The possible protective role of quercetin on induced cardiac Oxidative DNA Damage by repeated exposure to diesel exhaust nanoparticles in rats (a histological and immunohistochemical study)

Eman Mohammed Faruk1*, Aisha El Mansy1, Wardah Abdullah Mohammed Alasmari2 and Amal Mahmoud Elsafy Elshazly3

Abstract
Diesel exhaust nanoparticles (DENPs) are gaseous exhaust and one component of particulate matter (PM) air pollution which is found to cause health hazards in humans. Quercetin is widely known for its antioxidant and antitoxic potential. We aimed to detect the protective effect of quercetin in DENP-induced cardio toxicity. Fifty Male albino rats were divided into two groups, first group (ten rats) divided into two equal subgroups (one receive saline act as control and other receive quercetin, the second group (forty rats) divided equally into two subgroups (one was treated with repeated doses of DENPs [90µg/ rat and 180µg/ rat for 6 days intratracheally every two days] and other treated orally with quercetin (60 mg/kg B.Wt.) 1h prior to DENP exposure, DNA damage was examined by measuring serum 8-hydroxydeoguanosine (8-OHdG) levels followed by histological and immunohistochemical studies for nuclear factor-$\kappa$B (NF-$\kappa$B) expression in heart tissues. Tumor necrosis factor- $\alpha$ (TNF$\alpha$), interleukin-6 (IL-6) and serum level 8-Ohd Gare significantly higher in repeated doses of DENPs than the control group. Pretreatment with quercetin reduced significantly serum level 8-OhdG when compared to DENPs group. Cardiac myocytes disruption, vacuolation, inflammation and wide separation of its fibers in contrast to control group. Myocyte inflammations were significantly decreased with quercetin group compared with control group (decrease NF-$\kappa$B immunostaining). The present work yields experimental evidence that quercetin can reduce oxidative DNA damage and inflammation induced by DENPs, so suggested that quercetin possible cardioprotective effect against DENPs.

Keywords: Diesels exhaust particles, DNAdamage, quercetin, air pollution

Introduction
Various studies have seen a strong relationship between particulate matter (PM) air pollution and increase in the mortality and morbidity. In association with this, a relationship between the daily number of hospitalizations, deaths and everyday differential in the ambient PM air pollution concentration has been detected by experts [1,2]. Inhaled airborne PM was found to cause cerebrovascular and cardiac diseases such as ischemia [3]. Studies also showed that airborne PM increased circulatory neutrophil count (CNC) in the bloodstream of residents exposed to high PM concentrations [4]. PM10 deposition in alveoli leads to an infiltration of polymorphonuclear leukocytes (PMN) [4]. Studies by Terashima et al. [5] revealed that bronchial epithelial cells and alveolar macrophages exposed to PM10 lead to the release of hematopoietic cytokines. Suwa et al. [6] reported that the inhalation of air pollution leads to an increase in the pro-inflammatory cells concentration in the circulation because of increased production of cytokines.

Traffic-related particles such as diesel (DEPs) and petrol (DENPs) exhaust particles are the main components of PM air...
pollution. They mainly contain nano-size particles that can easily enter the lungs [7]. Studies on humans reported that exposure to DEP in a controlled manner resulted in elevated thrombus formation and endothelial dysfunction. Previous studies on lung cells and macrophages exposed to diesel exhaust nanoparticles (DENPs) indifferent concentrations demonstrated dose-dependent cytotoxicity, oxidative stress, inflammation, and DNA damage in cell lines [8]. Increased oxidative stress is detected in most types of HF, that resulting from ischaemic and non-ischaemic cardiomyopathy, pressure and volume overload, tachycardiomyopathy [9]. The side effects of oxidative stress in HF patients are vascular endothelial dysfunction [10], cardiac hypertrophy [11] and myocyte contractile dysfunction [12].

Oxidative stress results in damage to DNA, membranes, proteins and other macromolecules. The previous study showed that somatic DNA damage included in the pathogenesis of atherosclerosis and elevated levels of oxidative DNA damage in HF patients [13].

Quercetin is a major flavonoid found widely in various medicinal plants. It is one of the famous anti-oxidant and anti-inflammatory medicinal herbs [14]. Recently, quercetin has been detected as an inhibitor of interleukin 1 beta (IL-1β) induced intercellular adhesion molecule-1 (ICAM-1) expression and nuclearfactor-kappa B (NF-kB) activity in human A549 lung cells [15]. Indeed oral administration of quercetin in animal models was reported to inhibit experimentally induced pulmonary fibrosis, [16] airway inflammation [17] and emphysema [18]. Mi et al. [19] showed that quercetin suppressed oxidative damage characterized by lipid peroxidation, decreased spermatogonial cell numbers, vacuolated cytoplasm and condensed nuclei in spermatogonial cells of embryonic chicken exposed to 3-methyl-4-nitrophenol. However, to the finest of our acquaintance, the histological effect of quercetin on the cardiovascular effects of DENPs has not been addressed so far. Consequently, in the current study, we have evaluated the histological and immunohistochemical effect of DENPs (repeated exposure) 48 h after last 4 exposures to DENPs performed every 2nd day and the possible protective effect of pretreatment with quercetin on cardiac tissues.

Materials and methods

Chemicals

All fine chemicals were procured from Sigma-Aldrich, Egypt. 1-Quercetin (Sigma Aldrich, Egypt) (60 mg/kg b.wt) dissolved in olive oil and was given to animals by oral gavage 1h before each i.t. instillation of DENPs. Dose of quercetin was solved in olive oil and was given to animals by oral gavage 1h before each i.t. instillation of DENPs. Dose of quercetin was 1500rpm in orderto collect DEPs, as the method by Durga et al., [7]. They were suspended in sterile normal saline (NaCl 0.9%) containing tween 80 (0.01%) to decrease aggregation, Control group received only normal saline with 0.01% tween 80 [8]. The collected particles were less than 2.5µm in size. Transmission Electron Microscope (HR-TEM) (JEOL 3010) was used for morphological analysis and seen the presence of nano-size particles.

Animals and treatment

This study was reviewed and approved by our institutional animal ethical committee and the experiment was performed according to the approved protocols from the Institutional Animal Care Committee.

Fifty male albino rats of average weight 200–250 g were used after a 7 day acclimatization period in the current study. All animals were housed in cages in temperature (30±2°C), humidity and light controlled rooms. They were given water ad libitum and standard pellet food. Animals were randomly divided into two groups first group (ten rats) divided into two equal subgroups (one receive saline act as control and other receive quercetin, the second group (forty rats) divided equally into two subgroups, one of them treated with repeated doses of DENPs [90µg/ rat and 180µg/ rat for 6 days intratracheally every two days] othereone treated orally with quercetin (60 mg/kg b.Wt.) 1h prior to DENP exposure. Following this, sodium pentobarbital (intraperitoneally; 60mg/kg b.wt) was used for rats anesthesia. A 24-gauge cannula by the mouth was inserted into the trachea. Either the DENPs suspensions or normal saline only was intratracheallyinstilled using a sterile syringe [8]. The administration was done on days 1, 3, 4 and 7. Body and heart weights of each animal were measured at the end of the study.

Collection and analysis of blood

48h post the last i. instillation of DENPs the rats were anesthetized as describes above. Blood was collected from the tail vein in 4% EDTA tubes. Then was investigated for total cell counts and platelets using a fully automated hematological analyzer. The remaining blood sample was centrifuged at 900g for 15mins at 4°C and the plasma was separated. It was stored at -80°C for further analysis.

Determination of plasma IL-6 and TNFα

The concentration of rat IL-6, TNF-α, D-dimer, CRP, MIP-2 and PAI-1 levels were determined using ELISA kits (Invitrogen, India) [20].

Serum 8-OHdG Levels

Blood was collected and allowed to clot for 1 h before centrifugation at 3,000 rpm for 10 min. Then, the samples were stored at −80°C until analysis. Serum 8-OHdG levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Japan Institute for the Control of Aging, Fukuroi, Japan).

Histological studies

The heart specimens were fixed in 10% formalin, processed,
embedded in paraffin sections. Then sections were cut into 5 μm thicknesses using a microtome and mounted on slides to H&E stain for histological assessment [21] and immunohistochemical detection of TNF-êB expression, using a standard avidin-biotin peroxidase complex system according to the kit used (Neomarkers) followed by diaminobenzidine (DAB) visualization [22]. Sections were counterstained with hematoxylin [23].

Statistical analysis
The results were expressed as mean±SD. Data were analyzed by standard statistical analysis one-way ANOVA for multiple comparisons to determine significance between different groups. The results were considered statistically significant if ‘p’ value was .05 or less.

Morphometric study
The mean area percentage for TNFêB expression were quantified in 10 images for each group using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Results
Body weight and lung weight
The body weights of animals exposed to D1 and D2 significantly decreased by 38.39% and 27.89%, respectively, compared with the control. However, in quercetin-treated groups, the body weights were significantly increased by 60.19% and 38.47%, respectively, compared with D1 and D2 and were almost near normal control values (Figure 2A). In contrast, lung weights were increased by 126.02% (D1) and 116.14% (D2) when compared with the control group. However, lung weights of Q + D1 treated groups and Q + D2-treated groups were decreased significantly by 47.86% and 43.58% when compared with D1 and D2 groups and were near normal values (Table 1).

Total cell counts and platelet numbers in blood
Total cell count was significantly increased by 2.21 (D1) and 3.65 times (D2) by repeated i.t. exposure to DENPs. Pretreatment with quercetin decreased the levels by 0.39 and 0.29 times for Q + D1 and Q + D2 groups when compared with the D1 and D2 groups. In contrast to this, platelet levels were decreased upon DENPs exposure by 29.59% (D1) and 34.89% (D2) demonstrating the occurrence of in vivo platelet aggregation. However, quercetin pre-treatment partially reversed the decrease in platelet counts significantly (p<0.05) by 38.81% and 68.09% caused by the i.t. exposure to DENPs (Table 2).

Cytokine analysis in plasma
The concentration of plasma IL-6 and TNFα was seen increased by 128.11% and 250.25%. Prior treatment with quercetin reduced the levels by 48.18% and 32.06% respectively (Table 3).

Serum Levels of 8-OHdG
D1 and D2 group had significantly elevated serum levels of 8-OHdG compared with control subjects (D1 and D2: 5.6±3.1ng/mL and 6.3±1.6ng/mL vs control groups: 3.0±1.5ng/mL and 3.5±2.1ng/mL, p=0.0018). Decrease in serum 8-hydroxy-

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<th>Q + D2</th>
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<td>204±1.38a*</td>
<td>200±0.83a*</td>
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<td>Body weight at end of experiment BW (g)</td>
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<td>206±6.32</td>
<td>192±3.25*</td>
<td>198±8.65a*</td>
<td>205±0.20a*</td>
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<td>Heart weight at end of experiment (g)</td>
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<td>6.1±1.94</td>
<td>4.9±1.28a*</td>
<td>4.3±7.21a*</td>
<td>5.7±0.12a*</td>
<td>5.4±1.52b*</td>
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Table 1. Body and heart weight of animals exposed to saline (control) or diesel exhaust particles (D1/D2) with or without quercetin (Q) pretreatment. Data are mean±SD (n=8 in each group).

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<thead>
<tr>
<th>Group Parameters</th>
<th>C</th>
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<td>Hemoglobin (g/dL)Hb</td>
<td>12.5±0.39</td>
<td>12.4±0.12</td>
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<td>Erythrocytes (×10^6/µL) (TEC)</td>
<td>7.4±0.23</td>
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<td>Leukocytes (×10^3/µL) TLC</td>
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<td>6.6±0.27</td>
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<td>11.40±1.11a*</td>
<td>8.53±3.43a*</td>
<td>8.70±2.57b*</td>
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<td>Platelet (×10^3/µL)</td>
<td>227±52.03</td>
<td>216±87.55</td>
<td>175.50±75.7*</td>
<td>130±95.05a*</td>
<td>196±43.55a*</td>
<td>241±33.3b*</td>
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Table 2. Changes in hemoglobin (Hb, mg/dl), total erythrocyte count (TEC, 106/mm3), and total leukocyte count (TLC, •103/ mm3), Platelet (×103/µL) of rats quercetin exposed to saline (control) or diesel exhaust particles (D1/D2) with or without quercetin (Q) pretreatment. Data are mean±SD (n=8 in each group).

Note: C i.e control, Q i.e quercetin, D1 and D2 i.e DENP treated group and D + Q quercetin pretreated group *as compared with control normal group, a* as compared with D1 treated group and b* as compared with D2 treated group.
2-deoxyguanosine (8-OHdG) levels with quercetin pretreatment (7.2 ± 2.5 ng/mL and 5.1 ± 3.2 ng/mL (19%), p<0.05) (Table 4).

**Histological (H&E)**

The histological examination of cardiac tissues of control group ratsshowed normal cardiac muscle fibers that appeared in different directions and contained acidophilic sarcoplasm with central, single or oval nuclei. Cardiac muscle cells were connected to make fibers by intercalated discs and the space between the fibers were narrow (Figure 1).

The cardiac muscle fibers of group II D1 (DENP treated group) showed separation of myofibers that contain extravasated RBCs, and congested blood vessels with an area of hemorrhage could be seen and degenerated cardiac myocytes that appeared vacuolated with darkly stained nuclei (Figure 2).

Focal mononuclear cellular infiltration could also be seen in many areas of the degenerated cardiac muscle fibers of group II D2 (DENP treated group) with congested blood vessels (Figure 3B). There was an apparent hemorrhage of separated degenerated cardiac muscle fibers in the affected areas (Figure 3B). In D+Q quercitin pretreated group revealed that most of the cardiac muscle fibers appeared nearly similar to the control group with central vesicular nuclei. However, many wide interfiber spaces were still detected (Figure 4B). Some areas showed pale vacuolated cardiac muscle fibers with inflammatory cells infiltration between wide interfiber spaces (Figure 4B).

**Immunohistochemical stains (Inos & Kappa B)**

Immunohistochemical examination of Kappa B stained sections of cardiac tissue of control group revealed weak brown positive cytoplasmic reaction, Figure 5. While, Immunohistochemical examination of Kappa B stained sections of...
Figure 3A. A photomicrograph of a longitudinal section in the cardiac muscle from the group IIaD2 (DENP treated group) showing, degenerated and separated of the cardiac muscle fibers with focal mononuclear cellular infiltration (dark arrow). Notice hemorrhages between the myocytes (white arrow). (H&E × 200).

Figure 3B. A photomicrograph of a longitudinal section in the cardiac muscle from the group IIaD2 (DENP treated group) showing, degenerated of the cardiac muscle fibers with focal mononuclear cellular infiltration (arrow). Notice large areas of hemorrhage could be detected (H). (H&E × 400).

Figure 4A and B. A photomicrograph of a longitudinal section of the cardiac muscle from the group IIbD +Q quercetin pretreated group showing, nearly similar to the control group with central vesicular nuclei (white arrow). Notice wide interfiber spaces were still detected with inflammatory cells infiltration (dark white). (H&E X 400).

Cardiac tissue of D1 (DENP treated group) showed moderate brown positive cytoplasmic reaction, Figure 6. On the other hand, sections of cardiac tissue of D2 (DENP treated group) resulted in strong Immunohistochemical reaction in Kappa B stained sections, Figure 7 In D1 +Q quercetin pretreated group showed moderate to week brown cytoplasmic reaction in Kappa B stained sections, Figure 8 which more decreased with D2+Qgroup, Figure 9. Table 5.

Morphometric and statistical analysis

Optical density mean of Kappa B

There was a significant decrease in the Optical density mean of Kappa B immunoreactivity in group D1+Q (1.73±1.30) when compared with the control group (5.10±2.56), with a P value (0.006). While in D1 and D2 groups, the Optical density mean of Kappa B were (6.32±1.35) and (7.84±2.15) respectively was significantly increased as compared with the control group (Pvalue 0.001). No significant difference was found between control and quercetin group (P value 2.001) (Table 5).
Discussion

There is prominent evidence that inhalation exposures to air pollutants result in cardiopulmonary effects but the detailed mechanisms are not well understood. Various studies have also reported higher risks of lung infection and asthma in residents who reside near highways with increased traffic congestion [8].

In the present study, we began to investigate the relationship between exposure to DENPs (a component of air pollution) and the accumulating evidence of inflammation and cardio toxicity. The current study approach is seen more related to the daily human exposure circumstances than single exposure dose. The dose of DENPs administered to rats in the current study was chosen from previous studies which have seen to correct arrhythmia in rats [24, 25]. In general the results of our present study demonstrates that exposure to DENPs induces significant cardiac toxicity in rats and this is evident from (a) reduction in total body weight (b) increase in levels of pro-inflammatory cytokines in plasma, (c) decrease in the levels of platelets in blood, (d) increase in the serum level 8-OhdG which is biomarker of oxidative DNA damage and (e) histologically result revealed degeneration and inflammatory cell infiltration of the cardiac myocytes with apparent separation of its fibers.

Our results represented that repeated intratracheal exposure to DENPs causes an important inflammatory reaction in the cardiac tissue characterized by an elevation in the optical density of NF-κB immunostaining. Similar results were seen after a single dose and repeated dose administration of particles [8, 26-28]. Studies by Nemmar et al. [26] in humans showed...
an increase in a number of mast cells and neutrophils in the bronchial submucosa along with an increase in myeloperoxidase and interleukin 8 (IL-8) concentrations. Along with the influx of inflammatory cells, we found a statistically significant elevation in the levels of TNFα. This finding correlates with the data of previous studies that have illustrated an elevation in TNFα concentrations in particle exposed humans [29]. An increase in IL-6 concentration in plasma after the last administration was observed invivo. Similarly, studies by Nemmar et al. [27] demonstrated that IL-6 was elevated at 18 hours but not at the 4h time point or 24h. Our previous invitro studies showed an elevation in IL-6 concentration in human A549 lungs cells [30]. Although the mechanism of cardiovascular mortality and morbidity caused by nanoparticles is not still completely understood, various studies showed that systemic inflammation is the key step leading to these pathological changes through the production of inflammatory mediators. Our results showed that repeated administration of DENPs to rats caused systemic inflammation. Sallam et al. [31] and Park et al. [32] demonstrated in diabetic mice an elevation in CRP during inflammation. Studies reported that IL-6 is required for CRP expression [33]. Our study results were similar to human experiments that reported the high risk of cardiovascular effects associated with elevated levels of pro-inflammation and inflammatory cytokines such as CRP and TNFα [29,34]. Increased concentrations of CRP and TNFα play a key role in the pathology of plaque instability and atherosclerosis [35]. Our experimental results corroborate with previous findings which illustrated that long exposures to PM air pollution caused the influx of cytokines leading to systemic inflammation. Simultaneously, the markers for oxidative damage were also elevated [36,37]. In the current study, and we have determined the effect of DENPs on platelet numbers and all blood content. Our results showed that there was a significant reduction in platelet numbers in rats exposed to DENPs suggesting invivo platelet aggregation. Previous studies reported the decrease in platelets both in clinical studies [31] and in mice [38]. Weight loss is associated with increased oxidative DNA damage not only in healthy individuals but also in people with inflammatory diseases.

Our data represented that DENPs caused neutrophil influx into the heart tissue enhanced epithelial permeability in rats [36]. To the best of our knowledge no study till date has demonstrated the effect of quercetinon the cardiovascular parameters following DENP exposure. The chosen dose of quercetin has been stated to correspond to doses of quercetin in commercial products routinely consumed on the mg/kg scaling basis. Quercetin has been reported to exhibit numerous beneficial activities like antioxidant, anti-inflammatory, and anti-tumour activities. Human oral animal studies reported that quercetin interfered with the formation of inflammatory cytokines, p300 activity, accumulation of beta-amyloid and activities of cytokine p450 NF-kB [9]. Animal oral human studies demonstrated that quercetin is tolerated in oral doses of (60 mg/kg) without evident toxicity [18]. The chosen dose of quercetin has been stated to correspond to doses of quercetin in commercial products routinely consumed on the mg/kg scaling basis. Our data reported that pretreatment with quercetin inhibited the production of inflammatory cells and cytokines in plasma, which can play an important role in atherosclerosis and coronary thrombosis [39,40]. Previous studies demonstrated that quercetin blocks TNFα mediated NF-kB activity and inhibit TNFα dependent regulation of PPARy in mesangial cells [41]. Studies showed that in the mouse model, TNFα plays an important role in LPS induced PAI-1 regulation [42]. Studies also demonstrated that TNFαs a key regulator of PAI-1 in the adipose tissue of rats [40]. Hence, TNFα is a strong against for PAI-1 regulation, contributing to various diseases. Likewise, similar to our results Hou et al. [43] and Plomgaard et al. [44] reported that TNFα but not cytokine IL-6 regulates PAI-1 expression in the sub-cutaneous adipose tissue. This confirms the key role of TNFα in the observed effects. The reduction in platelet numbers by the repeated exposure to DENPs was partially reverted to quercetin. This suggests that DENPS exhibit a direct effect on platelet aggregation. DNA damage has a high incidence in human hearts under different diseases, such as myocardial ischemia and myocarditis [45,46]. In the present study, we found increased oxidative DNA damage in the serum of the rats taken DENPs, so oxidative stress is also a condition under which DNA damage occurs.

The serum 8-OHdG levels are affected by oxidative stress not only in heart but systemically. Elevation of circulating 8-OHdG levels may reflect other organ damage, such as endothelial dysfunction [47].

Conclusion
In conclusion, the present work showed that the repeated exposure to DENPs induced cardiac inflammation and oxidative DNA damage. Our results indicate that quercetin is a significant anti-inflammatory agent and might be effective for decreasing the oxidative DNA damage induced by DENPs.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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