Urban Particulate matter induces changes in gene expression in vascular endothelial cells that are associated with altered clot structure *in vitro*

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Abstract

Background: Particulate matter contained in ambient air pollution has been associated with cardiovascular diseases in a number of epidemiological studies.

Objective: The aim of this study was to investigate the potential for urban particulate matter to induce changes in clot structure through interaction with vascular endothelial cells.

Methods: We examined the structure of clots formed on human umbilical vascular endothelial cells that had been treated with various types of particles versus those formed on untreated cells. Particles used were standard reference particulate matter from diesel engine emissions (SRM2975) and urban ambient collection (SRM2787).

Results: There was a dose dependent increase in fibre density in clots formed on particle treated endothelial cells. It was also found that exposure to the particles induced increased expression of tissue factor and reduced expression of thrombomodulin genes as measured by RT-PCR and increased expression of von Willebrand factor and plasminogen activation inhibitor-1 as measured by ELISA.

Conclusions: These changes are consistent with increased pro-coagulant activity of air pollution particulate matter treated endothelial cells and suggest that particulate matter has the potential to promote clot formation through changes induced in endothelial genes controlling clot formation.

Introduction

Air pollution has been associated with adverse effects on the pulmonary and cardiovascular systems (1–4) with 3.6 million premature deaths being attributable to ambient air pollution each year in both rural and urban areas worldwide (1,5,6).

The components of air pollution vary depending on the meteorological conditions, time of the day, industrial operations, traffic density, etc. (3). Gaseous pollutants of air pollution include ozone (O_3), nitrogen oxides (NO_x), sulphur dioxide (SO_2), carbon monoxide (CO), carbonyl compounds, and organic solvents (1,7,8). The particulate matter (PM) in air pollution is a mixture of particles, with different sizes, shapes, surface area, chemical composition, solubility, and different origins that are suspended in the air (9). PM is categorized by aerodynamic diameter, including coarse particles with an aerodynamic diameter range less than 10 μm (PM_{10}), fine particles with a diameter less than 2.5 μm ($PM_{2.5}$), and ultrafine particles with a diameter less than 100nm ($PM_{0.1}$) (7,10). PM_{10} is identified as “inhalable particles” (5) as particles are able to enter into the lung through the respiratory tract (11). Coarse particles most likely deposit in upper and larger airways. Fine particulate matter can transfer into deeper respiratory tract, with deposition in the alveolar region (8,12) and the potential to affect the cardiovascular system (11). $PM_{0.1}$ deposits in the deeper alveolar region, where particles may interfere with cells, fluids, and tissues of the lungs due to their large surface area. Also, these ultrafine particles may be able to translocate to different organs by crossing into the circulatory and/or lymphatic systems (13,14).

The PM in urban air pollution has been associated with cardiovascular mortality and morbidity in a number of studies (1,4,15). There are several cardiovascular diseases

where exposure to PM has been shown to contribute to risk, including ischemic heart disease, heart failure, cerebrovascular disease, cardiac arrhythmias, peripheral arterial and venous diseases (1,5,15).

Some studies have shown that particulate matter can induce increased expression of C-reactive protein, fibrinogen, and reduced level of tissue Plasminogen Activator (tPA) in epidemiological studies (8,19–22). Associations between PM exposure and thrombosis/coagulation have been reported (16) and we have reported changes in the structure and function of clots formed from plasma of patients with DVT, but not from healthy controls (17,18).

To further investigate the mechanism for such changes we have analysed changes in clot structure formed on vascular endothelial cells, and how this may be attributed to changes in coagulation factors expressed by these cells when exposed to PM in vitro.

Materials and Methods

Particulate Matter Preparation

PM_{10} collected from an air intake filtration system of a major exhibition center in Prague, Czech Republic (SRM 2787) and diesel particulate matter (DPM) collected from an industrial diesel-powered forklift exhaust (SRM 2975) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). These are designated as PM_{10} and total DPM, respectively, in this paper.

To generate PM of smaller size, both particles were suspended in double distilled water, at a stock concentration of 1 mg/ml, and then centrifuged (Beckman Centrifuge,

UK) for 30 min at maximum speed, and filtered through a 0.2 μm membrane filter. Designated as $PM_{0.2}$, the filtered SRM 2787 particles made up 30% of the mass fraction of the original PM_{10}. The filtered SRM 2975 diesel particulate matter, referred to subsequently as filtered DPM constituted 35% of the total diesel particles.

The four types of particles were suspended in distilled water with stock concentration 1 mg/ml.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (Heidelberg, Germany). Cells were cultured in M199 media supplemented with 20% fetal bovine serum, 1% 1M Hepes, 1% antibiotic antimycotic solution (100x), 0.5% endothelial cell growth supplements solution (15 μg/ml), 0.5% Heparin (1000 U/ml), and sodium pyruvate solution. Cells were maintained in a humidified atmosphere at 37℃ and 5% CO_2. HUVECs were used only between passage 3 to passage 7.

Cytotoxicity Test

Cytotoxicity was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reduction assay (23).

For the treatment, cells were seeded into a 96-well plate with $2x10^4$ cells per well. After the cells reached 80 to 90% confluence, they were treated for 24 hours with 6 different concentrations of these four types of particles respectively. Particle suspension were further diluted with ECGM without serum to different concentrations (0 μg/ml, 0.01

µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 50 µg/ml). Following exposure, 10 µl of MTT solution was added to each well and incubated for 4 hours. Then, 100 µl of solubilising solution (10% sodium dodecyl sulphate in 0.01 M hydrogen chloride) was added to each well and incubated overnight. Optical density was read on a microplate reader at 540 nm, with a reference at 690 nm (ASCENT software).

Fibrin Clot Formation on HUVECs

Particle concentrations for clot formation experiment were chosen based on the MTT assay. The cells were seeded on ibidi µ-slide (5 x 10^4 cells per well). After reaching 80 to 90% confluence, the cells were treated for 24 hours with 6 different concentrations of these four types of particle suspension respectively which was prepared as same as in MTT assay.

After the treatment, the particle suspension was removed completely. Clot mixtures were prepared from either normal pooled plasma or purified fibrinogen mixed with 200 µl of activation mix (containing Alexa Fluor® 488 dye, $CaCl_2$, thrombin and M199 media). Once the activation mixture mixed with plasma or fibrinogen, they were immediately introduced into each well. Slides were incubated for 30 min allowing clot formation. The final concentrations of normal pooled plasma/fibrinogen, Alexa Fluor® 488 fibrinogen conjugate, thrombin and $CaCl_2$ were three times dilution/1 mg/ml, 50 µg/ml, 0.5 U/ml and 15 mM, respectively.

The 3D structure of the clot was visualized by laser scanning confocal microscopy on a LSM 700 T-PMT ZEISS microscope (ZEISS, Jena, Germany). Clot structure was viewed using 63x oil immersion lens with a 5-W argon laser and 488 nm laser filter.

The images were collected in the format of single z-scan with 512x512 pixels (x: 101.61 µm; y: 101.61 µm). Fibre density was calculated as the number of fibre bundles crossing a straight line of fixed length across the scanfield. All measurements were performed with Image J version 1.25s software.

Enzyme-Linked Immunosorbent Assay (ELISA)

After the cells were treated with different concentrations of particles for 24 hours, the cell supernatant was collected for protein quantification. Enzyme-Linked Immunosorbent Assay (ELISA) kits purchased from Abcam (Abcam, UK) were used to measure the concentrations of von Willebrand Factor (VWF) and Plasminogen Activator Inhibitor-1 (PAI-1) produced by endothelial cells. Cell supernatants were centrifuged before use. All standards, controls and samples (cell supernatants) were diluted with Standard Diluent Buffer 1:1 (50 µl : 50 µl) and added into each well in triplicates. Following the manufacturer's protocols, the plate was incubated and then washed with Wash Buffer for four times. Then, provided Conjugate Solution was added into each well. After 2h incubation, the plate was washed and followed by Streptavidin-HRP Working Solution addition to each well. After washing, Stabilized Chromogen was added and incubated for 30 min. Finally, the plate was read at 450nm immediately after Stop Solution addition.

Real Time Polymerase Chain Reaction (RT-PCR)

Relative gene expression in HUVECs was analysed using quantitative RT-PCR. Total RNA was isolated from HUVECs using an RNeasy kit (Qiagen, UK) as per manufacturer's protocol. Total RNA was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, UK).

Total RNA was reverse transcribed to cDNA using Taqman Reverse Transcription Reagents (Life Technologies, UK). GAPDH as household standard gene transcript, tissue factor (TF) and thrombomodulin (TM) primer pairs were obtained from Applied Biosystems (GAPDH: sense-5' AAG CCT GCC GGT GAC TAA C 3', antisense-5' GCA TCA CCC GGA GGA GAA AT 3'; TF: sense-5' AAA CCT CGG ACA GCC AAC AA 3', antisense-5' CCC GGA GGC TTA GGA AAG TG 3'; TM: sense-5' AGC CCC TGA ACC AAA CTA GC 3', antisense-5' GAA ACC GTC GTC CAG GAT GT 3'). SYBR Green Master Mix (Thermo Fisher, UK) was used in the RT-PCR for fluorescence quantification after double stranded DNA binding to the SYBR® Green. Reactions were performed in triplicate by the LightCycler® 480 Instrument II (Roche, UK). Results were confirmed in at least three independent experiments.

Plasmid Strand Break Assay

The plasmid strand break assay was used to investigate the free radical generating capacity of the particles. pBR322 Plasmid DNA was purchased from Thermo Fisher Scientific (Road Loughborough, UK). The plasmid was precipitated to remove the chelating agent, EDTA, before use.

The precipitated plasmid DNA was incubated with different concentrations of particles for 12 hours. After incubation, plasmid was transferred to the gel and gel

electrophoresis was run at 60 V for 2 hours. Strand breaks were quantified by BioRad QuantityOne software (Bio-Rad, UK) to calculate the proportion of supercoiled plasmid DNA remaining of each sample compared to the control.

Cellular Reactive Oxygene Species Detection Assay

Cellular reactive oxygen species (ROS) detection assay uses the cell permanent reagent 2', 7'-dichlorofluorescin diacetate (DCFDA), a fluorogenic dye to measure hydroxyl, peroxyl and other ROS activity within cells. DCFDA cellular ROS detection assay kit was purchased from Abcam (Cambridge, UK). HUVECs were exposed to 0.1, 1, 10, and 50 ug/ml of PM_{10}, $PM_{0.2}$, total DPM and filtered DPM respectively for for 4 hours. Cells were stained with DCFDA which could be deacetylated by cellular esterases to a non-fluorescent compound. Later, DCFDA could be oxidized by ROS into 2', 7'-dichlorofluorescein (DCF), a highly fluorescent compound. The accumulation of DCF in cells can be measured by an increase in fluorescence at 530nm when the samples is excited at 485nm. Fluorescence at 530nm was measured using the flow cytometer. Cells without staining and tert-butyl hydrogen peroxide (THBP) solution were used as negative and positive controls respectively.

Fibrin Clot Formation on HUVECs without Thrombin Addition

Particle concentrations for cell treatment and clot formation experiments were same as the previous fibrin clot formation on HUVECs. The cells were seeded on ibidi µ-slide (5×10^4 cells per well). After reaching 80 to 90% confluence, the cells were treated for 24 hours.

After the treatment, the particle solution was removed completely. Clot mixture was prepared from normal pooled plasma mixed with 200 µl of a mixture containing Alexa Fluor® 488, $CaCl_2$ and M199 media, but without thrombin. This mixture was added to plasma and immediately introduced into each well. Slides were incubated for 4 hours allowing clot formation. The final concentrations of Alexa Fluor® 488 dye and $CaCl_2$ were 50 µg/ml and 15 mM, respectively.

The visualisation and measurement methods of fibre density were same as clot formation with thrombin by using Image J.

Statistical Analysis

The data are reported as mean ± SEM. Comparisons between groups are analysed using two- sample (independent) t-test. We considered a p value of <0.05 to be statistically significant as indicated in the figures.

Results

Cytotoxicity

HUVECs were treated with five different concentrations of PM_{10}, $PM_{0.2}$, total DPM and filtered DPM for 24 hours. For most of the particles, no significant cytotoxicity was observed up to a concentration of 50 µg/ml (Fig.1). Only the PM_{10} sample induced a slight reduction in cell viability at this highest dose.

Clot Formation on treated cells

The fibrin clots formed using normal pooled plasma were formed on the cells that had been treated with PM for 24 h.

As seen in figure 2(A), the structure of the clot became significantly denser on cells treated with 10 µg/ml or 50 µg/ml of PM_{10} compared to control. As the concentration of filtered particles increased, figure 2 shows that the clots became more compact with more branched fibres. Results were similar for the other particle types (not shown). Figure 2(B) shows the density of clot fibres, quantified by counting fibre bundles across a line of equal length drawn across the images generated from the confocal micrographs. As the concentration of particles increased, the number of fibre bundles increased in a dose-dependent manner.

Gene expression

As TF and TM are membrane bound proteins, RT-PCR was used to quantify gene expression of these clotting factors by the endothelial cells after incubation with the particles for 24 hours (Figure 3).

After the cells were treated with PM_{10} and total DPM at 0.1 µg/ml, gene expression of TF was significantly higher compared to control. $PM_{0.2}$ caused significantly increased expression of TF gene from 1 µg/ml. Filtered DPM had less effect on TF gene expression in comparison with the other three types of particles as shown in Figure 4A. At 50 µg/ml, all four kinds of particles induced significantly higher TF mRNA expression than control. At this concentration, PM_{10} induced three times more TF gene

expression compared to control, whilst the other three particles induced 1.85, 1.72, and 1.49 times elevation of TF mRNA, respectively.

In terms of the gene expression level of TM, the cells without particle treatment had the highest level of TM mRNA expression (Figure 3B). As the concentration of particles increased, the gene expression of TM decreased in a dose-dependent manner. PM_{10}, $PM_{0.2}$ and total DPM showed significantly reduced TM mRNA expression at 1 μg/ml, with filtered DPM showing a significant reduction compared to control at 10 μg/ml. At 50 μg/ml, PM_{10} and total DPM inhibited the TM gene expression by 70% compared to control, whilst $PM_{0.2}$ and filtered DPM led to approximately 55% less TM mRNA expression by the HUVECs.

Although changes in gene expression were observed for TM and TF, we were unable to detect differences in protein level measured by ELISA (results not shown). TM protein levels were low with no difference seen between treated and untreated cells and TF protein levels were too low to detect.

Protein expression

ELISA was used to quantify the levels of VWF and PAI-1 secreted by endothelial cells after incubation with different concentrations of PM (Figure 4). As the concentrations of PM increased, the levels of VWF expressed by treated HUVECs increased in a dose-dependent manner (Figure 4A). At 50 μg/ml, PM_{10} caused the most VWF secretion compared to the other three particles, followed by $PM_{0.2}$, total DPM and filtered DPM.

There was also an increase in expression of PAI-1 when endothelial cells were stimulated by PM (Figure 4B). At 0.1 μg/ml of PM_{10}, the concentration of PAI-1 increased to approximately 250 pg/ml which was significantly higher compared to the control level of 200 pg/ml. Total DPM induced significant PAI- expression at 1 μg/ml. $PM_{0.2}$ and filtered DPM caused similar levels of PAI-1 expression after cells were treated with concentrations of 5 and 10 μg/ml.

Free radical generating capacity of particles

The plasmid strand break assay was used to detect the generation of free radicals released from the particles. After 12 hours incubation in the dark, there was a dose dependent increase in free radicals as shown by the increase in conversion of supercoiled plasmid DNA to the nicked form of plasmid (Figure 5). PM_{10} and $PM_{0.2}$ generated more free radicals compared to the DPM and filtered DPM, with about twice as much supercoiled plasmid converted to the nicked form at 50 μg/ml (60% vs 35%).

Cellular Reactive Oxygen Species Detection Assay

After HUVECs were incubated with four types of air particulate matter for 4 hours at multiple doses, the flow cytometry assay was used to analyse the ROS production in HUVECs. The results showed that after the cells incubated with the particles, the ROS was induced in a dose-dependent manner. The significant upregulation of ROS was seen at 50 μg/ml of these four particles (Figure 6).

Clot Formation on treated cells without thrombin addition

The fibrin clots using normal pooled plasma, but without thrombin, were formed on the cells that had been treated with PM for 24 h.

The four types of particles were all used to treat the cells at concentrations of 1 µg/ml and 50 µg/ml. The results showed that the clots formed on the cells treated with 50 µg/ml of PM_{10} were significantly denser compared to the control (figure 7). For the other three types of particles, the clots formed on the cells treated with different concentrations of particles showed no significant differences from control. Figure 8 generated from the confocal micrographs shows the density of clot fibres that the clots formed on the cells treated with the highest concentration of PM_{10} induced significant denser clots.

Discussion

We have previously shown that blood clot structure and function is modified in vitro by the presence of diesel PM (24) Although no changes were seen in the clotting of plasma from young healthy individuals exposed to ambient diesel exhaust (18), we found changes associated with traffic emissions exposure in clotting of plasma from patients with deep vein thrombosis (17). In this study we have explored the impact of urban and diesel PM on vascular endothelial cells and observed changes in expression of genes and proteins that lead to similar alterations in clot structure in clots formed on the treated cells in vitro. This is the first study to investigate the structure of fibrin clots formed on vascular endothelial cells after the cells had been exposed to air pollution particles.

Vascular endothelial cells were treated in serum free media with four preparations of PM at doses that did not induce cytotoxicity. Studies on human coronary artery endothelial cells (12) and human aortic endothelial cells (25) have also shown that diesel particles induced no significant toxicity at 50 µg/ml.

After endothelial cells were incubated with different concentrations of particles, clots subsequently formed on the cells showed altered structure compared to control clots. As the concentrations of particles increased, the clots formed became much denser, with increased number of fibres per clot area and smaller pores. It is important to note that unlike our previous experiments showing that clot structure was altered in the presence of PM (24), in the current experiments with vascular endothelial cells, the PM was not present in the culture media when the clot was formed. Observed changes were therefore due to coagulation factors expressed by the endothelial cells. There is strong evidence that air pollution is linked with cardiovascular diseases (CVD), especially thrombosis, and it is known that patients with CVD show altered fibrin clot structure (26,27). Okraska-Bylica et al. showed that patients with peripheral artery disease were characterized by thrombotic fibrin clot phenotype with 32% lower clot permeability (Ks) ($P < 0.001$) and 7% longer clot lysis time (t50%) ($P = 0.004$) compared with controls (26) and Palka et al. found that clots in plasma from patients with chronic heart failure, who were predisposed to thromboembolic complications, showed 23% lower permeability ($p < 0.0001$), 13% less clot compaction ($p < 0.001$), 15% faster fibrin polymerisation ($p < 0.0001$) and prolonged lysis time ($p = 0.1$) compared to controls (27). A review from Undas and Ariens also indicated that altered fibrin structure was found in patients with several diseases complicated by thromboembolic events, including patients with acute or prior myocardial infarction,

ischemic stroke, and venous thromboembolism. Even relatives of patients with myocardial infarction or venous thromboembolism display similar fibrin abnormalities (28).

Our results show that the exposure of the HUVECs to urban and diesel PM induces a pro-coagulant state in the endothelial cells, which increase expression of genes and proteins that promote coagulation (TF, vWF and PAI-1) whilst reducing expression of the anti-coagulant, TM.

Under normal physiological conditions, TF is only expressed in subendothelial cells such as vascular smooth muscle cells, and is produced by endothelial cells only when they are exposed to stimuli that activate the endothelial cells in response to the initiation of coagulation cascade (29). In this study, incubation of the endothelial cells with different concentrations of PM led to a dose-dependent increase in the gene expression of TF. This is consistent with results of Snow et al. (12) that soluble ultrafine PM (diameter less than 0.1 µm) induced 3.8 and 5.1 fold significant increases in TF gene expression after 50 and 100 µg/m^3 treatment, respectively, and those of Karoly et al. (30) showed that TF was upregulated in a dose-dependent manner in human pulmonary arterial endothelial cells treated with ultrafine PM. Expression of TF has also been shown to be increased in a macrophage cell line treated with PM_{10} across a similar dose range (31). Although protein levels of TF were too low to detect, the changes in gene expression for this transmembrane protein are consistent with inducing a pro-coagulant state.

Increased level of TF gene expression may be a potential mechanism of endothelial dysfunction and pathogenic role of PM and diesel particles in endothelial cell system. Evidence shows that increased levels of TF expression is associated with elevated

risks of procoagulability, inflammation and increased tendency of thrombosis (32–35). TF initiates the extracellular coagulation which provokes intracellular inflammation signalling. The coagulation factors (FVIIa, FXa, and thrombin) and fibrin are proinflammatory, all of which can activate the cells independently (32). Inflammation boosts coagulation through feedback upregulation on TF expression that sustains the coagulation TF pathway and coagulation dependent inflammation to refuel the coagulation-inflammation cycle. Therefore, regulation of TF expression is crucial in inhibition of coagulation-dependent inflammation (32).

Von Willebrand factor is a large glycoprotein which plays a pivotal role in haemostasis, circulating in human plasma at concentrations of 10 µg/ml. VWF is synthesized by vascular endothelial cells (36–38) and mediates platelet aggregation and adhesion to the site of vascular injuries, which is particularly important under high shear stress (37,38). There are two platelet receptors for VWF; glycoprotein (GP) Ibα in the GP Ib-IX-V complex and the integrin $\alpha_{IIb}\beta_3$ (GP IIb-IIIa complex) (39). In this study, concentrations of VWF increased in a dose-dependent manner after HUVECs exposed to increased concentrations of PM. VWF acts as a plasma carrier for factor VIII and protects it from degradation and cellular uptake- when not bound to VWF the plasma half-life of FVIII is reduced from 12 hours to 1 to 2 hours (38–40). Consequently, the levels of VWF and FVIII are closely correlated, so high levels of VWF leads to high levels of FVIII, which contributes to atherothrombotic diseases and venous thromboembolism (36,41,42).

The increase in PAI-1 we observed following PM treatment of the HUVECs is also consistent with thrombosis as this will act to decrease fibrinolysis. Plasmin is the main fibrinolytic enzyme which can be activated by two serine proteases, tPA and uPA.

Fibrinolysis is initiated when tPA and plasminogen both bind to the fibrin and plasminogen is partially cleaved to plasmin by tPA on the surface of fibrin. The elevated level of PAI-1 effectively supresses fibrinolysis through inhibiting tPA and uPA, resulting in less plasmin activation and impairment of fibrinolytic function, thus facilitating thrombosis (43). High plasma PAI-1 concentration is associated with many thrombotic disorders, and is considered to be a strong marker of reduced fibrinolytic function (43,44). Budinger et al. indicated that after exposed to $PM_{2.5}$, mice had increased levels of PAI-1 mRNA and protein compared to the mice only exposed to filtered air (45). In a panel study in Taiwan healthy young people had increased high-sensitivity C reactive protein, PAI-1, fibrinogen, and decreased heart rate variability associated with PM_{10} or $PM_{2.5}$ in single-pollutant models, after ambient exposure to air pollution (46). Mills et al. found lower levels of tPA secreted from endothelial cells after exposure to diluted diesel exhaust compared with controls in humans with impaired fibrinolytic function persisting for 6 hours after the exposure (47). The reduction of tPA was possibly due to the increased secretion of PAI-1. Reviews also indicated that elevated level of PAI-1 was closely associated with cardiovascular diseases (43,48–51).

In contrast to the three coagulation factors above that showed increased expression from HUVECs after PM treatment, the TM gene expression decreased following exposure to the PM, although we were unable to detect differences in protein levels in cell lysates and cell supernatant (results not shown). TM is a transmembrane protein produced by endothelial cells that transforms thrombin from a pro-coagulant converter of fibrinogen to fibrin (among other procoagulant reactions) to an anti-coagulation activator of protein C. Activated protein C synergistically deactivates the coagulation

cascade by supressing the activities of FVa and FVIIIa and reducing thrombin generation (52,53). TM acts as a potent anticoagulant on intact, healthy endothelial cells. In addition to the anti-coagulant function, thrombin/thrombomodulin complex also inhibits fibrinolysis by activating the thrombin activatable fibrinolysis inhibitor (TAFI) (52). TAFIa is activated by thrombin from TAFI. TAFIa removes COOH-terminal lysine residues from partially degraded fibrin and causes impaired fibrinolysis (54). The reduced expression of TM that we observed following treatment of the cells with PM may cause low level of activated protein C. In a cross-sectional study conducted in 2009-2010, it was reported that participants who were exposed to high levels of PM_{10} over six months had reduced levels of serum TM compared to those exposed to low levels of PM_{10}, although the difference was not significant (55).

There were differences in the magnitude of the changes in gene/protein expression observed for different types of PM tested here. PM from different sources and of different size fractions can contain various amounts of chemicals such as organic PAH and nitro-PAH and inorganic metals such as Cu, Fe and Zn. It is known that ROS is a common cause of endothelial dysfunction, and can be generated from Fenton chemistry by transition metals such as iron and copper that are adsorbed to the surface of PM (56). We have previously found that the free radical generating capacity of PM may be related to differences in cellular toxicity (57). We have also shown that inclusion of hydroxyl radical scavengers in the media can reduce the impact of PM on clot structure (24). We therefore used the plasmid strand break assay to test the free radical generating activity of the PM and DCFDA flow cytometry assay to detect the ROS formed in cells in this study. It was found that the particles were able to generate significantly more free radicals as determined by the ability to induce strand breaks in

supercoiled DNA in the strand break assay. Also, after HUVECs were exposed to 50 µg/ml of particles, intracellular ROS was significantly increased compared to control. This raises the possibility that the ROS generating activity of the PM was played an important role in the induction of the changes observed in our study.

In this study, our results are limited to HUVEC's and do not necessarily apply to endothelial cells from other vascular beds. Future studies on human saphenous vein endothelial cells, or endothelial cells of arterial origin should be performed.

Conclusion

Many studies have confirmed that patients with thrombotic diseases had abnormal fibrin clot structure with thinner fibres, more compact arrangements and prolonged lysis time. These features are in accordance with the clots formed here from plasma samples on HUVECs after those cells had been exposed to various PM.

Our results show that PM can induce changes in expression of genes in HUVECs that may explain the ability of PM to alter clot structure and function in vitro. This is important because healthy endothelial cells have several functions such as anti-coagulation, anti-inflammation, anti-oxidation and pro-fibrinolysis and disruption of these functions by PM may contribute to the pathogenic effects of particulate exposure in vivo.

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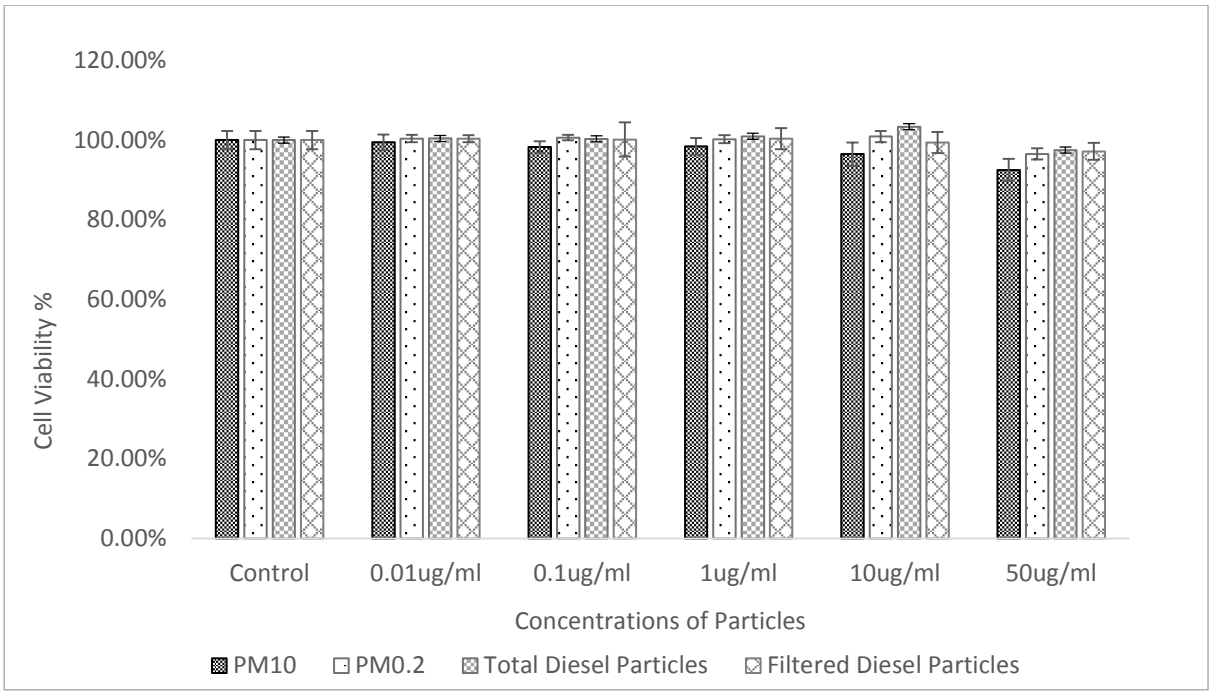
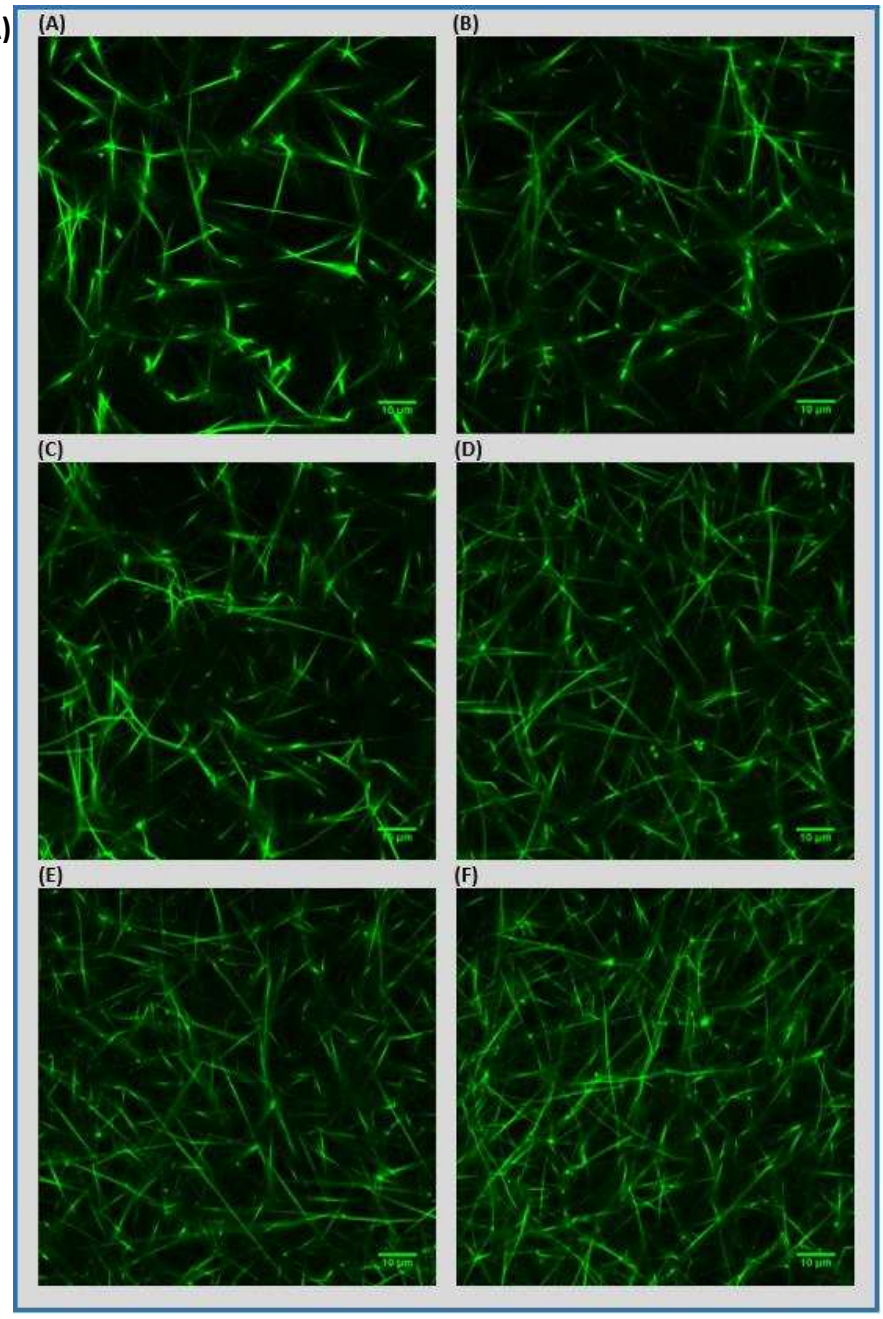


Figure 1. Cytotoxicity of endothelial cells after 24H particles exposure (n=10)

MTT assay was performed to detect the particle cytotoxicity. HUVECs were treated with different concentrations of the four different particles for 24 hours.

Values are mean +/- SE.

(A)



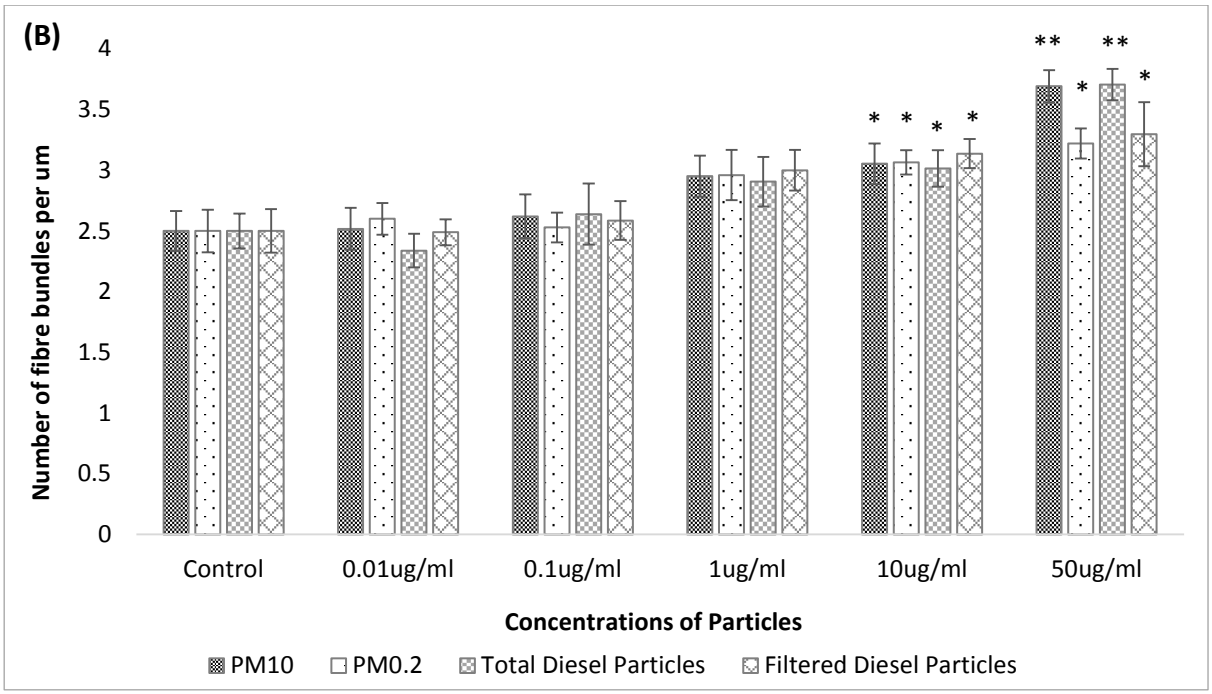


Figure 2. (A) Fibrin clots formation on HUVEC after PM_{10} 24H treatment (Normal Pooled Plasma)

Laser scanning confocal microscope assay was used to measure the fibrin clot structure. After the cells were treated with different concentrations of particles for 24 hours, particles were washed off and fibrin clots were formed with thrombin on the cells using plasma samples. The image represents the clot structure formed on the cells that had been treated with different concentrations of PM_{10} (A: Control; B: 0.01 µg/ml; C: 0.1 µg/ml; D: 1 µg/ml; E: 10 µg/ml; F: 50 µg/ml).

(B) Density of clots formed from normal pooled plasma samples on cells treated with different concentrations of particles (n=5)

The clots formed from plasma were analysed using J Image software. The number of fibre bundles were counted on a line of equal length drawn across the image.

Values are mean +/- SE.

Significant differences from control are indicated by: *p<0.05; **p<0.001

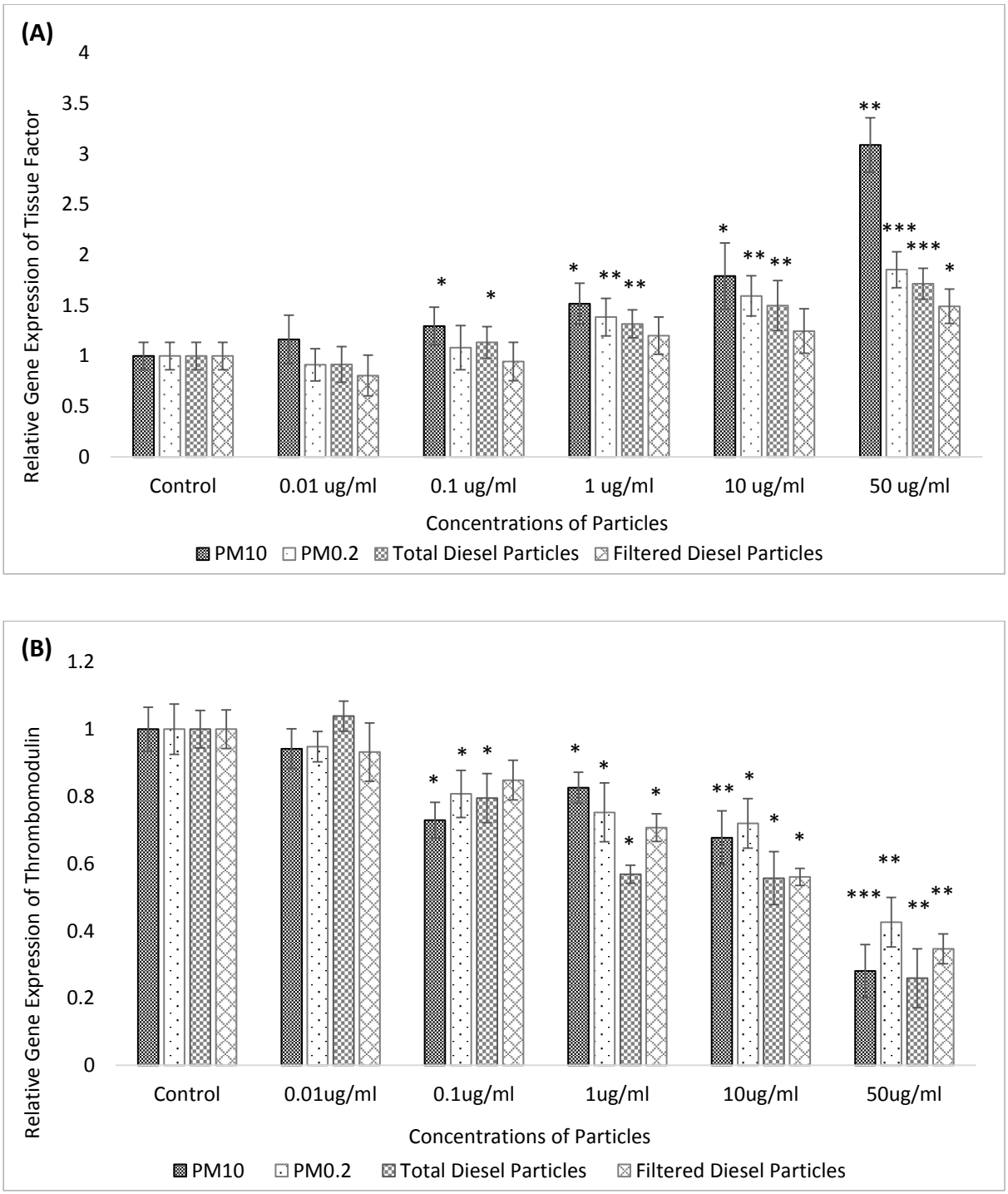


Figure 3. Gene expression of Tissue Factor (A) and Thrombomodulin (B) in endothelial cells after treatment with the different particles (n=3)

Relative gene expression level of TF and TM were determined by real-time PCR. Relative gene expression compared to GAPDH gene expression is shown.

Values are mean +/- SE.

Significant differences from control are indicated by: *p<0.05; **p<0.001; ***p<0.0001

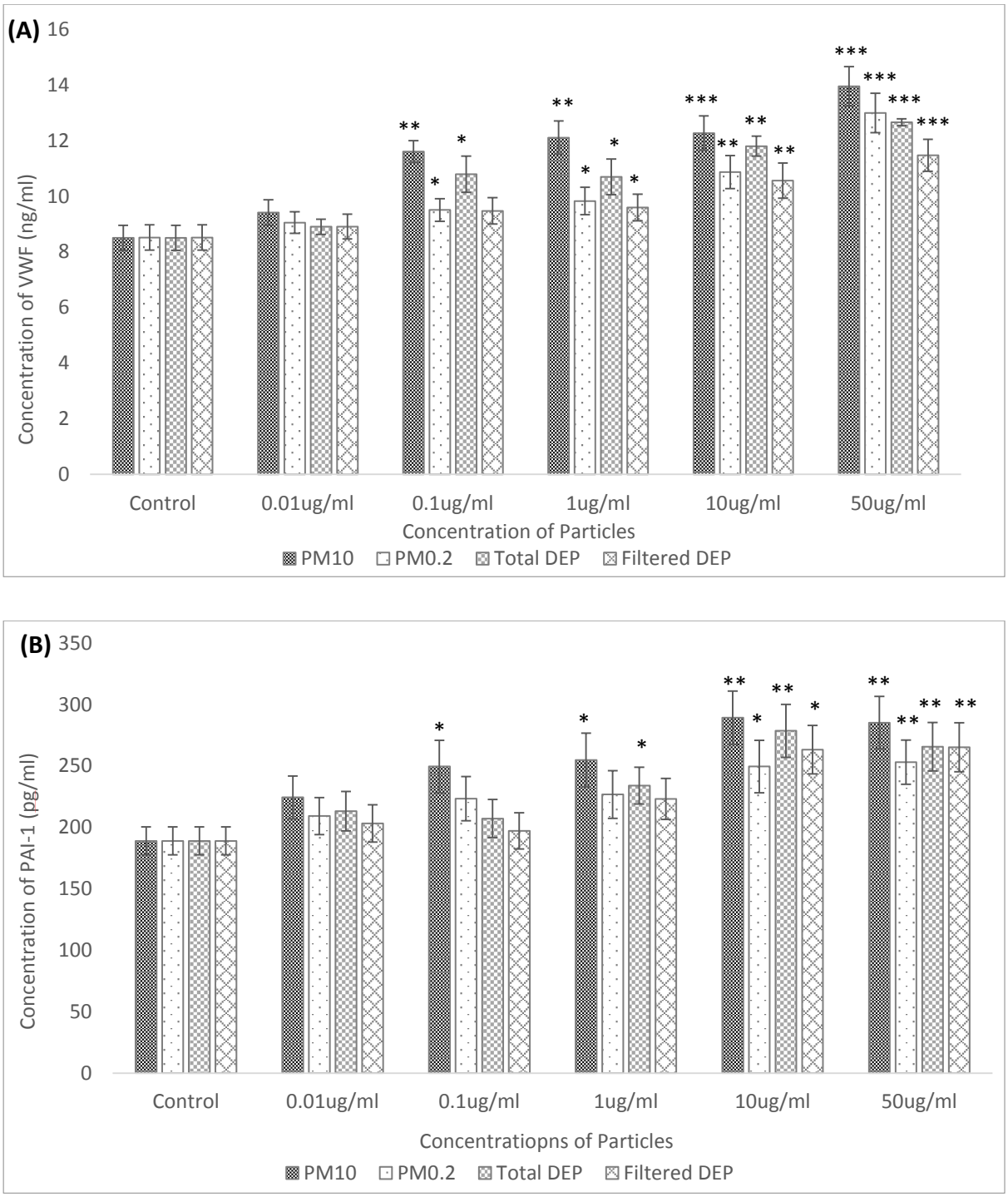
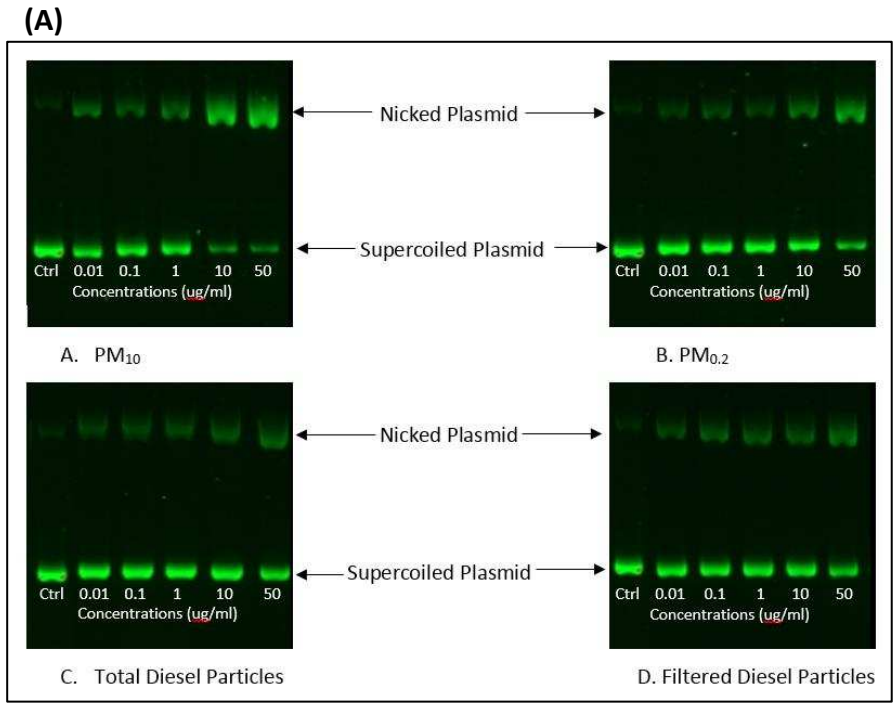


Figure 4. Concentrations of von Willebrand factor (A) and Plasminogen Activator Inhibito-1 (B) from endothelial cells after treatment with different concentrations of particles (n=5)

After the cells were treated with different concentrations of particles for 24 hours, the VWF and PAI-1 concentrations were measured by ELISA.

Values are mean +/- SE.

Significant differences from control are indicated by: *p<0.05; **p<0.001; ***p<0.0001



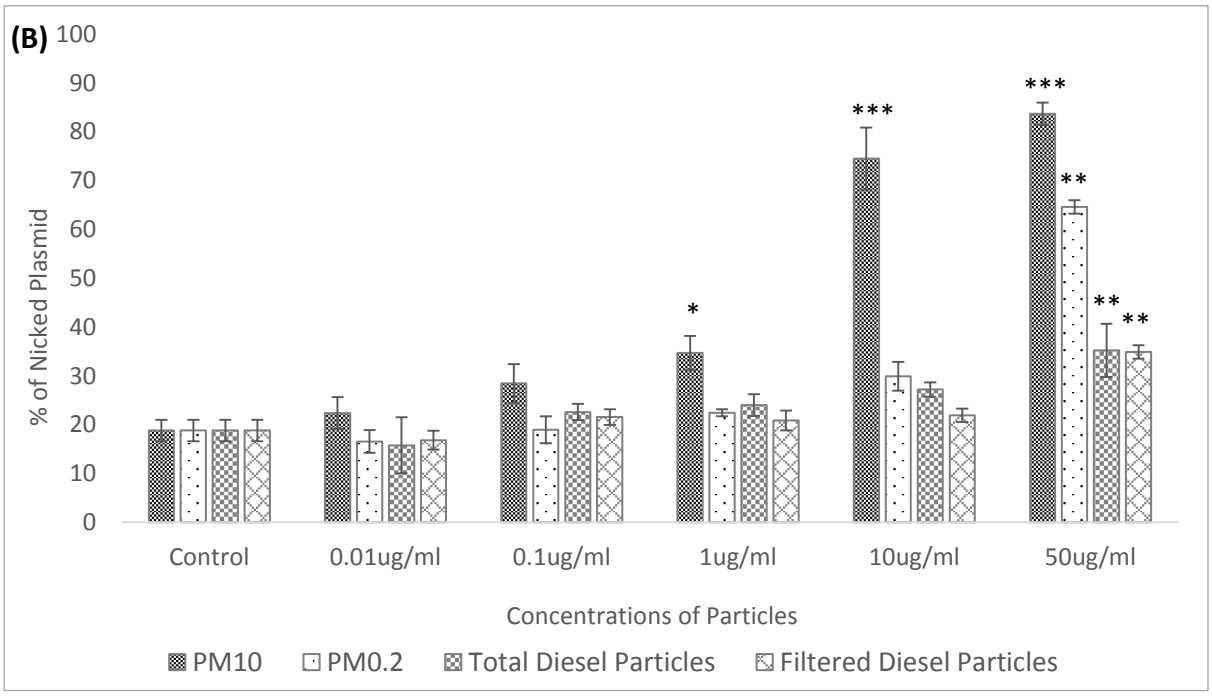
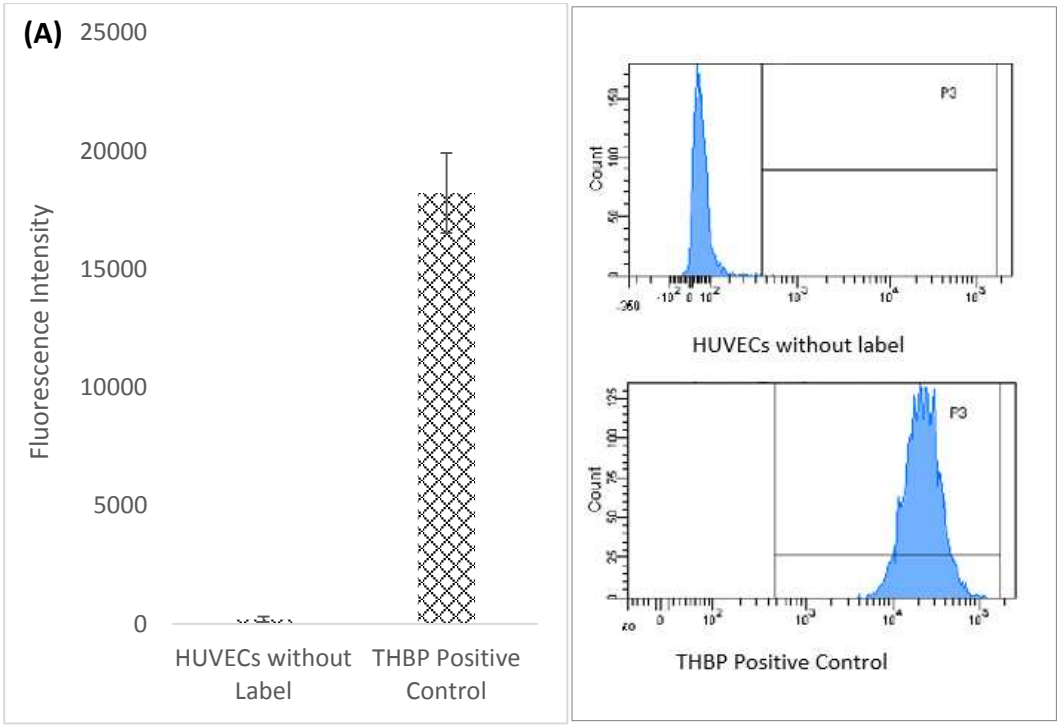


Figure 5. Induction of single stand breaks in pBR322 DNA following incubation with different particles; (A) Gel electrophoresis; (B) Percentage of nicked plasmid caused by particles (n=3)

Supercoiled plasmid DNA was incubated with different particles at concentrations from 0 to 50 µg/ml for 12 hours in the dark. The amount of DNA in nicked or supercoiled forms was visualised by gel electrophoresis with ethidium bromide staining (A). The induction of strand breaks was assessed and expressed as the percentage of nicked DNA observed (B).

Values are mean +/- SE.

Significant differences from control are indicated by: *p<0.05; **p<0.001; ***p<0.0001



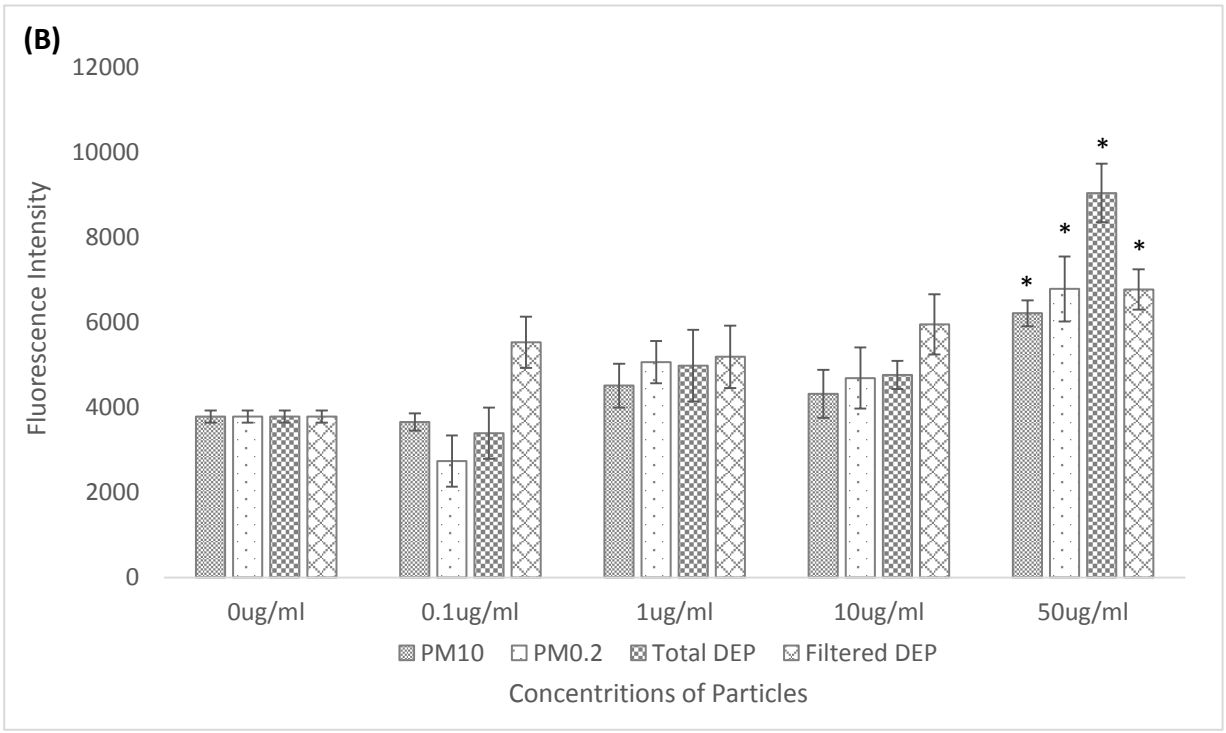


Figure 6 (A) DCFDA flow cytometry assay results (n=3).

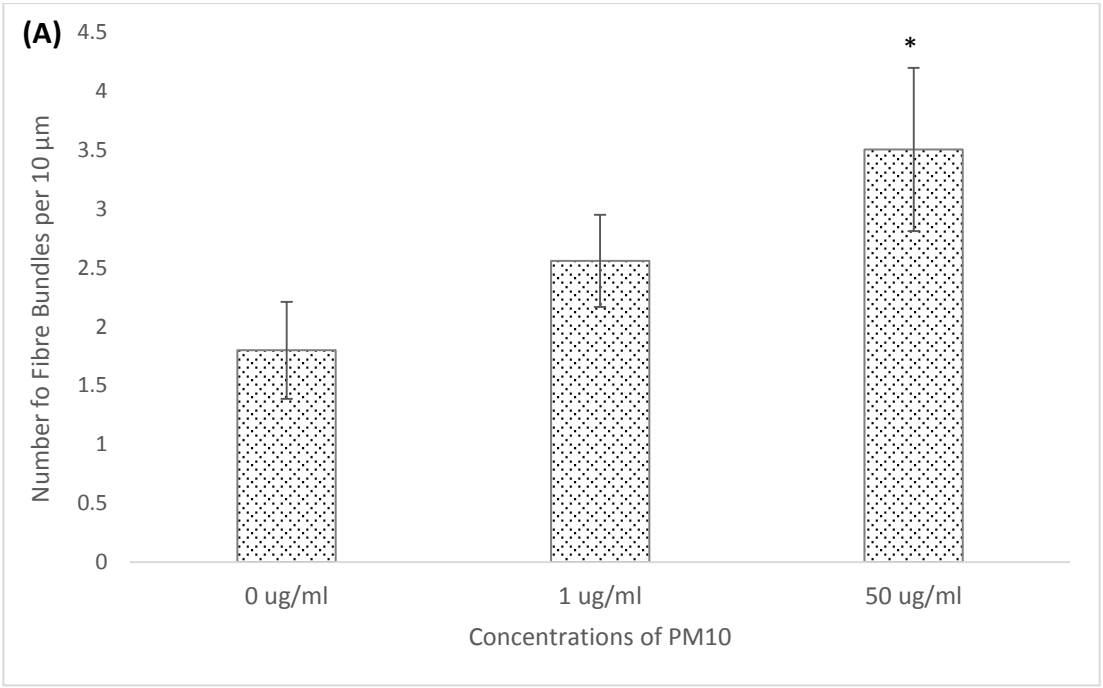
Labeled and unlabedled HUVECs were treated with 30 µM TBHP and then analysed by flow cytometry.

(B) Effect of air particulate matter on ROS production in HUVECs (n=3).

Labelled HUVECs were treated with different concentrations of four types of air pollution particles respectively for 4 hours. The samples were analysed by flow cytometry.

Values are mean +/- SE.

Significant differences from control are indicated by: *p<0.05.



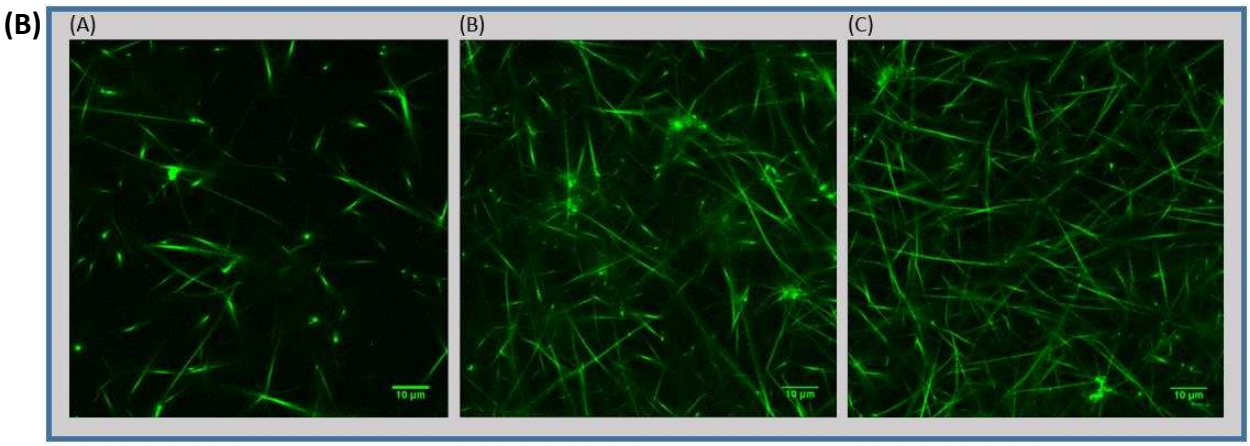


Figure 7. (A) Density of clots formed from normal pooled plasma samples (without thrombin addition) on cells treated with different concentrations of PM_{10} (n=5)

The clots formed from plasma were analysed using J Image software. The number of fibre bundles were counted on a line of equal length drawn across the image.

Values are mean +/- SE.

Significant differences from control are indicated by: *p<0.05

(B) Fibrin clots formation on HUVEC (without thrombin) after PM_{10} 24H treatment (Normal Pooled Plasma)

Laser scanning confocal microscope assay was used to measure the fibrin clot structure. After the cells were treated with different concentrations of particles for 24 hours, particles were washed off and fibrin clots were formed on the cells using plasma samples. The image represents the clot structure formed on the cells that had been treated with different concentrations of particles (A: Control; B: 1 µg/ml of PM_{10}; C: 50 µg/ml of PM_{10}).