Article

Evaluation of the Effects of Airborne Particulate Matter on Bone Marrow-Mesenchymal Stem Cells (BM-MSCs): Cellular, Molecular and Systems Biological Approaches

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Abstract: Particulate matter (PM) contains heavy metals that affect various cellular functions and gene expression associated with an array of acute and chronic diseases, in humans. However, their specific effects on the stem cells remain unclear. Here, we report the effects of PM collected from Jeddah city on bone marrow mesenchymal stem cells (BM-MSCs) on proliferation, cell death, related gene expression and systems biological analysis aiming to understand the underlying mechanisms. Two different sizes ($PM_{2.5-10}$) were tested in *vitro* at various concentrations (15 to 300 µg/ml) and durations (24 to 72 h). PMs induced cellular stress including membrane damage, shrinkage and death. Lower concentrations of $PM_{2.5}$ increased BM-MSCs proliferation, while higher concentrations decreased it. PM_{10} decreased BM-MSCs proliferation in a concentration-dependant manner. The X-Ray Fluorescence spectrometric analysis showed that PM contains high levels of heavy metals. Ingenuity Pathway Analysis (IPA) and hierarchical clustering analyses showed that heavy metals were associated with signalling pathways involving cell stress/death, cancer and chronic diseases. qRT-PCR results showed differential regulation of the apoptosis genes (BCL2, BAX); upregulation of inflammation associated genes (TNF-α and IL-6) and downregulation of cell cycle regulation gene (P53). We conclude that PM could affect different cellular functions and predispose to debilitating diseases.

Keywords: Particulate matter; BM-MSCs; cell proliferation; cell death; qRT-PCR; IPA

1. Introduction

Air pollution is a major environmental risk factor that plagues both developing and developed nations. Toxic air pollutants can be due to natural causes such of giant volcanic emissions, forest fires, gas emanating from radioactive rocks or manmade *viz*. industrial, chemical or traffic emissions. Irrespective of the cause, toxic air pollutants adversely affect the health of an individual leading to morbidity and mortality [1]. The World Health Organization (WHO) reports that three million premature deaths occurred worldwide in 2012, due to air pollution. South-East Asia and Eastern Mediterranean countries show rising levels of urban air pollution which has become a matter of great concern [2]. According to the WHO record on the annual mean concentrations of fine particulate matter from urban areas in 2014, the Kingdom of Saudi Arabia (KSA) is identified to be on the top of the list with 127 µg/m3 (WHO, Public Health and Environment, Ambient Air Pollution Database, 2014).

Visible smoke or dust can be avoided to some extent, but it is practically impossible to protect us daily from getting exposed to the invisible suspended particulate matter (PM). PMs are therefore hazardous to health and their size range from very fine 0.5 µm to coarse 100 µm in diameter [3]. PM with an aerodynamic diameter equal or less than 10 µm size (PM_{10}) can gain easy access to the human body and affect different organ systems [4]. Dermatological (irritations, rashes, pigmentations) [5], ocular (corneal irritation, conjunctivitis)[6], respiratory (cough, allergic rhinitis, chronic obstruction of the airways) [7-9], cardiovascular (palpitations, atherosclerosis) [10,11], neurological (cerebrovascular accidents) [12-16], immunological (allergy, inflammation) [17,18], metabolic (diabetes mellitus, dyslipidaemia) diseases [19,20] and cancers [21,22] are associated with PM.

At the cellular level, $PM_{2.5-10}$ µm induce cytotoxic effects including DNA oxidative damage and stimulation of pro-inflammatory factors [2]. Bronchial epithelial cells exposed to PM_{10} led to the activation of cholesterol and lipid synthesis [23]. PM are also reported to cross the fetomaternal interface leading to developmental delay and/or malformations [2,24]. Stem cells play an active role not only during the embryonic development but also in later life where they are associated with continuous turnover of cells of the skin, haematopoietic and the intestinal system [25]. In addition, the adult stem cells continue to reside in special niches within various tissues and contribute to their repair whenever needed. As stem cells have prolonged survival capacity, they are vulnerable to long-standing toxic effects of biological, chemical or environmental agents. The effects of PM directly on stem cells hitherto remains unexplored. As such in the present study, we aimed at evaluating the effects of the $PM_{2.5}$ and PM_{10} collected from Jeddah city, on bone marrow-derived mesenchymal stem cells (BM-MSCs) *in vitro* with regard to their proliferation and cell death. Furthermore, the predictive effects of PM on the various signalling pathways were analysed using the Ingenuity Pathway Analysis (IPA).

2. Materials and Methods

2.1. Particle collection, extraction and analysis

Particle sample collection, extraction, and their component analysis was performed as reported earlier [23]. All the dust samples were collected from King Abdulaziz University (KAU) campus, located in south Jeddah. The particles collected on Teflon were analysed using a non-destructive X-Ray Fluorescence (XRF) spectrometer to determine the metal concentrations. Particles collected on polypropylene filters were extracted using a modified aqueous extraction protocol described in Duvall et al, 2008 [26] and tested *in vitro*.

2.2. Culture of bone marrow mesenchymal stem cells (BM-MSCs)

Bone marrow aspirates were obtained from patients attending surgical procedures at the Department of orthopaedics, KAU Hospital, Jeddah, Saudi Arabia, following Institutional Ethical Committee approval [11-557/KAU] and informed patient consent. In-house derived primary cultures of BM-MSCs that were characterized earlier were used in the present study. BM-MSCs were maintained in culture using Dulbeccos's modified Eagle's medium (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 2 mM Gluta-Max (Life Technologies, Carlsbad, CA) and antibiotic solution (penicillin, 100 u/mL; streptomycin 100 µg/mL - Sigma, St. Louis, MO) under standard culture conditions of 37°C and 5% carbon dioxide (CO_2) in atmospheric air. Basic fibroblast growth factor (bFGF; Peprotech, UK) at 5 ng/mL was added to culture medium to facilitate BM-MSCs expansion.

2.3. Cell morphology:

BM-MSCs from early passages (P3-P4) were seeded at a density of 2×10^4 cells/well in 24- well tissue culture plates and incubated in a complete culture medium under standard culture conditions. PM_{10} or $PM_{2.5}$ were added to the wells at different 15, 25, 50, 150, 300 µg/ml concentrations and cultured for 24 h, 48 h and 72 h. The cells were observed regularly and any changes in cell morphology were imaged using inverted phase contrast optics (Nikon Instruments, Tokyo).

2.4. Cell proliferation:

BM-MSCs from early passages (P3-P4) were seeded at a density of 2 x 10^4 cells/well in 24- well tissue culture plates and cultured as above. The cells were exposed to similar concentrations of PM_{10} or $PM_{2.5}$ and time durations as above. At the end of the respective time points, changes in cell proliferation if any were analysed using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide assay (MTT; Sigma, St. Louis, MO). Briefly, 10 µl of MTT reagent was added to 100 µl of the freshly changed medium and incubated for four hours. The tetrazolium salt gets reduced due to the action of mitochondrial dehydrogenases to form water-insoluble formazan crystals, which were dissolved using the solvent and absorbance at 570 nm was obtained using spectrophotometry (SpectraMax® i3x, Molecular Devices, Sunnyvale, CA).

2.5. Ingenuity (IPA) pathway analyses of heavy metals in the dust particles

Dust particles collected from Jeddah region has been reported to contain heavy metals namely Chromium (Cr), Manganese (Mn), Cobalt (Co), Arsenic (As), Lead [14], Cadmium (Cd), Nickel (Ni), and Strontium (Sr) [27]. The Ingenuity Pathway Analysis (IPA) Knowledgebase (Qiagen, USA) was used to decipher the influence of these heavy metals on specific genes regulated in BM-MSCs. The gene list obtained for each heavy metal was then subjected to Core Analyses in IPA and the results were further clarified using Fisher Exact Test ($P<0.05$) to get involved pathways and networks. Additionally, the results from core analysis results were compared using the Comparison Analyses Module in IPA, and these results were further clarified using Benjamini-Hochberg Correction ($P<0.05$) to identify cellular and disease-specific pathways. Heatmaps and hierarchical clustering (complete linkage) were then generated based on the log2 transformed ratios, using Genesis Software (Release 1.7.7, Institute for Genomics and Bioinformatics, Graz University of Technology, Austria) [28].

2.6. Quantitative real-time gene expression analysis (qRT-PCR):

Total RNA was extracted from controls and treated samples ($PM_{2.5}$ µm and PM_{10} µm; at 150 µg/ml concentration for 48 hr) using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. On column DNase-I treatment was included in the protocol. Quantity and quality of the isolated RNA were assessed using Nanodrop (Nanodrop Technologies, Wilmington, DE). First strand complementary DNA (cDNA) was synthesized with random hexamers using the reverse transcription system (Promega, Madison, WI). Primers were obtained from previously published work and the primer sequences are given in Table-1. qRT-PCR was carried out with SYBRGreen master-mix (Life Technologies, Carlsbad, CA) using the ABI StepOnePlus™ (Applied Biosystems, Foster City, CA). Relative quantitation was done using the comparative $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analysis:

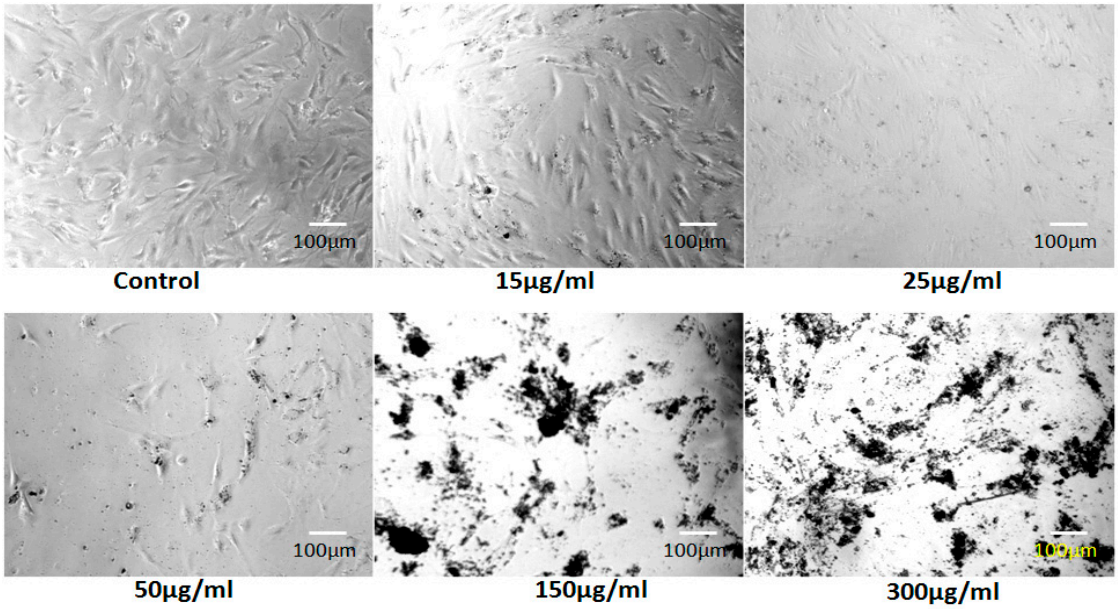
Statistical analysis was performed using 'statistical package for social sciences (SPSS) version 21. The differences between the experimental and control groups were analysed using one-way ANOVA. The results were expressed as a mean ± SEM (standard error of the mean) from a minimum of three experimental replicates. The asterisk (*) indicates statistical significance of $P < 0.05$.

3. Results

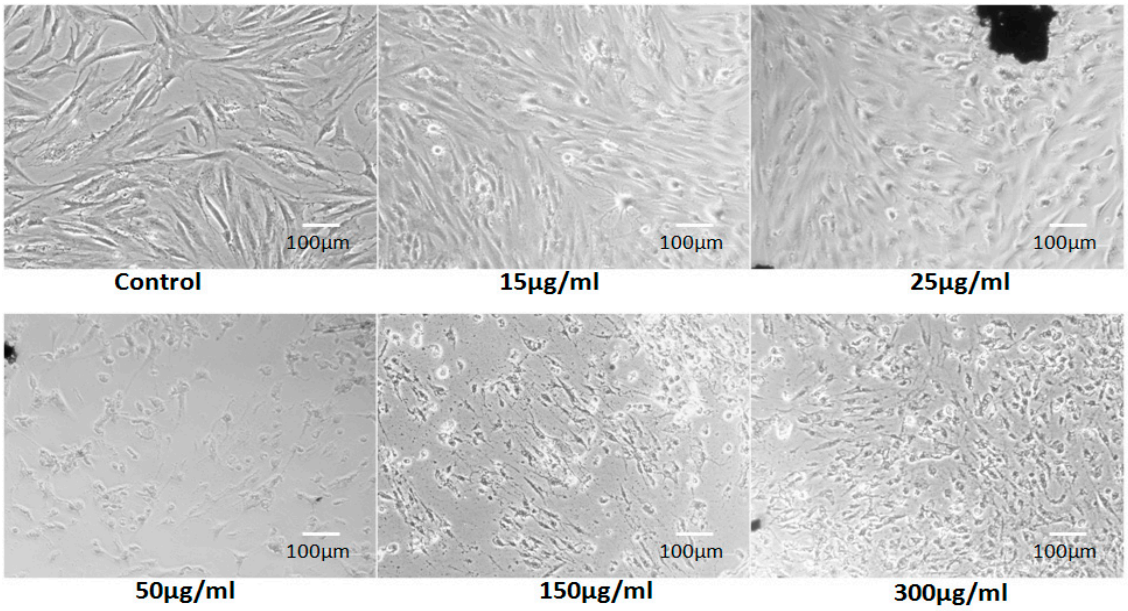
3.1. Cell morphology:

Phase contrast microscopy of the BM-MSCs exposed to $PM_{2.5-10}$ and cultured for 24 h, 48 h or 72 h, showed a general increase in cell numbers with $PM_{2.5}$, and a decrease with PM_{10} compared to their respective controls. The PMs were tethered to the cell surface and the cellular morphology was obscured especially with PM_{10}. Increase in the concentrations of PM and their exposure time led to a proportionate increase in cell death, that was more evident in PM_{10} compared to $PM_{2.5}$. The cells

showed features of stress including cell shrinkage and membrane disruptions leading on to their death (Figure 1a, 1b).



(a)



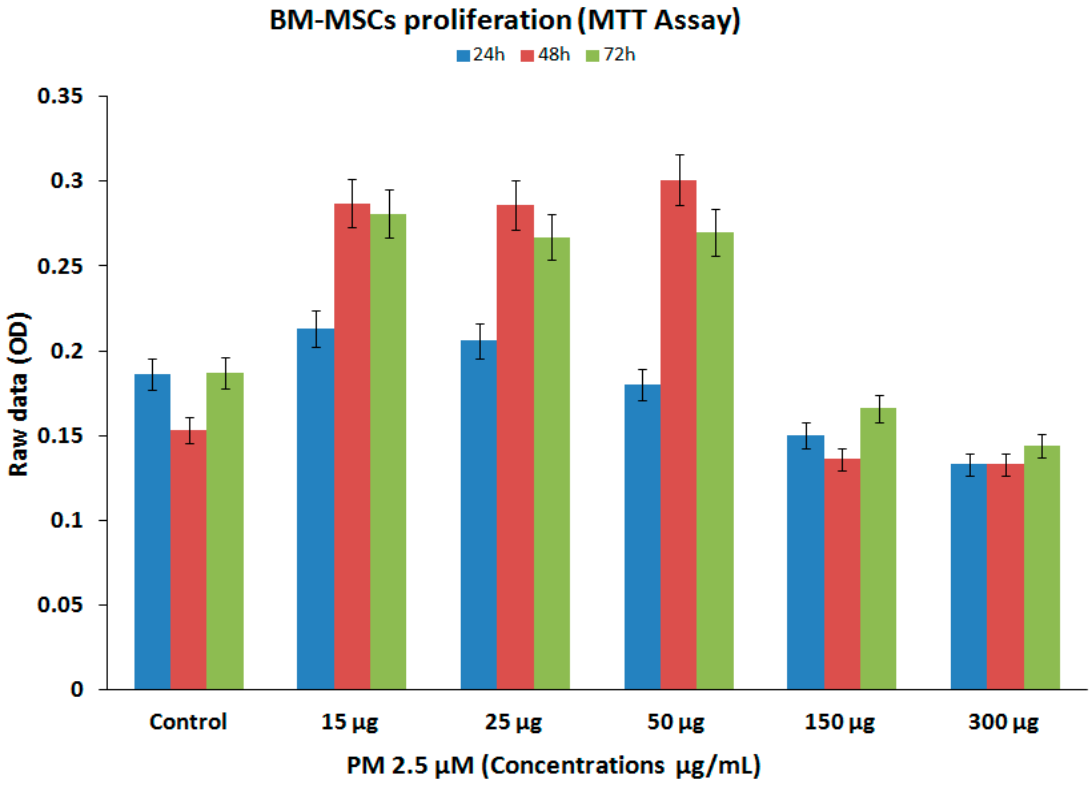
(b)

Figure 1. Cell morphology: Phase contrast images of bone marrow mesenchymal stem cells (BM-MSCs) treated with the airborne particulate matter of two different sizes PM2.5µM and PM10µM (Figure 1a,b), demonstrated the adherence of the PMs to the BM-MSCs even obscuring their complete morphology, which was more pronounced with PM10µM than with PM2.5µM. The increase in concentrations of PMs led to decreases in cell numbers. The BM-MSCs exhibited morphological changes such as cell shrinkage, thinning, fragmentations leading to cell death. These morphological changes were more evident with PM2.5µM. Arrows indicate dead cells which appear round and translucent (Magnification 100X).

3.2. Cell proliferation (MTT assay):

The BM-MSCs exposed to $PM_{2.5}$ demonstrated increases in cell proliferation at lower concentrations (15 µg/ml, 25 µg/ml, 50 µg/ml) and moderate decreases in proliferation at higher concentrations (150 µg/ml, 300 µg/ml). The mean percentage increases for $PM_{2.5}$ at 24 h, 48 h and 72 h were 14.52%, 87.58%, 50.27% for 15 µg/ml; 10.75%, 86.93%, 42.78% for 25 µg/ml; and 96.73%, 44.39% for 50 µg/ml respectively, and all these increases were statistically significant ($P < 0.05$). The mean percentage decreases for $PM_{2.5}$ at 24 h, 48 h and 72 h were 19.35%, 11.11%, 11.23% for 150 µg/ml and 28.49%, 13.07%, 22.99% for 300 µg/ml respectively, and all these decreases were statistically significant ($P < 0.05$) (Figure 2a).

In contrast, the BM-MSCs treated with PM_{10} demonstrated only decreases in cell numbers at higher concentrations compared to their untreated control. The mean percentage decreases for PM_{10} at 24 h, 48 h and 72 h were 14.04%, 23.71%, 14.90% for 50 µg/ml; 15.73%, 22.68%, 31.73% for 150 µg/ml and 20.79%, 31.96%, 38.46% for 300 µg/ml respectively. However only the decreases observed at 24 h for the highest concentration (300 µg/ml) as well as the decreases observed at 48 h and 72 h for all three higher concentrations (50 µg/ml, 150 µg/ml, 300 µg/ml) were statistically significant ($P < 0.05$) (Figure 2b).



(**a**)

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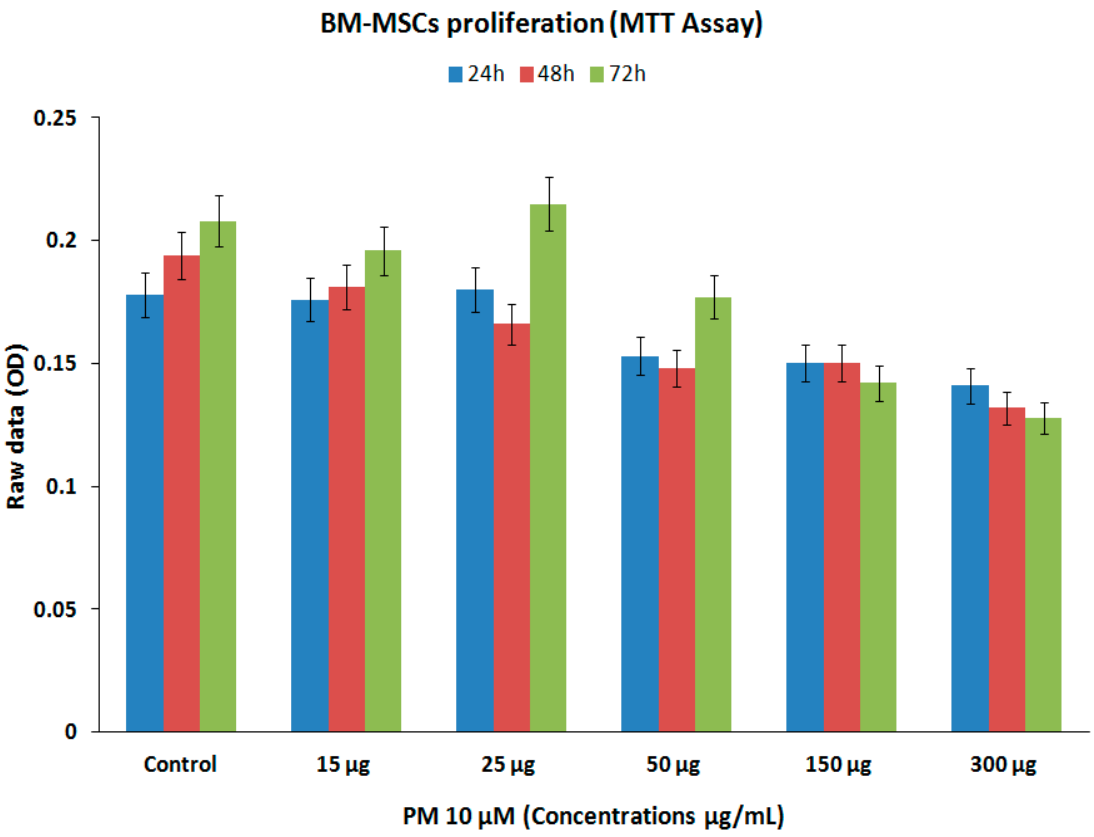

(**b**)

Figure 2. Cell proliferation/inhibition: MTT assay of BM-MSCs treated with PM at different concentrations (15, 25, 50, 150 and 300 µg/ml) at 24 h, 48 h and 72 h showed increases in cell proliferation with PM2.5µM (Figure 2a), except for the highest concentration; while PM10µM demonstrated inhibition of cell proliferation compared to untreated controls (Figure 2b). The values are expressed as mean ± SEM from triplicate samples of three independent experiments. The observed increases or decreases in cell proliferation with PMs were statistically significant. Asterisk '*' indicate statistical significance of $p<0.05$.

3.3. IPA analysis for associated genes and networks:

PM from the Jeddah city was reported to be rich in a significant number of heavy metals[28] . Using the core IPA analysis, genes that are differentially regulated in BM-MSCs and other primary cells and tissues by heavy metals present in the dust particles were identified. Besides, the comparison analysis of the core IPA results showed that heavy metals present in dust particles differentially regulate various canonical pathways such as cancer signaling, cell cycle regulation, stress, and injury signaling, inflammatory cytokine signaling and an array of disease-specific pathways (Figure 3A-D). The heatmap and complete hierarchical clustering (HCL) analyses showed that the heavy metals, such as Cd, Pb, As, Mn, Ni, Cr, and Co upregulated genes associated with cancer signaling. The genes related to ovarian cancer (OVC), colorectal cancer (CC), estrogen-dependent breast cancer (ER-BC), prostate cancer , Glioblastoma (GB), chronic myeloid leukemia (CML), as well as Wnt and ERK/MAPK signaling pathways were potentially upregulated (Figure 3A). Also, the genes responsible for the regulation of cell cycle, stress and injury were significantly upregulated by Cd, Pb, As, Cr, and Mn (Figure 3B). Interestingly, our results showed that, except Strontium (Sr), all the heavy metals upregulated the expression of proinflammatory cytokines such as TNFα, IFNγ, IL-1α, IL-1β, IL-2, IL-6 and IL-18 (Figure 3C). The disease-specific genes in Type I and Type II of Diabetes Mellitus (TIDM and TIIDM), Rheumatoid Arthritis (RA), Huntington Disease

(HD), Amyotrophic Lateral Sclerosis (ALS), and Systemic Lupus Erythematosus [29] were differentially regulated by Cd, As, Ni, Co, and Mn (Figure 3D).

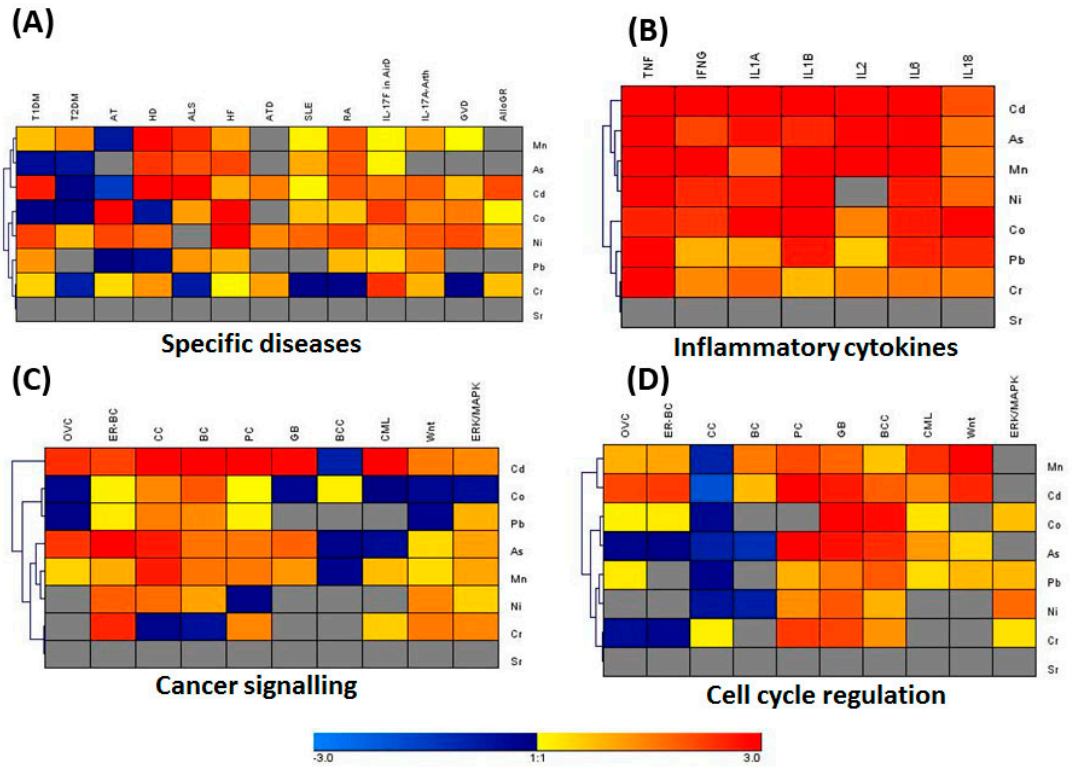

Figure 3. The core analysis module in the Ingenuity Pathway Analysis [42]. Knowledgebase was used to obtain genes that are differentially regulated by heavy metals present in the dust particles. Besides, comparison analysis module of IPA was utilized to compare the results obtained using the core analysis for each heavy metal. Hierarchical clustering was done based on the comparison analysis results using Genesis Software in specific diseases, immune regulation (inflammatory cytokines), cancer signalling and cell cycle regulation.

3.4. Gene expression analysis of the proinflammatory markers (qRT-PCR):

Gene expression analysis for some of the inflammation, cell death and cancer/cell cycle regulation related genes were done using qRT-PCR. The inflammation related markers namely, TNF-α and IL-6 were upregulated compared to control (Figure 4). TNF was increased by 1.34 and 5.80-fold following treatment with $PM_{2.5}$ and PM_{10} respectively. IL-6 was increased by 3.54 and 5.90-fold following treatment with $PM_{2.5}$ and PM_{10} respectively. Gene expression that showed more than two-fold upregulation was statistically significant. The anti-apoptotic BCL2 gene showed mild upregulation, while the pro-apoptotic BAX was downregulated. In addition, the tumour suppressor gene namely P53, which is involved in cell cycle regulation was also downregulated compared to the control (Figure 4). However, the associated fold increases or decreases of these genes were not statistically significant.

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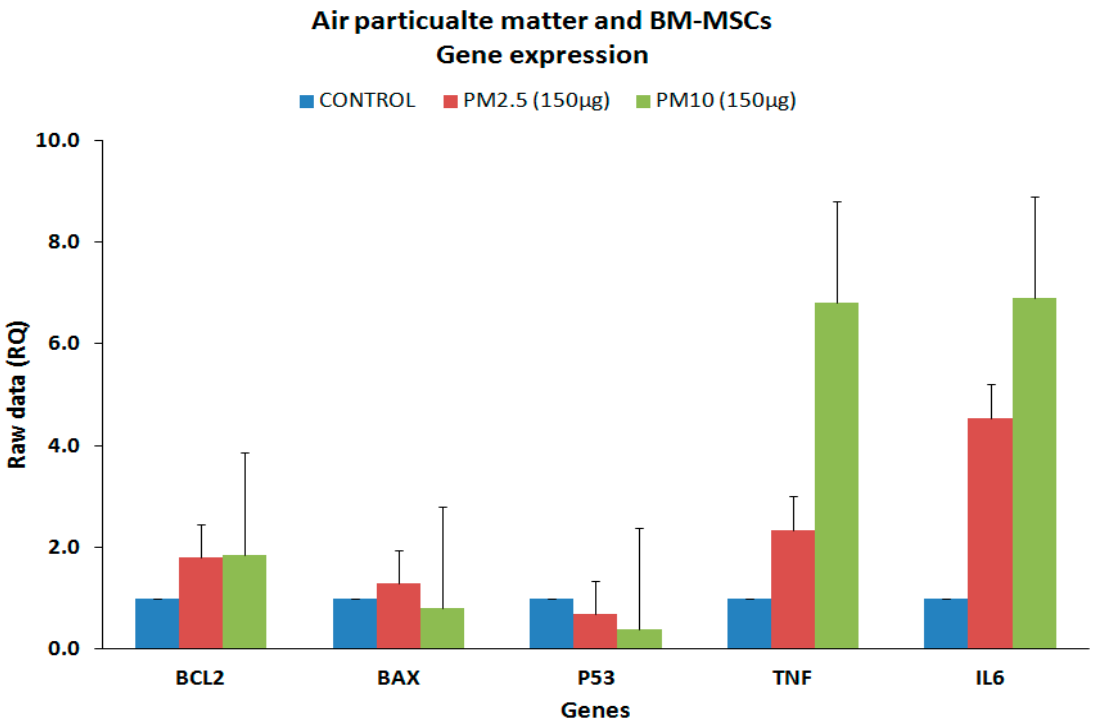

Figure 4. Gene expression analysis. qRT-PCR analysis of BM-MSCs showing the BCL2, BAX, P53, TNFα and IL-g gene expression profile following treatment with 150 µM/mL of PM2.5µM and PM10µM for 48h. GAPDH was used as the internal control and the data quantified using the comparative $2^{-\Delta\Delta Ct}$ method. The values are expressed as mean ± SEM from triplicate samples of two independent experiments.

4. Discussion

Airborne PMs collected from Jeddah city in Saudi Arabia, contained high levels of Cr, Mn, Sr, Co, As, Pb, Cd, Ni and Va and were implicated in many, allergic, inflammatory, genetic and epigenetic disorders [20,30-33]. In the current study, we evaluated the direct effects of these PMs on cellular functions, especially on the stem cells (BM-MSCs), using two different sizes of ($PM_{2.5}$ and PM_{10}). Our results revealed that several cellular morphological changes including cell shrinkage, thinning, and fragmentations (led to cellular death) were induced. An earlier *in vitro* study tested urban particles on alveolar macrophages reported its reversal to a more immunoreactive phenotype and induction of apoptosis upon incubation for 24 h at 100 µg/mL and 200 µg/mL concentrations respectively [20]. In addition, alveolar epithelial cell line (C10) exposed to higher concentrations (50 µg/cm^2) of $PM_{2.5}$, resulted in increase in the sub G0/G1 phases indicative of both apoptotic and necrotic cell death [34]. Increased expression of two death domain proteins namely, receptor interacting protein (RIP) kinases and Fas Associated protein with Death Domain (FADD) complex acting in concert with caspase-8 were implicated in the $PM_{2.5}$ induced apoptosis [34]. Though no direct apoptotic assays were done in this study, the morphological characteristics indicated that the cell death could partly be due to apoptosis. However, in the present study the TNF-α gene expression was increased with both $PM_{2.5}$ and PM_{10}, and interestingly, RIP is reported to interact either with TNF receptor signalling or with FADD to bring about apoptosis [35].

BM-MSCs proliferation was increased upon treatment with lower concentrations of $PM_{2.5}$ in our present study. Alveolar epithelial cells exposed to 10 µg/cm^2 of $PM_{2.5}$ for 24 h increased the cells in 'S' phase of the cell cycle which was attributed to injury/inflammation leading on to compensatory proliferation [34]. Human alveolar basal adenocarcinoma cell line (A549) and human non-small lung carcinoma cell line (H1299) exposed to conditioned medium of $PM_{2.5}$ (50 µg/cm^2 for 72 h)

demonstrated an increase in their proliferation [30]. Increased cell proliferation of these cancer cell lines was contributed by interleukin 1-beta (IL1β) mediated mitogen associated protein kinase (MAPK) signalling [30]. In contrast to the lower concentrations of $PM_{2.5}$, higher concentrations of both $PM_{2.5}$ and PM_{10} decreased BM-MSCs proliferation. Significant decrease in cell viability was reported with alveolar macrophages tested with urban particles *in vitro* at 200 μg/ml concentration for 24 h [20]. Similarly, the alveolar epithelial cells exposed to 50 μg/cm² of $PM_{2.5}$ for 24 h decreased the cells in 'S' phase of the cell cycle [34]. Furthermore, the human colon cancer cell line HCT116 and the human embryonic kidney cell line HEK293T, exposed to PM_{10} at 400 μg/mL and 60 μg/ml concentrations respectively for 48 h demonstrated 50% inhibition in their proliferation [27]. Results from all the above studies and ours therefore indicate that inhibition in cell proliferation is associated with higher concentrations of PM. Mostly, these decreases in cell proliferation were associated with cell death either due to apoptosis and/or necrosis. However, the expression of BCL2, BAX and P53 genes in our study did not support apoptotic pattern of cell death indicating a role for other mediators of cell death. Although, interaction with TNF receptor with RIP and FADD is reported to cause apoptosis [35], it is indeed necessary to undertake either an early time point of study for apoptosis or screen for additional pathways of cell death in our subsequent studies.

Chronic exposure of airborne PMs, can cause detrimental health effects, by decreasing normal cells including stem cells that offer general protection and promote cancer cell growth and migration even at smaller concentrations. Heavy metals, among others, are the most important toxic pollutants in the PM that affect many cellular functions and signalling pathways contributing to a disease. The systems biological analysis, using the IPA knowledgebase, helped us to decipher the disease-associated functions of heavy metals present in the PMs. The heavy metals have been found to trigger the production of proinflammatory cytokines such as TNFα, IFNγ, IL-1α, IL-1β, IL-2, IL-6 and IL-18 causing both acute and chronic inflammation. Besides, the heavy metals present in the PMs were shown to induce signalling pathways implicated in an array of cancers afflicting humans. More importantly, the inflammation is the basis for various diseases like allergy, asthma, rheumatoid athritis, sytemic lupus erythematosus, Huntington's disease, cardiovascular diseases, HD, Alzheimer's disease, cardiovascular diseases, diabetes mellitus and a wide variety of cancers [36]. This was in tandem with our current gene expression analysis, which showed increased expression of inflammatory cytokines namely TNF-α and IL-6 following exposure to both $PM_{2.5}$ and PM_{10}. These results are in agreement with a previously reported studies in which $PM_{2.5-10}$ increased the expression levels of the inflammatory markers including TNF-α and IL-1β in endothelial progenitor cells [37], IL-6 and IL-8 in monocytes [38], IL-6 in Kupffer cells [39] and TNF-α, IL-6 and IL-8 in lung epithelial cells [40]. The mechanism through which the airborne particulates induced apoptosis and increased the expression level of the inflammatory markers was attributed to triggering the formation of the reactive oxygen species (ROS) [37,40,41].

5. Conclusions

Airborne PM in Jeddah contain different heavy metals and environmental exposure can be detrimental to health. The present study provides an insight into the effects of PM on stem cells, that normally participate in the repair mechanisms against injury, inflammation and diseases. *In vitro* effects of two different sizes of PM ($PM_{2.5}$ and PM_{10}) indicated that lower concentrations increased and higher concentrations decreased BM-MSCs cell proliferation. The PM also increased pro-inflammatory cytokines, which are usually associated with various acute and chronic disease pathogenesis. The IPA analysis identified that PM augment inflammation and cancer signalling pathways. The actual mechanism of cell death evidenced in the present study and the predicted signaling pathways needs further investigation.

Abbreviations

ALS: Amyotrophic Lateral Sclerosis
As: Arsenic
bFGF: Basic fibroblast growth factor

BM-MSCs: Bone-marrow mesenchymal stem cells
CC: Colorectal cancer
Cd: Cadmium
cDNA: Complementary DNA
CML: Chronic myeloid leukemia
Co: Cobalt
CO_2: Carbon Dioxide
Cr: Chromium
ER-BC: Estrogen-dependent breast cancer
FADD: Fas Associated protein with Death Domain
GB: Glioblastoma
HCL: Heatmap and complete hierarchical clustering
HD: Huntington Disease
IPA: Ingenuity Pathway Analysis
Mn: Manganese
MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide assay
Ni: Nickel
NSCLC: Non-small-cell lung cancer cells
OVC: Ovarian cancer
ORS: oxygen reactive species
Pb: Lead
PC: Prostate cancer
PM: Particulate matter
qRT-PCR: Quantitative real-time gene expression analysis
RIP: Receptor interacting protein
ROS: Reactive oxygen species
RA: Rheumatoid Arthritis
SLE: Systemic lupus erythematosus
SPSS: Statistical package for social sciences
Sr: Strontium
TIDM: Type I Diabetes Mellitus
TIIDM: Type II Diabetes Mellitus
WHO: World Health Organization
XRF: X-Ray Fluorescence

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Author Contributions: MAE, GK, MAA, MS and MIK designed the study and supervised execution of the work. GK, RK, HA, PNP and MAE executed the work and analysed the data. MAE and GK wrote the manuscript. MC, MA, PNP, MQ and MG contributed to the manuscript preparation and did the proofreading. All authors approved the final version of the manuscript.

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