the metabolism (33). Then the lungs were removed immediately; right lungs were fixed in 10% buffered formaldehyde for histopathologic evaluation and the left lungs were washed with saline to remove residual blood, placed into tubes, frozen with liquid nitrogen and stored at –70°C. The frozen lung tissues were thawed and homogenized in phosphate buffer (pH 7.4) using a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany). The supernatant was divided into two to three aliquots, placed in separate tubes, and stored at –70°C.

Tissue Oxidative/Nitrosative Stress Evaluation. Oxidative stress and antioxidant enzymes were detected in lung tissue as described previously (34, 35). In brief, first the protein content of the lung was assayed so the subsequent data could be expressed per tissue protein. Lipid peroxidation was measured with the thiobarbituric acid (TBA) reaction. This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to TBA with malondialdehyde (MDA) at 535 nm. The final estimated MDA levels were presented as mmol/g of protein in lung tissue. Copper/zinc-superoxide dismutase (Cu/Zn-SOD) activity was assayed using the nitroblue tetrazolium (NBT) method, in which NBT was reduced to formazan by the superoxide radical, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity was expressed as U/mg of protein. Glutathione peroxidase (GPx) activity was measured using a method in which GPx was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed up at 340 nm at 37°C. The absorbance at 340 nm was recorded for 5 mins. The activity was the slope of the lines as mmol of NADPH oxidized per minute. GPx activity was expressed as U/mg of protein. Inducible NOS activity was determined as previously described (36). In brief, tissue iNOS activity was measured by determining the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline and the estimated NOS activity was expressed as pmol citrulline per mg protein per minute.

Urinary NO_x and 8-Isoprostane Measurement. During the study, all animals were placed in metabolic cages to collect 24-hour urine samples. Urine was used to measure nitrites plus nitrates (NO_x; end products and indicator of nitric oxide metabolism) *via* ion chromatography (Dionex, Sunnyvale, CA) and 8-isoprostane (indicator of lipid peroxidation) levels with the corresponding enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemicals, Ann Arbor, MI).

Histopathological Evaluation. The formaldehyde-buffered lung and kidney tissues were embedded in paraffin and at least seven cross-sections were taken from each lung in 4–5 μm thickness; the sections were stained with hematoxylin and eosin (H&E). Histopathological examination was performed by a pathologist blinded to the study groups.

Statistical Analysis. The results are expressed as mean ± standard deviations for urine measurements (NO_x and 8-isoprostane) and as median and range (minimum-maximum) for lung tissue parameters (iNOS, MDA, GPx, and SOD) and $P < 0.05$ was accepted as statistically significant. All numeric data were analyzed first using the Kruskal-Wallis test to find whether there was a difference between groups and then the Mann-Whitney *U* test was performed to analyze the two groups consecutively. Time-dependent changes within study groups (for urinary NO_x and 8-isoprostane values) were evaluated using the Friedman test.

Results

In the PQ only group, 5 of 10 animals died by day 7; three of them died within the first 24 hrs after PQ injection and the last two died by 72 hrs. In the Dex only treated group, only three animals died; all deaths occurred after the cessation of Dex treatment. No other deaths were recorded (Fig. 2).

Nitrosative Stress Markers. Urinary NO_x levels were measured daily and are illustrated in Figure 3A. In all groups, PQ caused an increase in urinary NO_x levels. High NO_x levels were sustained in PQ only group until the end of the study (5.6 ± 0.34 vs. 1.06 ± 0.15 mg/dl in control animals at day 7; $P < 0.05$). The NO_x decrement became apparent after the second day in PQ+Dex group and again increased after final dose of Dex (4.6 ± 0.39 mg/dl; $P < 0.05$ vs. control). In rats given Dex+Mel, a moderate increase in urinary NO_x was measured only with PQ treatment; NO_x levels did not increase after cessation of Dex+Mel (1.1 ± 0.19 mg/dl; $P > 0.05$ vs. control, $P < 0.05$ vs. PQ group).

Lung iNOS levels at day 7 were highest in PQ group (Fig. 4A) (27.88 [21.68–35.12] pmol citrulline/mg protein/min; $P < 0.05$ vs. control). Sustained elevated iNOS levels were also encountered in Dex treated rats (23.57 [19.48–30.15] pmol citrulline/mg protein/min; $P < 0.05$ vs.

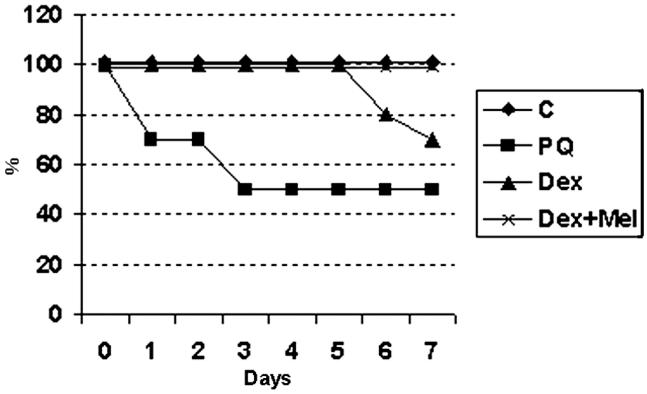

Figure 2. The percentage of survivors in the groups. Three animals died in the early days and an additional two deaths occurred later in the PQ group. Three deaths in the Dex group occurred after cessation of the drug. In the Dex+Mel combined and the control groups, no deaths were seen.

control). Conversely, Dex+Mel resulted in reduced iNOS expression (5.02 [2.28–8.41] pmol citrulline/mg protein/min; $P < 0.05$ vs. PQ group). iNOS expression and urinary NO_x levels were near normal (1.44 [0.83–1.77]) in Dex+Mel treated rats.

Oxidative Stress Markers. Urinary 8-isoprostane was dramatically increased by PQ treatment (Fig. 3B) (602 ± 38.1 vs. 118 ± 20.8 pmol/ml in control animals at day 7; $P < 0.05$). Dex treatment caused a slight reduction, but after the fourth day they tended to increase (801 ± 45.3 pmol/ml; $P < 0.05$ vs. control). Combination of Dex+Mel successfully suppressed 8-isoprostane levels, and reduced levels continued until the study ended (204 ± 45.3 pmol/ml; $P > 0.05$ vs. control, $P < 0.05$ vs. PQ group).

MDA levels of lungs were highly elevated in the PQ only rats and in those given Dex (0.89 [0.77–0.97] and 0.81 [0.75–0.89] mmol/g protein, respectively; $P < 0.05$ vs. control for both). MDA levels in Dex+Mel combination treated rats were significantly lower than either the PQ and Dex groups (0.64 [0.56–0.67] m*M*/g protein; $P < 0.05$ vs. both PQ and Dex groups). The value for the Dex+Mel treatment rats did not differ from that of the untreated controls ($P > 0.05$ vs. control values = 0.56 [0.54–0.61] mmol/g protein) (Fig. 4B).

Lung Antioxidant Enzymes. The antioxidant enzyme SOD at day 7 was still higher in all groups vs. control: 10.11 (8.59–12.97), 9.25 (8.11–10.54), and 10.69 (8.12–12.76) U/g protein for PQ, Dex, and Mel+Dex groups, respectively, against control values of 6.5 (5.48–7.11) U/g protein ($P < 0.05$ for all). GPx activity, however, was depressed with PQ only and Dex-treated rats (30.76 [25.65–38.88] and 31.76 [29.33–37.34] U/g protein for PQ and Dex, respectively; $P < 0.05$ vs. control values of 50.41 [45.34–56.23]). Dex+Mel combination treatment elevated GPx levels more than Dex only (49.03 [42.37–53.12] U/g protein; $P < 0.05$ vs. PQ group) (Fig. 4C and D).

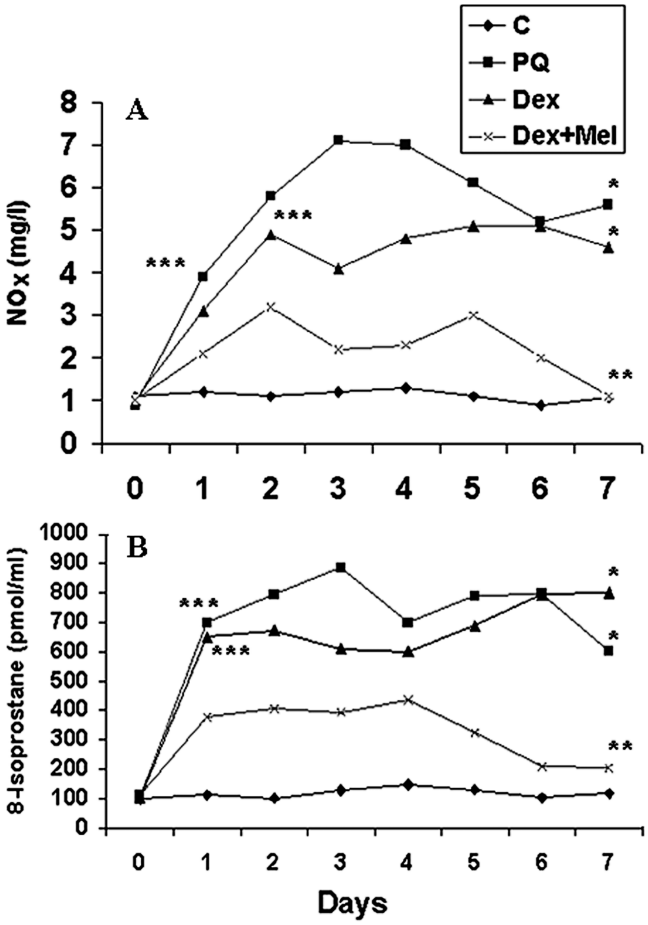

Figure 3. Urinary NO_x and 8-isoprostane levels (means) during study. (A) NO_x levels of the PQ group continued to increase up to the third day, and afterward tended to decrease. Similar but lower results appeared in the Dex group. However, after cessation, NO_x levels tended to a moderate elevation. In the combination group, NO_x levels elevated moderately and did not continue to elevate after cessation of the drugs. Hypothetically, melatonin metabolites may be not only antioxidants but also iNOS inhibitors. Another explanation may be that reduced tissue damage can be accompanied by reduced iNOS induction. (B) In the PQ group, the levels remained higher at all times. The Dex group showed decreased levels first, then continued with compromised results. The Dex+Mel combination group showed lower levels during the study. *$P < 0.05$ vs. control, **$P < 0.05$ vs. PQ, ***$P < 0.05$ within group.

Histopathological Outcome. Histopathological evaluation of rat lung following PQ revealed that the development of lesions predominantly appeared in parenchymal region. There was severe inflammatory cell infiltration and diffuse alveolar collapse. Thickened alveolar walls were also common. In addition, significant edema and alveolar hemorrhage were observed after PQ treatment (Fig. 5). Lung parenchyma showed inflammation including neutrophil infiltration into the airways and alveoli. The significant lung lesions included congestion, hemorrhage, and accumulation of hemorrhagic exudate, parenchymal debris, and pulmonary edema. All treatments improved lung morphology; in Dex group, inflammatory cells and edema were less when compared with PQ only. Dex+Mel treatment

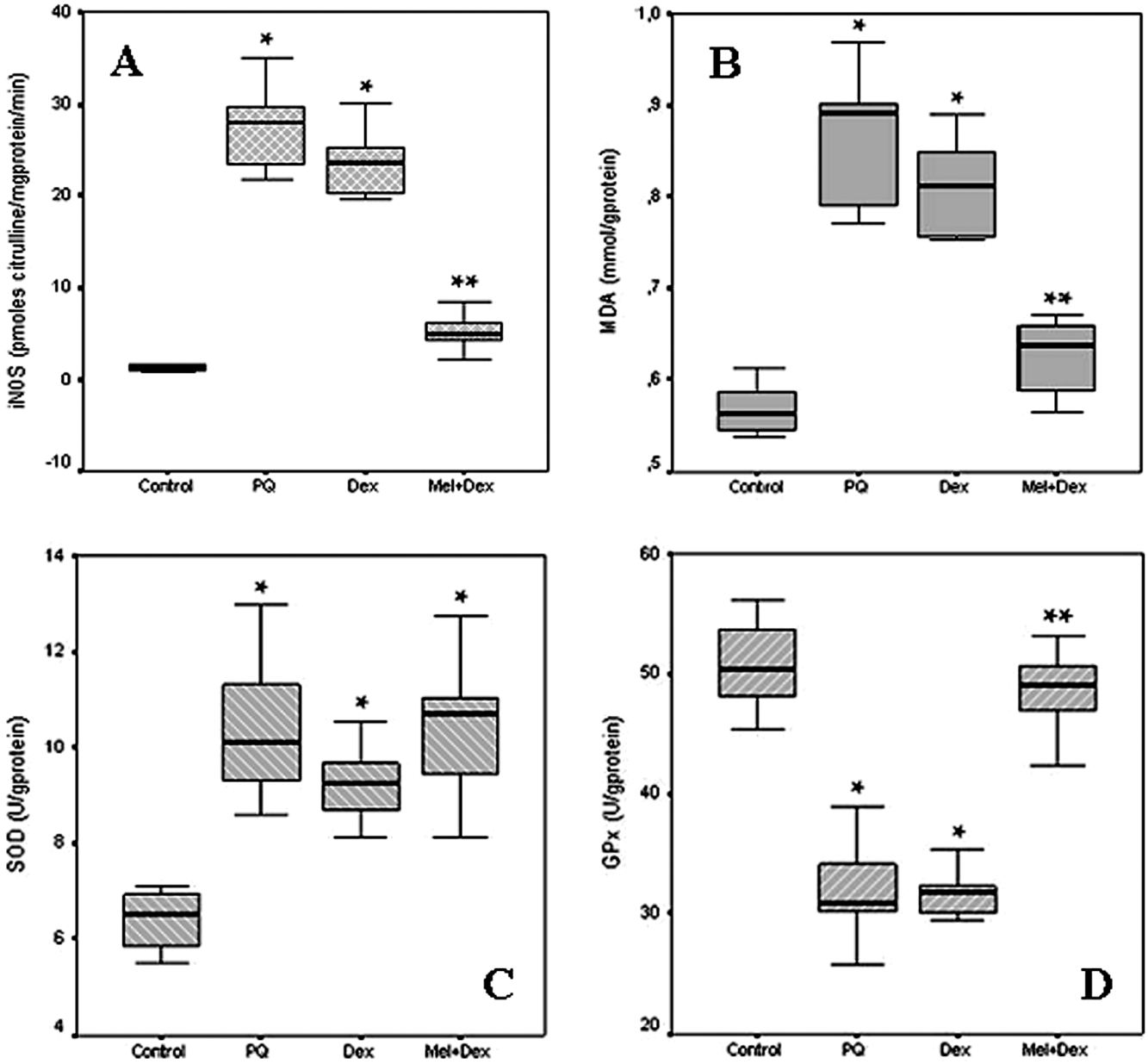

Figure 4. Lung tissue iNOS, MDA, SOD, and GPx levels at day 7. (A) PQ caused strong iNOS induction. A small reduction appeared in the Dex group and this reduction became statistically significant in the combination group as compared with the PQ group. Note that the final drugs were given at day 3 and the measurements performed at day 7. (B) PQ exposure caused severe lipid peroxidation. Dex did not decrease the levels as compared with the PQ group ($P > 0.05$). Dex+Mel resulted in significantly reduced MDA levels as compared with PQ. There was no significant difference between the combination and the control group MDA levels. (C) SOD activity was found to be increased and (D) GPx to be decreased in PQ-exposed as well as the Dex-treated groups. The combination treatment with melatonin did not affect SOD levels but was able to reverse GPx activity. *$P < 0.05$ vs. control, **$P < 0.05$ vs. PQ.

was associated with dramatic histological improvement in many of the criteria including less edema, alveolar hemorrhage, and airway pathology. Furthermore, many of the lung regions of the Dex+Mel–treated rats could not be distinguished from control lungs.

Discussion

To date, the management of PQ poisoning is mainly supportive, and a reliable treatment is in desperate need. Thus, novel treatment strategies according to toxicant mechanism are warranted to decrease the mortality rates. Because of the molecular toxicity mechanisms of PQ, several groups of chemicals, in particular antioxidants, have gained popularity against PQ toxicity (37). However, no protocol has yet been accepted as a standard treatment strategy.

Corticosteroids have been widely used as antiinflammatory (including NF-κB inhibition) drugs in clinical practice and in several emergencies. Steroids have been shown to reduce morbidity and mortality if used at an early

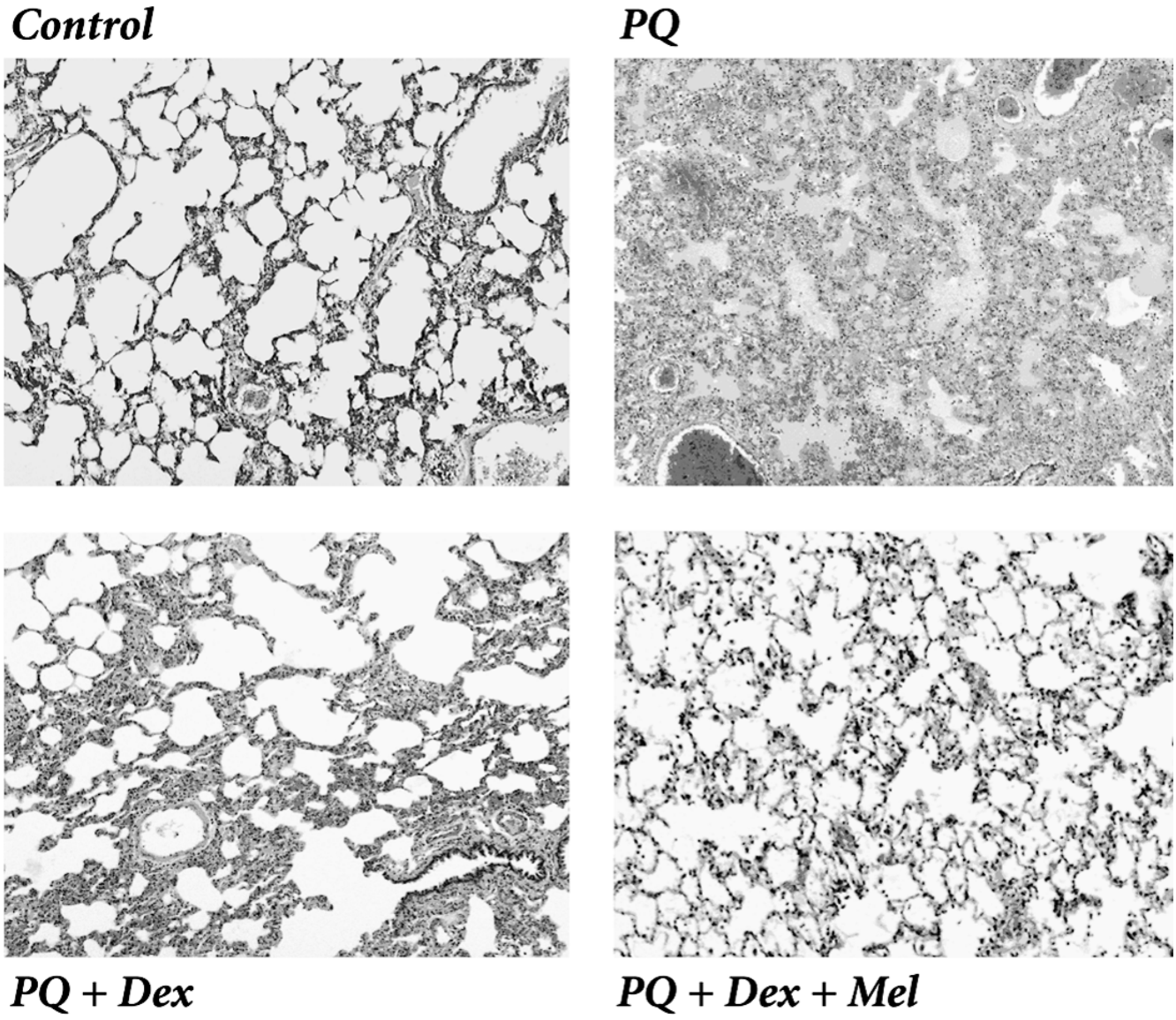

Figure 5. Representative histological photographs of the lung tissues of rats investigated at the seventh day after PQ exposure. In the lung tissue of control animals alveolar cavities are normal; there is no edema and hemorrhage. In the representative figure of the PQ group, interstitial edema and hemorrhage as well as septal thickening is apparent in many areas. A dense inflammatory cell infiltration is present in the alveolar cavity, mucosal epithelium, and airways. Many airways are larger than those of the control group. Administration of Dex alleviated the PQ symptoms; however, although there were improvements in particular inflammatory cell infiltration, the tissues of the Dex-treated animals still have noticeable damage. Results of the Dex+Mel combination clearly revealed impressive protection. The alveolar cavities, vascular bed, and airways are nearly normal. Interstitial edema and hemorrhage nearly disappeared. Inflammatory cell infiltration and septal thickening were not observed. The pathologist could not distinguish most of the slides from control. H&E stain; magnification ×50 for each panel.

phase of PQ-induced lung injury both experimentally (19, 20, 38) and clinically (39, 40). However, several reports indicate that treatment is not beneficial for those who died within the first week after PQ exposure, and the effectiveness of steroids is still in debate (41, 42). Considering steroids as multifunctional antiinflammatory agents, particularly in early stage of toxication, may be reasonable. Given that PQ may be reduced by antioxidants or antiinflammatory agents, melatonin in combination with steroids may exert a more positive outcome owing to their synergistic effects and because melatonin reduces both ROS and peroxynitrite-dependent early toxicity and the consequent inflammatory process (7). In the current study, six doses of Dex improved mortality and morbidity ratio as compared with PQ-intoxicated animals. Nevertheless, the observation that the contribution of Mel+Dex resulted in no deaths is of major importance. Histopathological outcome supports the improved survival ratios of Dex+Mel combination.

An experimental study using single high-dose Dex (100 mg/kg) showed that Dex treatment decreased lung MDA and another oxidative stress parameter (e.g., protein carboxylation) (20). However, using such a high dose is not realistic. In another experimental report, Dex was used at a dose of 0.01 mg/kg as an antiinflammatory drug in a

mouse model of carrageenan-induced pleurisy (42). Although the causative agents of lung injury are different, the pathophysiologic process of the acute lung injury is similar (e.g., ROS, NO, peroxynitrite, cytokines, and transcription factors). However, the difference between two doses was 10,000-fold. The latter work with 0.01 mg/kg Dex did not show antiinflammatory effects and failed to reduce lung injury. In our experiment, 1 mg/kg Dex (100-fold higher than the former and 100-fold lower than the latter dose) resulted in moderate antioxidative effects. However, the result of the combination treatment is impressive. Combining Dex with melatonin exerted very important benefits against almost all parameters including urinary NO_x and 8-isoprostanes, as well as tissue iNOS, MDA, and GPx levels.

Daily measured urinary 8-isoprostane and NO_x levels showed that nitro-oxidative stress was present as a consequence of PQ treatment. Because these parameters reflect total body nitro-oxidative stress, not only lung but also kidney, liver, and other tissues may be affected by PQ intoxication. Furthermore, an inconsistency appeared between urinary 8-isoprostane and lung MDA levels. In Dex-treated rats, MDA levels were somewhat depressed, but 8-isoprostane levels were increased. This finding may indicate that other tissues such as kidney, liver, or spleen may still be experiencing oxidative stress (20). Urinary NO_x levels suggest a rise in NO synthesis possibly *via* stimulation of iNOS. Yeh *et al.* (43) showed that PQ stimulates NO synthesis, and excess NO production is a key factor in the induction of tissue injury. In their study they reported elevated iNOS 16 and 24 hrs after PQ exposure. At day 7 after PQ treatment, we also found high iNOS relative to the control (Fig. 3A).

Melatonin is known to support tissue GPx levels. This beneficial effect was also clear in our study. Melatonin has also been shown to induce GPx mRNA at the transcriptional level (44). This is important because oxidative stress caused by PQ can lead to significant depletion of glutathione levels in tissues (24) and, conversely, inhibition of glutathione production intensifies PQ toxicity (45–47). Based on the above observations, it can be inferred that maintenance of glutathione levels helps ameliorate PQ toxicity. Nevertheless, exogenous administration of glutathione to prevent oxidative stress by PQ or other oxidants is not successful because of its rapid hydrolysis in the circulation and its limited ability to cross cell membranes (37). Thus, melatonin may be important by recycling glutathione. Several studies using N-acetyl-cysteine (NAC), a precursor of glutathione and also a free radical scavenger, has been shown to increase glutathione levels *in vivo* when administered before (48), or after (43) PQ exposure.

In the present study, higher SOD levels were observed in all groups except control (Fig. 4C). This finding can be interpreted to mean that oxidative stress is still present in all groups. This explanation is supported by the GPx levels; reduced levels of GPx in the PQ and Dex groups suggest that the tissue is under ongoing oxidative stress. Higher SOD accompanied by lower GPx can be more dangerous; SOD can reduce superoxide to hydrogen peroxide, but if hydrogen peroxide accumulates because of reduced GPx levels, it can undergo a Fenton reaction to produce the hydroxyl radical. This may have occurred in the Dex group. However, in the combination group, both higher SOD and GPx can reduce such a risk. Furthermore, melatonin has a high metal binding capacity including Fe^{+3}, thus blocking the formation of hydroxyl radical *via* the Fenton reaction. This may cause reduced oxidative cellular damage as seen in MDA levels of Dex+Mel group (Fig. 4B).

Although not measured in the current work, the combination of Dex+Mel has potent antiinflammatory potential (42). The benefits of this combination may be summarized as attenuation of infiltration of PMN leukocytes in the lung, reduction in adhesion molecules (e.g., selectins and cellular adhesion molecules), iNOS suppression, and inhibition of lipid peroxidation (18). Both compounds have potential as transcription factor inhibitors (e.g., NF-κB) and cytokine suppressors (e.g., TNF-α and IL-1β); these are involved in PQ toxicity.

The missing melatonin-only group has to be emphasized as a limitation of the study. Nevertheless, previous literature has already demonstrated the beneficial effects of melatonin alone against PQ-toxicity (23–25). More recent studies also reported limiting and preventing effects of melatonin against bleomycin toxicity, another cytotoxic agent, particularly on its lung injuring properties (49–51). Therefore, the present study was focused on testing the ability of melatonin as an adjuvant for the conventionally used treatment method rather than as a treatment agent alone. The results were encouraging; therefore further research with different dosages and combination alternatives should be performed. The importance of this study is also related to some other situations that share similar pathophysiological mechanisms. For example, in case of mustard toxicity (e.g., sulfur mustard–induced lung toxicity and cyclophosphamide-induced hemorrhagic cystitis) the combination of Dex+Mel may show more promising results. In addition to mustard agents, the side effects of doxorubicin and acetaminophen also share the same toxicity mechanisms. Thus, the combination of these two drugs may be useful in these situations. Another importance relates to the fact that the combination with steroids, which have side effects, may allow their use in larger amounts of melatonin which is safe and has little side effects. Also, melatonin can be administered *via* any of several routes (e.g., orally, sublingually, etc.) The molecule has a long shelf-life and is inexpensive like steroids (52). Since PQ toxicity is still an important public health problem, especially in developing countries (53), further investigations are warranted to test this combination in preventive medicine and clinical practice.

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