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RESEARCH PAPER

Contrasting actions of diesel exhaust particles on the pulmonary and cardiovascular systems and the effects of thymoquinone

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BACKGROUND AND PURPOSE
Acute exposure to particulate air pollution has been linked to acute cardiopulmonary events, but the underlying mechanisms are uncertain.

EXPERIMENTAL APPROACH
We investigated the acute (at 4 and 18 h) effects of diesel exhaust particles (DEP) on cardiopulmonary parameters in mice and the protective effect of thymoquinone, a constituent of *Nigella sativa*. Mice were given, intratracheally, either saline (control) or DEP (30 μg·per mouse).

KEY RESULTS
At 18 h (but not 4 h) after giving DEP, there was lung inflammation and loss of lung function. At both 4 and 18 h, DEP caused systemic inflammation characterized by leucocytosis, increased IL-6 concentrations and reduced systolic blood pressure (SBP). Superoxide dismutase (SOD) activity was decreased only at 18 h. DEP reduced platelet numbers and aggravated *in vivo* thrombosis in pial arterioles. *In vitro*, addition of DEP (0.1–1 $\mu g \cdot mL^{-1}$) to untreated blood-induced platelet aggregation. Pretreatment of mice with thymoquinone prevented DEP-induced decrease of SBP and leucocytosis, increased IL-6 concentration and decreased plasma SOD activity. Thymoquinone also prevented the decrease in platelet numbers and the prothrombotic events but not platelet aggregation *in vitro*.

CONCLUSIONS AND IMPLICATIONS
At 4 h after DEP exposure, the cardiovascular changes did not appear to result from pulmonary inflammation but possibly from the entry of DEP and/or their associated components into blood. However, at 18 h, DEP induced significant changes in pulmonary and cardiovascular functions along with lung inflammation. Pretreatment with thymoquinone prevented DEP-induced cardiovascular changes.

Abbreviations
BAL, bronchoalveolar lavage; DEP, diesel exhaust particles; DMSO, dimethyl sulphoxide; PM, particulate matter; PMN, polymorphonuclear cells; SBP, systolic blood pressure; SOD, superoxide dismutase

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Introduction

Although air pollution consists of a heterogeneous mixture of gaseous and particulate matter, adverse cardiovascular events are most strongly associated with exposure to fine particulate matter (diameter < 2.5 μm, $PM_{2.5}$) (Brook *et al.*, 2010). An important component of $PM_{2.5}$ is particulate matter generated during the combustion of diesel fuel (Brook *et al.*, 2010). These particles, and particularly the nanoparticulate fraction (diameter < 100 nm), of which the combustion-derived particulates of diesel exhaust are an important component, penetrate deeply into the respiratory tract and can carry large amounts of toxic compounds, such as hydrocarbons and metals, on their surfaces (Vermylen *et al.*, 2005; Brook *et al.*, 2010).

While epidemiological evidence strongly supports the association between particulate air pollution and cardiopulmonary effects, the mechanism is not fully understood (Vermylen *et al.*, 2005; Brook *et al.*, 2010). It has been suggested that inhaled particles may lead to systemic inflammatory responses through the release of IL-6, TNFα or histamine and oxidative stress within the lungs and/or systemically (Vermylen *et al.*, 2005; Brook *et al.*, 2010). Additional experiments showed that exposure to air pollution is associated with rapid changes in autonomic nervous system balance, favouring sympathetic nervous system activation and parasympathetic withdrawal (Vermylen *et al.*, 2005; Brook *et al.*, 2010). Other lines of evidence also suggest that inhaled nanoparticulates can rapidly cross the alveolar–capillary barrier and directly affect the cardiovascular system (Nemmar *et al.*, 2004b; Vermylen *et al.*, 2005; Brook *et al.*, 2010).

Epidemiological and clinical studies reported the occurrence of a cardiovascular effect of particulate air pollution within a few hours of exposure (Peters *et al.*, 2001; 2004; Harrabi *et al.*, 2006; Mills *et al.*, 2007;). Indeed, acute exposure to diesel exhaust particles (DEP) caused a systemic and inflammatory response in the airways and impaired the regulation of vascular tone and endogenous fibrinolysis in healthy human volunteers (Salvi *et al.*, 1999; Mills *et al.*, 2005). Other human exposure studies have shown that exposure to particulate air pollution caused cardiovascular effects without significant effects on the respiratory tract (Samet *et al.*, 2009). We have investigated the contribution of lung inflammation and/or direct particle translocation in the extrapulmonary effects of inhaled particles (Nemmar *et al.*, 2003a,b; 2004a; 2005b; 2007b; 2009a; 2010b). To evaluate the effect of translocated particulate air pollution, several studies have assessed the direct effect of particles *in vivo* (Nemmar *et al.*, 2007a; Nemmar and Inuwa, 2008), *ex vivo* (Nemmar *et al.*, 2005a) and *in vitro* (Alfaro-Moreno *et al.*, 2008). We have recently reported that systemic administration of DEP affected blood pressure and caused lung inflammation assessed by bronchoalveolar lavage (Nemmar *et al.*, 2007a; 2009b; 2010a; Nemmar and Inuwa, 2008). However, with respect to pulmonary exposure, in all the animal models we studied, DEP resulted in lung inflammation in hamsters (1, 6 and 24 h) (Nemmar *et al.*, 2003a,b; 2004a), rat (24 h) (Nemmar *et al.*, 2010b) and mice (24 h) (Nemmar *et al.*, 2009a). However, no measurement of pulmonary function has been reported. Thus, it is not clear whether pulmonary exposure to particulate air pollution can affect systemic and cardiovascular variables, without causing detectable pulmonary inflammation.

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) is the main active principle in the oil of *Nigella sativa* seeds and has broad anti-inflammatory activities (Ali and Blunden, 2003), and it has been reported to prevent pulmonary inflammation in a mouse model of allergic asthma (El Gazzar *et al.*, 2006) and to attenuate the inflammatory response in activated mast cells by blocking transcription and production of TNFα (El Gazzar *et al.*, 2007). To our knowledge, however, no study, has addressed the effect of thymoquinone on the cardiovascular and pulmonary effects of DEP until now.

We have tested here whether and to what extent, DEP given to the lung could induce acute cardiovascular effects, in the absence of detectable effects on lung inflammation and function. We have also assessed the protective effect of thymoquinone against such effects. Thus, in the present study, we have assessed the acute (4 and 18 h) effects of DEP on a comprehensive set of indices of cardiovascular function, including blood pressure, thrombosis in pial arterioles, platelet aggregation and systemic inflammation and respiratory endpoints, including pulmonary inflammation and airway resistance measured invasively using forced oscillation. Our data show that, at 4 h, in the absence of detectable pulmonary inflammation, DEP induced systemic inflammation, decreased blood pressure and caused thrombosis in pial arterioles. However, at 18 h, DEP caused both pulmonary and cardiovascular effects. Moreover, and interestingly, pretreatment with thymoquinone significantly ameliorated DEP-induced pulmonary and cardiovascular events.

Methods

Particles

DEP (SRM 2975), obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD), were suspended in sterile normal saline (NaCl 0.9%) containing Tween 80 (0.01%). To minimize aggregation, particle suspensions were always sonicated (Clifton Ultrasonic Bath, Clifton, NJ) for 15 min and vortexed before their dilution and before intratracheal (i.t.) administration. Control animals received normal saline containing Tween 80 (0.01%). We have previously (Nemmar *et al.*, 2007a) analysed the size of DEP used in the present study by transmission electron microscopy and found a substantial amount of ultrafine (nano) sized particle aggregates and larger particle aggregates (<1 μm in largest diameter).

Animals and treatments

All animal care and experimental procedures were in accordance with the protocols of the Institutional Animal Care and Research Advisory Committee and were approved by the Institutional Review Board of the United Arab Emirates University, Faculty of Medicine and Health Sciences. Male TO mice (30–35 g, HsdOla : TO, Harlan, UK) were housed in light (12 h light / 12 h dark cycle) and temperature-controlled (22 ± 1°C) rooms. They had free access to commercial laboratory chow and were provided tap water *ad libitum*. Mice were anaesthetized with sodium pentobarbital (60 $mg \cdot kg^{-1}$, i.p.), placed supine with extended neck on an angled board. A Becton Dickinson 24-gauge cannula was inserted via the mouth into

the trachea. Either the DEP suspensions (30 μg·per mouse) or saline-only were instilled i.t. (40 μL) via a sterile syringe and followed by an air bolus of 50 μL.

Systolic blood pressure (SBP) measurement

Four or 18 h after the i.t. administration of DEP, SBP was measured using a computerized noninvasive tail-cuff manometry system (ADInstrument, Colorado Springs, CO). To avoid the effects of procedure-induced anxiety on SBP, mice were trained for five consecutive days before the experimental procedure (Ying *et al.*, 2009).

Airway reactivity to methacholine

In separate animals, airway hyperreactivity responses were measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). Airway resistance (*R*) was assessed after increasing exposures to methacholine. Mice were anaesthetized with an i.p. injection of pentobarbital (70 $mg \cdot kg^{-1}$). The trachea was exposed, and into it an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of 10 $mL \cdot kg^{-1}$ at a frequency of 150 $breaths \cdot min^{-1}$ and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged with methacholine aerosol, generated with an in-line nebulizer and administered directly through the ventilator for 5 s, with increasing concentrations (0, 0.625, 1.25, 2.5, 5 and 10 $mg \cdot mL^{-1}$). Airway resistance (*R*) was measured using a 'snapshot' protocol each 20 s for 2 min. The mean of these six values was used for each methacholine concentration, unless the coefficient of determination of a measurement was smaller than 0.95. For each mouse, *R* was plotted against methacholine concentration (from 0 to 10 $mg \cdot mL^{-1}$) (Vanoirbeek *et al.*, 2004).

Blood collection and analysis of bronchoalveolar lavage (BAL) fluid

Four or 18 h after the i.t. administration of either saline or DEP, the animals were anaesthetized, as described above, and blood was drawn from the inferior vena cava in EDTA (4%). A sample was used for platelets and white blood cells (WBC) counts using an ABX VET ABC haematology analyzer with a mouse card (ABX Diagnostics, Montpellier, France). The remaining blood was centrifuged at 4°C for 15 min at 900× *g*, and the plasma samples were stored at –80°C until further analysis.

Mice were then killed with an overdose of sodium pentobarbital. The trachea was cannulated, and lungs were lavaged three times with 0.7 mL (a total volume of 2.1 mL) of sterile NaCl 0.9%. The recovered fluid aliquots were pooled. No difference in the volume of collected fluid was observed between the different groups. BAL fluid was centrifuged (1000× *g* for 10 min, 4°C). Cells were counted in a Thoma haemocytometer after resuspension of the pellets and staining with 1% gentian violet. The cell differentials were microscopically performed on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade, Brussels, Belgium). The supernatant was stored at–80°C until further analysis.

In the BAL fluid, total protein was measured spectrophotometrically (Sigma, St. Louis, MO), and the concentration of IL-6 (R&D Systems, Minneapolis, MN) was determined using ELISA kits.

Histopathology

After the lavage, the right lung was fixed with 10% formaldehyde (Vanoirbeek *et al.*, 2004). A pathologist examined slices (5 μm sections) from all lung lobes and evaluated lung injury without knowledge of the animals' treatment.

The degree of interstitial infiltration by inflammatory cells, in response to deposition of DEP within lung tissues after either 4 or 18 h, was evaluated by counting the number of polymorphonuclear cells (PMN) and macrophages in 10 high-power fields of lung tissue sections and then dividing the total by 10. An Olympus microscope BX41, 40× objective lens and 10× eyepiece and Image J software were used (Olympus, Melville, NY). The resulting numbers are expressed as number of cells in each high-power field. The calculation was made on random 10 high-power fields in each section in all groups.

Determination of IL-6 concentrations and superoxide dismutase (SOD) activity in plasma

The activity of SOD was measured spectrophotometrically (Cayman Chemical, Ann Arbor, MI), and the concentration of IL-6 (R&D Systems) was determined using ELISA kits.

Experimental pial cerebral arterioles thrombosis model

In a separate experiment, *in vivo* thrombogenesis in pial arterioles was assessed either 4 or 18 h after i.t. instillation of either DEP or saline, according to a previously described technique (Nemmar *et al.*, 2009a). Briefly, the trachea was intubated after induction of anaesthesia with urethane (1 $mg \cdot g^{-1}$ body weight, i.p.), and a 2F venous catheter (Portex, Hythe, UK) was inserted in the right jugular vein for the administration of fluorescein (Sigma). After that, a craniotomy was first performed on the left side, using a microdrill and the dura was stripped open. Only untraumatized preparations were used, and those showing trauma to either microvessels or underlying brain tissue were discarded. The animals were then placed on the stage of a fluorescence microscope (Olympus) attached to a camera and DVD recorder. A heating mat was placed under the mice, and body temperature was raised to 37°C, as monitored by a rectal thermoprobe connected to a temperature reader (Physitemp Instruments, NJ,). The cranial preparation was moistened continuously with artificial cerebrospinal fluid of the following composition (mM): NaCl 124, KCl 5, NaH_2PO_4 3, $CaCl_2$ 2.5, $MgSO_4$.4, $NaHCO_3$ 23 and glucose 10, pH 7.3–7.4. A field containing arterioles 15–20 μm in diameter was chosen. Such a field was photographed before and during the photochemical insult, which was carried out by injecting fluorescein (0.1 mL·per mouse of 5% solution) via the jugular vein, which was allowed to circulate for 30–40 s. The cranial preparation was then exposed to stabilized mercury light. The combination produces endothelial injury in the arterioles. This, in turn, causes platelets to adhere at the site of endothelial damage and then aggregate. Platelet aggregates and

thrombus formation grow in size until complete vascular occlusion. The time from the photochemical injury until full vascular occlusion (time to flow stop) in arterioles were measured in seconds. At the end of the experiments, the animals were killed by an overdose of urethane.

Platelet aggregation in mouse whole blood

The platelet aggregation assay in whole blood was performed, with slight modification, as described before (Nemmar *et al.*, 2008). After anaesthesia, blood from separate animals was withdrawn from the inferior vena cava and placed in citrate (3.2%), and 100 µL aliquots were added to the well of a Merlin coagulometer (MC 1 VET; Merlin, Lemgo, Germany). The blood samples were incubated at 37.2°C with either saline (control) or DEP (0.1–1 µg·mL^{-1}) for 3 min and then stirred for another 3 min. At the end of this period, 25 µL samples were removed and fixed on ice in 225 mL cellFix (Becton Dickinson, Mountain View, CA, USA). After fixation, single platelets were counted in a VET ABX Micros with mouse card (ABX Diagnostics, Montpellier, France). The degree of platelet aggregation following DEP exposure was expressed as follows: the number of platelets counted after exposure to different concentrations of DEP was divided by the number of platelets observed in saline-treated blood (control) and multiplied by 100. Thus, the results were expressed as % of control.

Effect of thymoquinone pretreatment on pulmonary and systemic parameters

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone; Sigma) was dissolved initially in dimethyl sulphoxide (DMSO) followed by dilution with normal saline. The final concentration of DMSO was 0.5%. No effect of the vehicle has been found on the different parameters investigated.

Mice were pretreated twice with thymoquinone (6 mg·kg^{-1}; i.p.), 24 and 1 h before i.t. saline or DEP (30 µg), and the pulmonary and cardiovascular parameters described above were evaluated. For the *in vitro* experiment, untreated blood was incubated for 3 min with various concentrations of thymoquinone (0.01–0.1 mg·mL^{-1}) before adding DEP 1 µg·mL^{-1} to the blood. Then platelet aggregation described above was assessed.

Statistics

Data are expressed as means ± SD. Comparisons between groups were performed by the following methods: one-way ANOVA followed by Newman–Keuls multiple-range tests, or two-way ANOVA followed by Bonferroni multiple-range tests, as indicated. *P* values less than 0.05 were considered significant.

Results

Cell composition and number in BAL fluid

Depending on the i.t. treatment, the cells found in BAL were primarily macrophages and PMN (Figure 1). Lymphocytes were not found in control mice BAL. No other cells were observed microscopically.

Four hours following their i.t. administration, DEP neither induced any cellular influx nor caused an increase of either IL-6 or total protein concentrations in BAL. Thymoquinone pretreatment did not affect either the BAL cell numbers or the IL-6 and total protein concentrations. In contrast, 18 h after DEP exposure, a marked and significant increase in macrophages and PMN numbers and total protein and IL-6 concentrations were observed. The latter effects were significantly prevented by pretreatment with thymoquinone.

Histology

Sections of lung from saline-treated mice, taken at 4 h after DEP, showed a normal appearance under light microscopy (Figure 2A, B). Lung sections from DEP-treated mice showed the presence of particulates within the intralveolar and interstitial tissue with infiltration of only a few inflammatory cells (Figure 2C, D). Likewise, morphometric lung analysis showed only slight and non-significant increases of macrophage and PMNs (Figure 3).

Similarly, the lungs of the thymoquinone + saline group (Figure 2E, F) showed a normal appearance, and those of the thymoquinone + DEP group (Figure 2G, H) demonstrated the presence of particles within the intralveolar and interstitial tissue with infiltration of a few inflammatory cells (Figure 3).

At 18 h, lung sections from saline-treated mice showed a normal appearance (Figure 2I, J). However, lung sections from the DEP-treated mice showed the presence of particulates within the intralveolar and interstitial tissue with a marked and significant inflammatory cellular infiltration consisting mainly of PMN and macrophages (Figures 2K, L, 3). While the lungs of the thymoquinone + saline group (Figure 2M, N) showed a normal appearance, those of the thymoquinone + DEP group (Figure 2O, P) demonstrated the presence of particles within the intralveolar and interstitial tissue and a significant reduction in the number of interstitial inflammatory cells that had infiltrated, following DEP exposure (Figures 2O, P, 3).

Airway hyperreactivity to methacholine

Figure 4 shows airway resistance, measured by the FlexiVent, to increasing concentrations of methacholine, after exposure to either saline or DEP with and without thymoquinone pretreatment. The results obtained at 4 h were highly variable, and no statistical differences in airway resistance were found between the different groups (Figure 4A).

However, 18 h following the exposure of DEP, we observed a significant and dose-dependent increase in the airway resistance, compared with the saline-treated group. No statistical differences were observed between the saline and thymoquinone + saline groups. Interestingly, thymoquinone pretreatment significantly prevented DEP-induced enhancement of airway resistance after increasing concentrations of methacholine (Figure 4B).

Leukocyte numbers in whole blood, IL-6 and SOD levels in plasma

DEP induced a significant increase in the number of leukocytes in whole blood and IL-6 concentration in plasma compared with the saline-treated group (Figure 5A, B). Pretreatment of control mice with thymoquinone affected

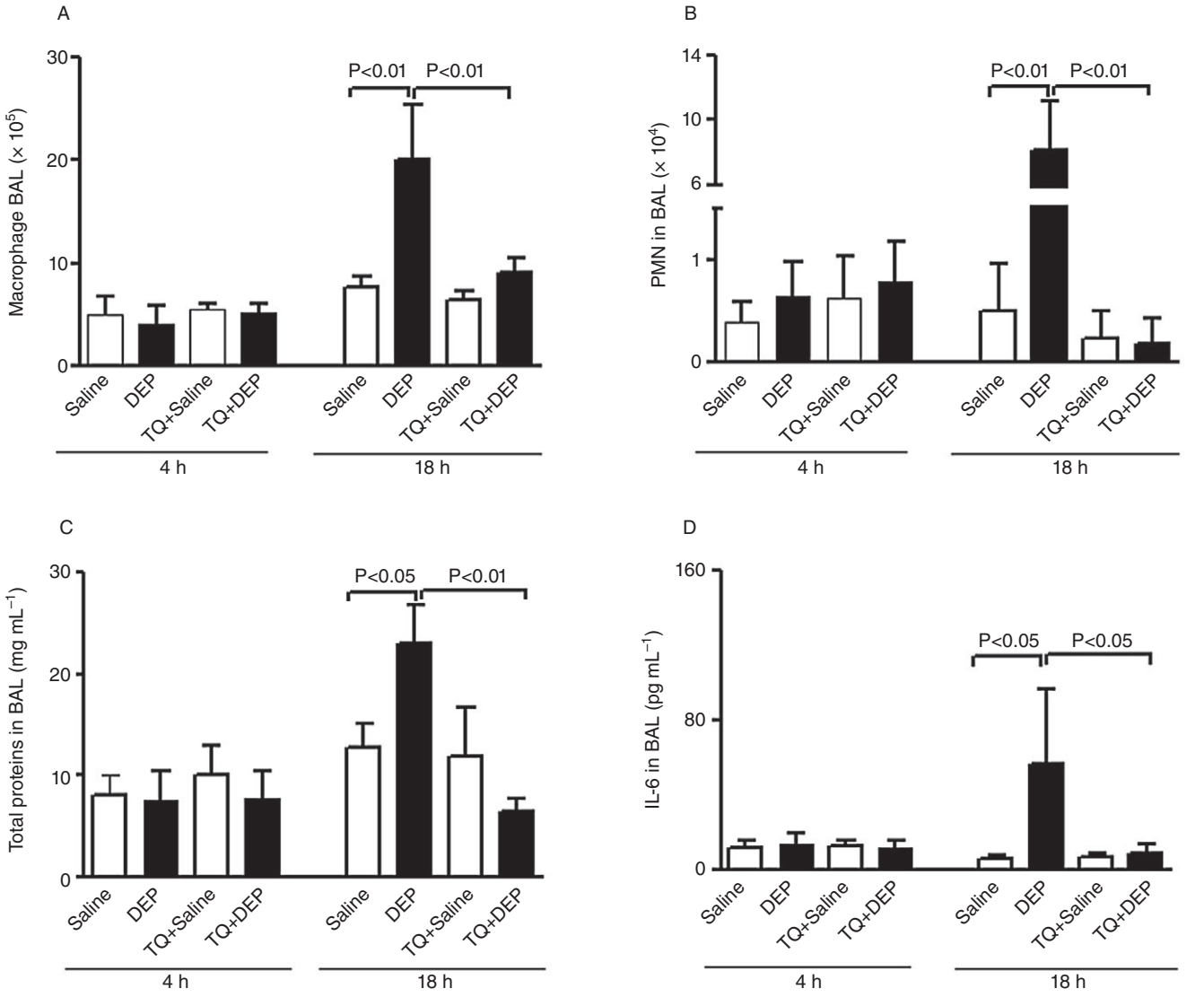

Figure 1

Numbers of macrophages (A) and PMN (B), and total proteins (C) and IL-6 (D) concentrations in bronchoalveolar lavage, 4 or 18 h after i.t. instillation of saline or DEP (30 μg·per animal) with or without thymoquinone (TQ) pretreatment. Data are mean ± SD (n = 8 in each group); two-way ANOVA followed by Bonferroni multiple-comparison test.

neither the number of leukocytes nor IL-6 concentrations. However, thymoquinone pretreatment significantly reduced the DEP-induced increase in leukocyte numbers and IL-6 increase in plasma (Figure 5A, B).

At 4 h after DEP, the plasma SOD activity was unchanged (Figure 5C). Similarly, thymoquinone did not affect the plasma SOD levels (Figure 5C). However, 18 h after DEP exposure, a significant decrease of SOD was observed (Figure 5C). Thymoquinone alone at this time did not significantly affect the plasma SOD levels but prevented the effect of DEP on SOD activity (Figure 5C).

SBP

Figure 6 illustrates the effect of i.t. administration of DEP on SBP in mice. Compared with the blood pressure in the control group, mice exposed to DEP exhibited a significant decrease in SBP. Interestingly, while thymoquinone + saline did not affect SBP, thymoquinone pretreatment significantly prevented the decrease of SBP caused by DEP at both 4 and 18 h.

Platelet numbers in blood and photochemically induced thrombosis in pial arterioles

Platelet counts in blood were significantly decreased 4 and 18 h following DEP administration, compared with control mice (Figure 7A), indicating platelet aggregation *in vivo*. This effect was significantly prevented by pretreatment with thymoquinone (Figure 7A).

Consistent with the results on platelet numbers, Figure 7B illustrates that the i.t. instillation of DEP induced

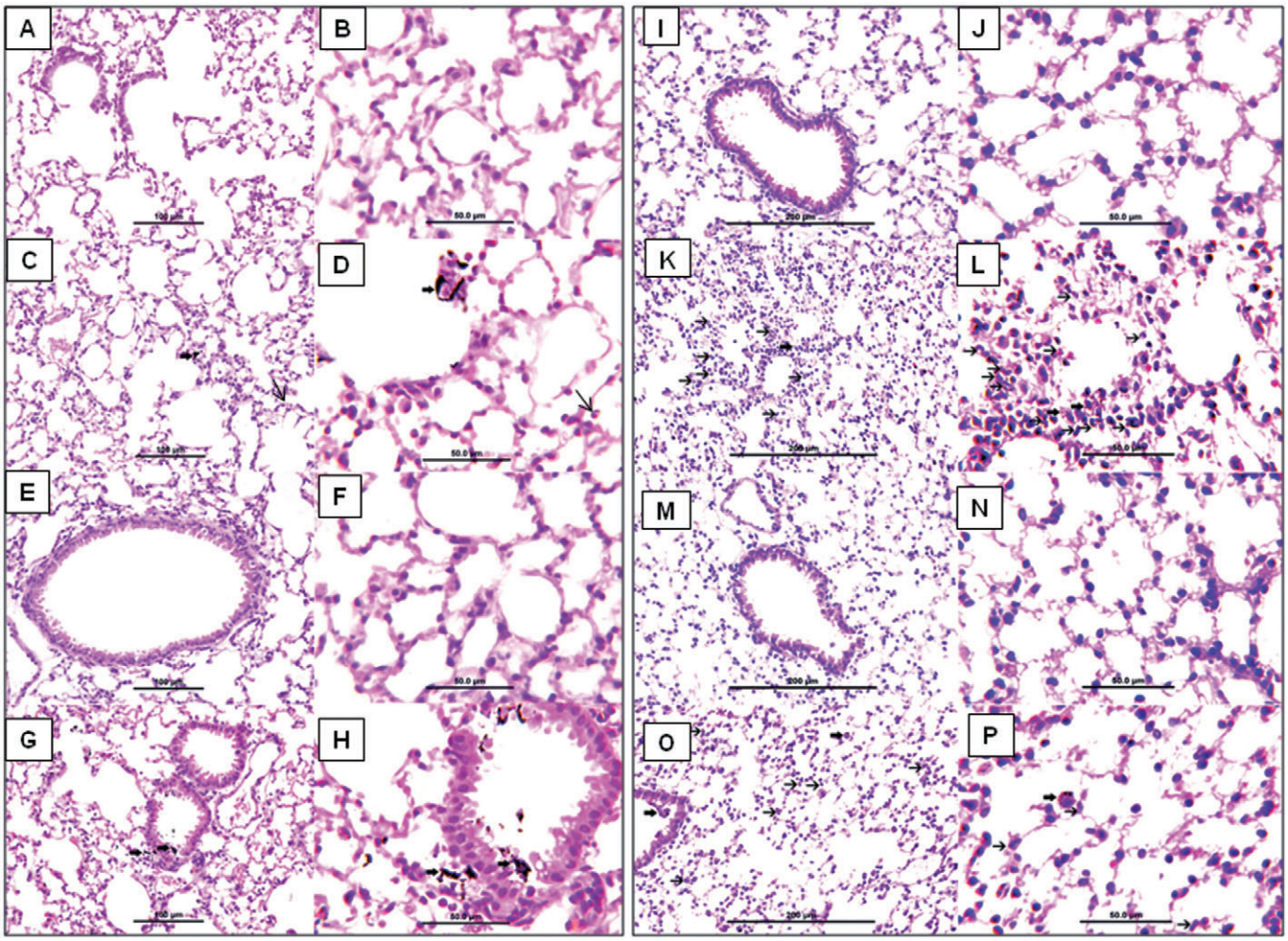

Figure 2

Representative light microscopy sections of lung tissues of mice 4 or 18 h after administration of saline 4 h (A, B), saline 18 h (I, J), DEP 4 h (30 μg per animal, C, D), DEP 18 h (K, L), thymoquinone + saline 4 h (E, F), thymoquinone + saline 18 h (M, N), thymoquinone + DEP 4 h (G, H) or thymoquinone + DEP 18 h (O, P). The micrographs show DEP deposition within the intralveolar and interstitial tissue and pulmonary macrophages and the presence of PMN cell infiltration.

a shortening of the occlusion time in pial arterioles in a photochemically injured vessel. Thymoquinone pretreatment significantly reversed the DEP-induced shortening of the occlusion time in pial cerebral arterioles (Figure 7B).

In vitro *platelet aggregation in whole blood*

Figure 8A shows that low concentrations of DEP (0.1–1 μg·mL^{-1} blood) caused platelet aggregation in a dose-dependent manner. The effect was significant at concentrations of 0.5 μg·mL^{-1} ($P < 0.05$) and 1 μg·mL^{-1} ($P < 0.01$). Thymoquinone pretreatment (0.01–0.1 mg·mL^{-1}) did not affect platelet aggregation in whole blood *in vitro*. For the sake of clarity, only the highest dose tested (0.1 mg·mL^{-1}) is shown in Figure 8B. Pretreatment with thymoquinone did not affect the pro-aggregatory effect of DEP, excluding any direct effect of thymoquinone on platelet aggregation (Figure 8B).

Discussion

We conclude from our results that the cardiovascular effects of DEP at 4 h were not associated with pulmonary inflammation but were more likely to be due to the entry into blood of DEP and/or their associated components. However, at 18 h, both lung inflammation and cardiovascular functions were affected by DEP. We chose to study the effects of DEP at 4 and 18 h because these times are particularly relevant, both epidemiologically and clinically, to the cardiovascular effects of particulate air pollution within a few hours of exposure (Peters *et al.*, 2001; 2004; Harrabi *et al.*, 2006; Mills *et al.*, 2007).

Pulmonary function and histology were adversely affected at 18 h (but not 4 h) after DEP exposure. Using the same type of particles, dose and mouse strain, but a later time, at 24 h, pulmonary exposure to DEP (15 and 30 μg·per mouse) induced a dose-dependent macrophage and PMN

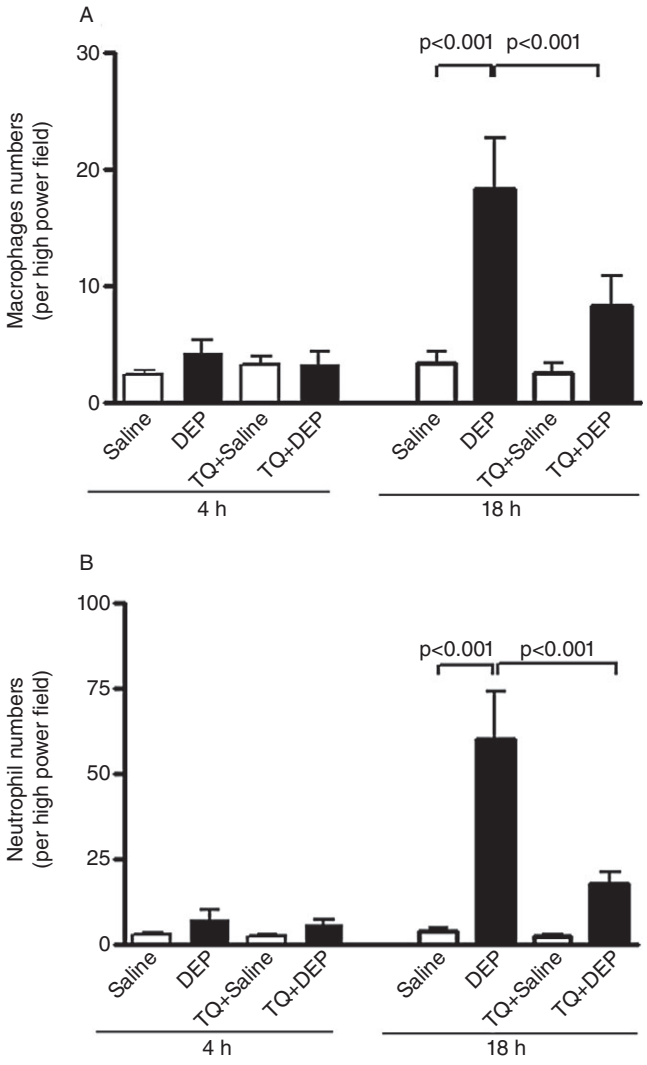

Figure 3

Numbers of macrophages (A) and PMN (B) obtained by morphometric analysis of microscopy sections of lung tissues of mice 4 or 18 h after i.t. instillation of saline or DEP (30 μg·per animal) with or without thymoquinone (TQ) pretreatment. Data are mean ± SD (n = 8 in each group); two-way ANOVA followed by Bonferroni multiple-comparison test.

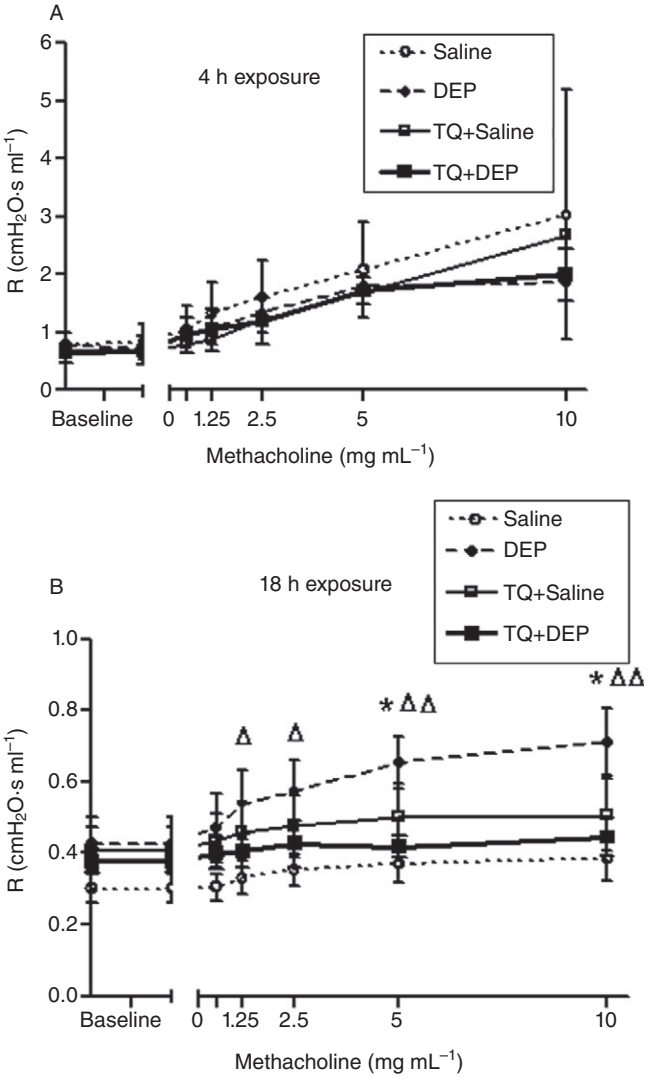

Figure 4

Airway hyperresponsiveness. The airway resistance (R), after increasing concentrations of methacholine (0–10 mg·mL^{-1}), was measured via the forced oscillation technique (FlexiVent) 4 or 18 h after i.t. instillation of saline or DEP (30 μg·per animal) with or without thymoquinone (TQ) pretreatment. Data are mean ± SD (n = 8 in each group); two-way ANOVA followed by Bonferroni multiple-comparison test. *P < 0.001, significant differences between DEP and saline groups for the same methacholine concentration. ΔP < 0.01, significant differences between DEP and TQ + DEP groups for the same methacholine concentration. ΔΔP < 0.001, significant differences between DEP and TQ + DEP groups for the same methacholine concentration.

influx into the BAL fluid with elevation of total proteins (Nemmar *et al.*, 2009a). Cho *et al.* (2009) reported no significant increase of BAL PMN at 4 and 18 h following i.t. instillation of either fine or ultrafine airborne particulate matter in mice. Previously, PMN influx in the alveoli has been shown, which significantly increased in a time-dependent manner (peak reached at 24 h) in rats exposed to carbon black particles (6 and 24 h) (Li *et al.*, 1997) and hamsters exposed to DEP (1, 6 and 24 h) (Nemmar *et al.*, 2003b). These discrepancies suggest that the kinetics of the initiation of lung inflammation following particle exposure is dependent on species, strains and nature of particles.

Mechanisms of air pollution–induced cardiopulmonary morbidity and mortality are uncertain (Mills *et al.*, 2009). Inhaled particles cause lung inflammation with release of inflammatory mediators (Nemmar *et al.*, 2003b; 2004b) and also particle translocation (Nemmar *et al.*, 2001; 2002a; Oberdorster *et al.*, 2002). Consequently, it is hard to distinguish between these two processes (Nemmar *et al.*, 2003b; 2010a). In the animal models we previously studied, pulmonary exposure to DEP caused lung inflammation in hamsters (1, 6 and 24 h), rats (24 h) and mice (24 h) (Nemmar *et al.*, 2003b; 2009a, 2010a,b). Here, we did not observe pulmonary effects 4 h following the exposure to DEP, but this negative result

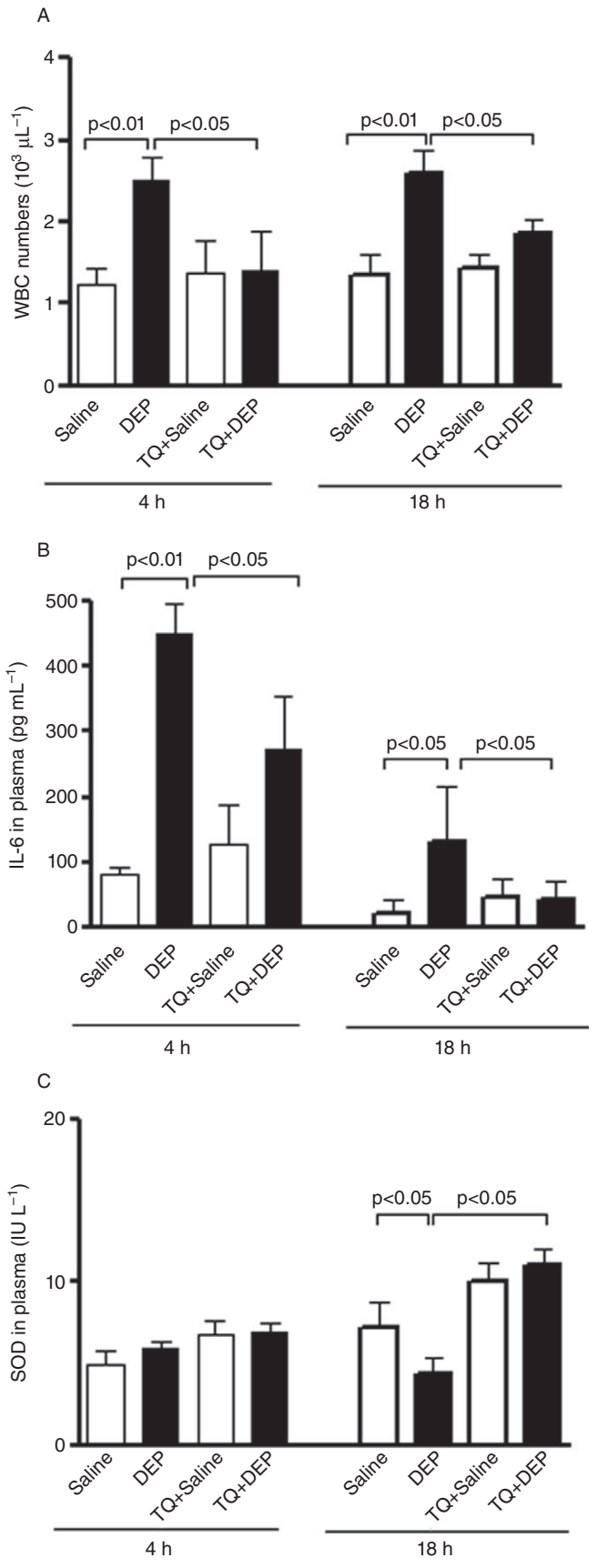

Figure 5

WBC numbers (A), IL-6 (B) and SOD (C) levels in plasma, 4 or 18 h after i.t. instillation of saline or DEP (30 µg·per animal) with or without thymoquinone (TQ) pretreatment. Data are mean ± SD (n = 8 in each group); two-way ANOVA followed by Bonferroni multiple-comparison test.

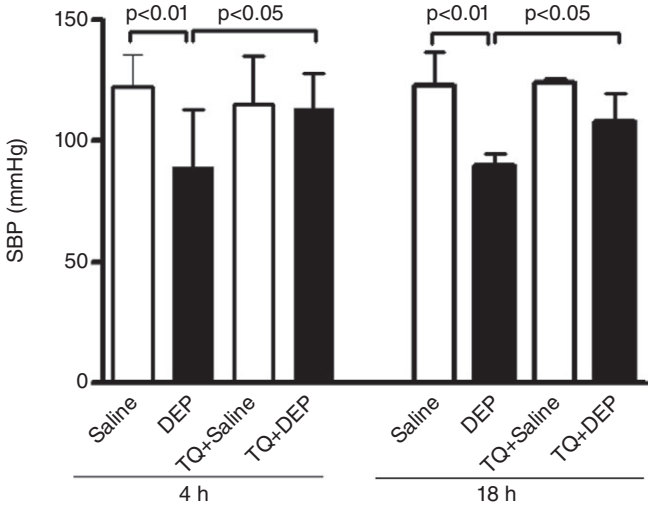

Figure 6

SBP, 4 or 18 h after i.t. instillation of saline or DEP (30 µg·per animal) with or without thymoquinone (TQ) pretreatment. Data are mean ± SD (n = 8 in each group); two-way ANOVA followed by Bonferroni multiple-comparison test.

encouraged us to verify whether, and to what extent, systemic inflammation and cardiovascular effects can occur in the absence of pulmonary inflammation. In humans, exposure to particulate air pollution caused cardiovascular effects without significant effects on the respiratory tract (Samet *et al.*, 2009).

In either the absence (4 h) or presence (18 h) of lung inflammation, exposure to DEP caused systemic inflammation characterized by an increase of leukocyte numbers and IL-6 concentration in plasma. The SOD activities decreased only at the 18 h time point. Recently, Dubowsky *et al.* (2006) provided epidemiological evidence for positive associations between air pollution and indicators of systemic inflammation such as leukocyte numbers, C-reactive protein and IL-6. In animals, 24 h following particulate air pollution exposure, IL-6 levels in the BAL fluid and in blood samples were increased (Mutlu *et al.*, 2007; Nemmar *et al.*, 2009b). In mice, PM_{10} exposure increased pulmonary inflammatory mediators, which entered the circulation, contributing to the systemic inflammation with downstream effects, such as vascular dysfunction (Kido *et al.*, 2011). Our results concur with this finding at 18 h but not at 4 h. The results obtained at 4 h could be explained by translocation of DEP themselves and/or their associated components into the systemic circulation, as has been reported in humans and experimental animals (Nemmar *et al.*, 2001; 2002a; Oberdorster *et al.*, 2002; Elder *et al.*, 2006).

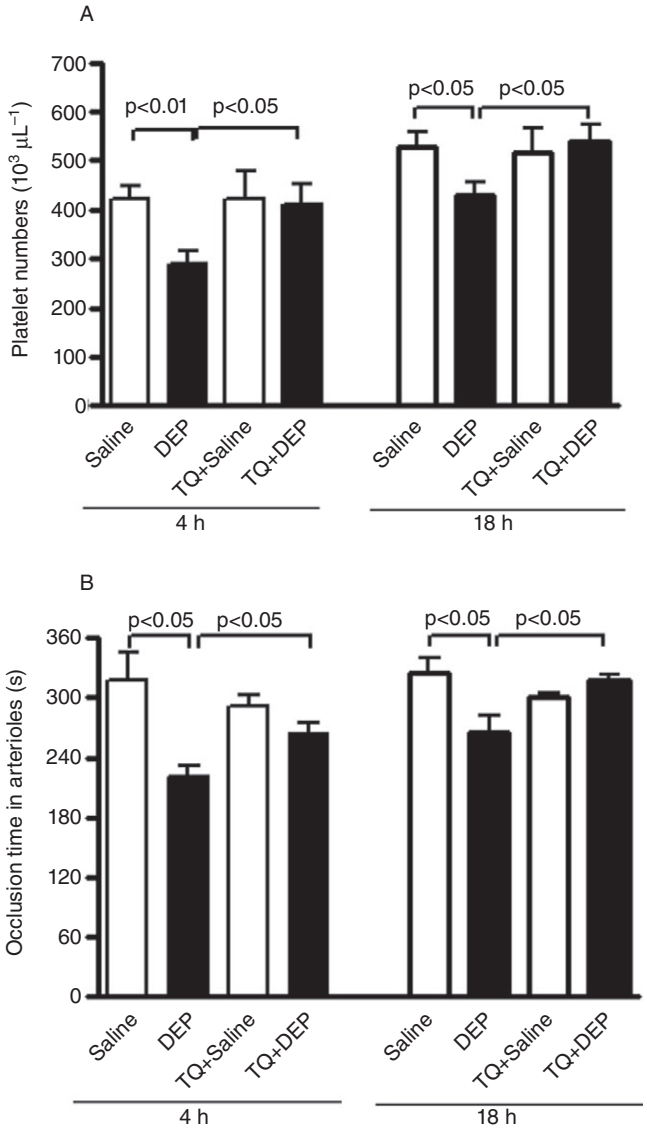

Figure 7

Platelet numbers (A) and occlusion time in pial arterioles (B), 4 or 18 h after i.t. instillation of saline or DEP (30 µg·per animal) with or without thymoquinone (TQ) pretreatment. Data are mean ± SD (n = 8 in each group); two-way ANOVA followed by Bonferroni multiple-comparison test.

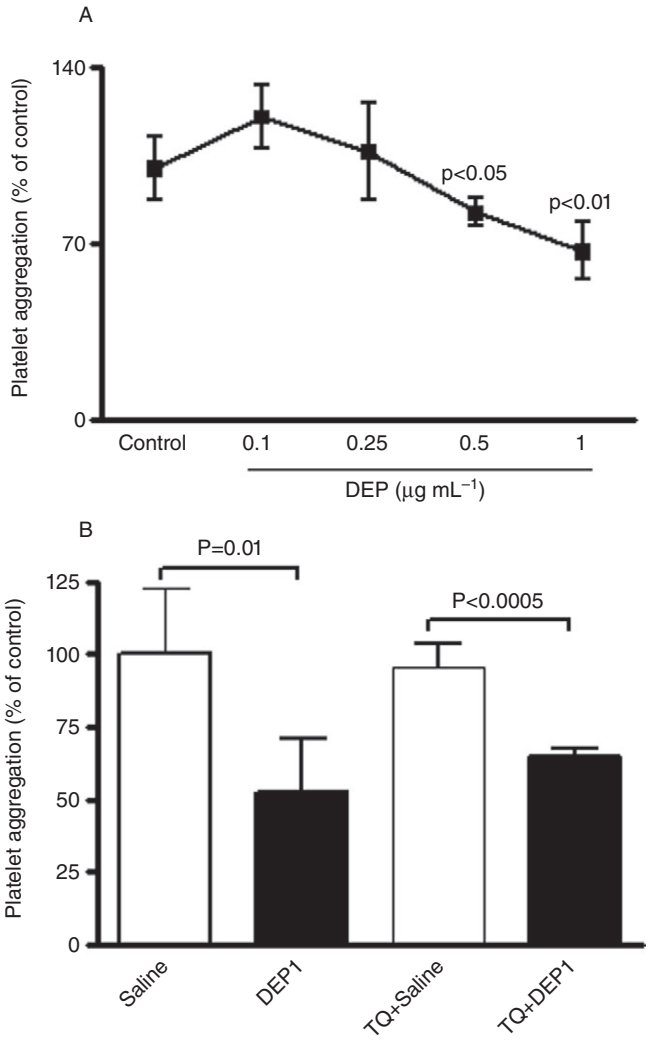

Figure 8

Concentration-dependent effects of DEP on platelet aggregation in whole blood *in vitro* (A) and effect of saline or DEP (1 µg·mL^{-1}) with or without thymoquinone (TQ; 0.1 mg·mL^{-1}) pretreatment on platelet aggregation in whole blood *in vitro* (B). The degree of platelet aggregation following DEP exposure was expressed in percent of control. Data are the mean ± SD (n = 3–5); one-way ANOVA followed by Newman–Keuls multiple-comparison test.

Plasma SOD activity was significantly decreased at 18 h but not at 4 h post-DEP exposure. DEP, 24 h following their i.t. instillation, caused oxidative stress leading to systemic inflammation and pretreatment with a cysteine prodrug, L-2-oxothiazolidine-4-carboxylic acid, prevented these effects through its ability to balance oxidant–antioxidant status (Nemmar *et al.*, 2009a). Nevertheless, the levels of IL-6 were not affected in BAL and plasma, 24 h following DEP exposure (Nemmar *et al.*, 2009a). Here, at 4 h after exposure, IL-6 was increased markedly but by 18 h, the concentration of IL-6 in the DEP group was decreased, compared with that at 4 h, while higher than those in the control group ($P < 0.005$). This suggests that, after DEP, IL-6 concentrations in plasma reach a peak at 4 h, decrease by 18 h and return to control levels at 24 h. However, in BAL, the IL-6 concentration increased only at 18 h but not at either 4 or 24 h. These results highlight the importance of determining the time course of release of inflammatory mediators following exposure to particulate air pollution.

SBP significantly decreased at both 4 and 18 h following i.t. administration of DEP. While epidemiological and experimental studies found that particles may affect blood pressure in different ways (Delfino *et al.*, 2005), our observation concurs with reports suggesting that the exposure of rats to either instilled $PM_{2.5}$ or inhaled concentrated ambient particles, decreased blood pressure at 1–2 h post exposure (Chang *et al.*, 2004; Rodriguez Ferreira Rivero *et al.*, 2005).

Our findings concur with another study in which blood pressure fell after exposure to PM_{10} (10 μg·m^{-3}) (Harrabi *et al.*, 2006). This association was significant only for a short time – in the order of few hours – following the increase of the atmospheric level of PM_{10}. However, one should take into account that the dose used in the present study was higher than the PM_{10} concentrations reported by Harrabi *et al.* (2006). The decrease of SBP could be related to systemic inflammation and increase of IL-6 levels as there was an association between decreased blood pressure and raised IL-6 in humans with sepsis and cats exposed to endotoxin (Tateishi *et al.*, 2007; Declue *et al.*, 2010).

I.t. administration of particles increases the risk of thrombosis (Nemmar *et al.*, 2002b; 2003a, 2004a; Mutlu *et al.*, 2007), and the i.t. instillation of DEP promoted thrombosis in pial cerebral venules (Nemmar *et al.*, 2009a). We used pial venules because a more stable thrombus is produced in the conditions of low blood flow, encountered in venules (Nemmar *et al.*, 2009a). The present photochemical injury model of thrombosis in the pial cerebral arterioles of mice represents an improvement over our previous study. In this model, damage to endothelial cells causes platelets to adhere at the site of endothelial damage and then aggregate (Nemmar *et al.*, 2009a). A decrease of platelet number following exposure to particulate air pollution has been reported in humans and animals, suggesting *in vivo* platelet aggregation (Ruckerl *et al.*, 2007; Nemmar *et al.*, 2009a). This effect was confirmed both by a prothrombotic effect of DEP in pial arterioles *in vivo* and a direct proaggregatory effect of DEP in whole blood *in vitro*, suggesting that translocated particles can either directly or indirectly (through systemic inflammation) activate platelets and promote thrombotic events. The possible involvement of the endothelial changes and reduction of fibrinolysis in the pial model of thrombosis remains to be determined. In fact, inhaled DEP have been reported to impair regulation of vascular tone and endogenous fibrinolysis in humans (Salvi *et al.*, 1999; Mills *et al.*, 2005)

Because of the occurrence of systemic inflammation following the exposure to DEP and subsequent cardiovascular effects, we aimed to assess the possible protective effect of thymoquinone, an active principle of the volatile oil of black seeds (from *N. sativa*) that exhibits diverse pharmacological activities (Ali and Blunden, 2003), including antioxidant (Erkan *et al.*, 2008) and anti-inflammatory actions (El Gazzar *et al.*, 2006; El Mezayen *et al.*, 2006). However, thymoquinone has not been used in the context of prevention of particulate-induced systemic and cardiovascular complications.

Pretreatment with thymoquinone prevented pulmonary inflammation and the increase of airway resistance caused by DEP at 18 h. Moreover, it inhibited the increase of blood leukocyte numbers and IL-6 concentrations in plasma at 4 and 18 h. In addition, it prevented the decrease of SOD activity in plasma at 18 h. Pretreatment with thymoquinone exerted an antioxidant effect and decreased IL-6 expression in human proximal tubular epithelial cells, stimulated with advanced glycation end products (Erkan *et al.*, 2008). Along with the prevention of the increase in WBC numbers and IL-6 concentration, and the decrease of SOD, thymoquinone significantly reversed the fall in SBP. Patients with sepsis have depressed heart rate variability in association with high IL-6 blood level and a lower blood pressure (Tateishi *et al.* (2007). Thus, we can hypothesize that the effect of DEP on SBP could result from the effects of raised IL-6. Further experimental studies using IL-6 knockout mice are required to answer this question. The effects of DEP at 4 h on SBP were more likely caused by the systemic inflammation caused by DEP, rather than by the DEP *per se*. In support of this possibility is that pretreatment with the anti-inflammatory thymoquinone prevented the systemic inflammation and the effect on SBP of DEP. At 18 h, the observed systemic inflammation and cardiovascular effects of DEP could result either from pulmonary inflammation and/or the direct translocation of DEP or their associated components into the systemic circulation. In fact, at this time point, both pulmonary and systemic inflammation, and the change in IL-6 and SOD levels, were all prevented by thymoquinone.

The decrease of platelet numbers *in vivo* and the prothrombotic effect caused by DEP were also prevented by thymoquinone. As thymoquinone failed to prevent platelet aggregation *in vitro*, a direct effect on platelets is less likely. We would therefore suggest that the prevention of thrombotic events by thymoquinone *in vivo* resulted from its ability to inhibit systemic inflammation and IL-6 (at 4 and 18 h) release, and SOD decrease (at 18 h). IL-6 levels and the oxidative stress have been correlated in humans with risk factors for cardiovascular disease, such as WBC count, and blood viscosity (Woodward *et al.*, 1999) and the development of thrombosis in mice (Mutlu *et al.*, 2007; Nemmar *et al.*, 2009a). Thus, the observed *in vivo* thrombotic events may be due to the systemic inflammatory response and not an effect of the DEP *per se*. Indeed, platelet aggregation *in vivo* was reversed by the administration of thymoquinone. Whilst DEP caused aggregation *in vitro*, thymoquinone had no effect, suggesting that the mechanisms at play here are different. It is not known whether particles with different chemical composition and/or size would elicit a similar response, and this will be investigated in future experiments.

The i.t. instillation has been shown to be a reliable, convenient and valid, though admittedly not perfect, mode of administration of foreign compounds into the airways (Driscoll *et al.*, 2000). Additional studies using inhalation exposures are needed to confirm our findings. The dose of particles (30 μg·per mouse) that we used here was based on our previous study (Nemmar *et al.*, 2009a), which was comparable with those used in previous animal models of particulate air pollution (Nemmar *et al.*, 2003a,b; Mutlu *et al.*, 2007; Kido *et al.*, 2011). However, when considering this level in relation to human exposure, the dose used in our experiments was relatively high (Brook *et al.*, 2010).

In conclusion 4 h after DEP exposure, the cardiovascular effects did not appear to result from pulmonary inflammation but possibly from the blood translocation of DEP and/or their associated components. However, by 18 h after DEP, pulmonary and cardiovascular functions, as well as lung inflammation, were all affected by DEP. Pretreatment with thymoquinone ameliorated DEP-induced cardio-pulmonary effects. Our data provide biological plausibility to the clinical and epidemiological studies, reporting cardiovascular effects following exposure to particulate air pollution, in the absence or presence of pulmonary inflammation.

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Conflict of interest

None of the authors have any conflict of interest.

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