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Impairment of Mitochondrial-Nuclear Cross Talk in Lymphocytes Exposed to Landfill Leachate

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ABSTRACT: Landfill leachate, a complex mixture of different solid waste compounds, is widely known to possess toxic properties. However, the fundamental molecular mechanisms engaged with landfill leachate exposure inducing cellular and sub-cellular ramifications are not well explicated. Therefore, we aim to examine the potential of leachate to impair mitochondrial machinery and its associated mechanisms in human peripheral blood lymphocytes. On assessment, the significant increase in the dichlorofluorescein (DCF) fluorescence, accumulation of 8-Oxo-2′-deoxyguanosine (8-oxo-dG), and levels of nuclear factor erythroid 2–related factor 2 (Nrf-2) strongly indicated the ability of the leachate to induce a pro-oxidant state inside the cell. The decrease in the mitochondrial membrane potential and alterations in the mitochondrial genome observed in leachate-exposed cells further suggested the disturbances in mitochondrial machinery. Moreover, these mitochondrial-associated redox imbalances were accompanied by the increased level of NF-κβ, pro-inflammatory cytokines, and DNA damage. In addition, the higher DNA fragmentation, release of nucleosomes, levels of polyadenosine diphosphate ADP-ribose polymerase (PARP), and activity of caspase-3 suggested the involvement of mitochondrial mediated apoptosis in leachate exposed cells. These observations were accompanied by the low proliferative index of the exposed cells. Conclusively, our results clearly indicate the ability of landfill leachate to disturb mitochondrial redox homeostasis, which might be a probable source for the immunotoxic consequences leading to plausible patho-physiological conditions in humans susceptible to such environmental exposures.

KEYWORDS: environmental health, municipal solid waste, immunotoxicity, redox signaling, DNA damage and repair

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Introduction

Management of municipal solid waste is a serious problem in India and other developing countries.[1] With increasing urbanization and industrialization, the rate of solid waste generation has also significantly increased throughout the world. As per the 2012 report of the World Bank, globally, almost 1.3 billion tons of solid waste is produced per year which by 2025 is supposed to rise to 2.2 billion tons.[2] In India, around 14.3 million tons per day of solid waste is generated from which only about 33 800 tons per day (23.73%) is being efficiently processed. The inefficient management and unscientific disposal of this solid waste material can cause severe impact on environment and human health.[3] Wide array of technologies such as landfilling, recycling, anaerobic digestion, and incineration are being used in the management of solid waste.[4] Due to simple formation and economic advantages, landfilling is the most pervasive strategy for solid waste management in both developing and developed countries.[5] It is commonly seen in developing countries that most of the landfill sites are devoid of a proper management system. This potentially results in production of the toxic leachate.[6,7]

The leachate is an aqueous stream containing innumerable organic and inorganic compounds generated due to percolation of rainwater through waste and moisture containing by-products (toxic and non-toxic) of biochemical reactions occurring within the landfill site.[8–10] Quality and quantity of leachate are mostly affected by the surface water runoff, wetness of top soil, intrinsic dampness of buried solid waste, environmental conditions, and biological modifications of the waste.[11] Landfill leachate is a plausible source for polluting underground and surface water ecosystems.[12] It is highly complex in nature consisting of several toxic and carcinogenic substances which include organic compounds, macro-inorganic compounds (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, NH^{4+}), heavy metals (Cd^{2+}, Cr^{3+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}), xenobiotic organic compounds (aromatic hydrocarbons, phenols, halogenated pesticides), and microorganisms which makes it extremely lethal.[13] When amalgamated, these chemicals could get bio-accumulated in aquatic organisms, eventually reaching to animals and humans through the food chain.[14] Existing literature strongly suggests the toxic potential of landfill leachate on certain cell types.[15,16] In fact, studies have been accounted for the adverse health consequences of landfill leachate which include low birth weight, increment of birth absconds, and certain forms of cancer among population residing nearby to landfill sites.[17,18] Therefore, more consideration has been paid to the health impacts of landfill leachate and their conceivable mechanisms.

Moreover, exposure to certain environmental pollutants disturbs the cellular mechanisms and also leads to the induction of mitochondria-mediated signaling pathways. Evidences

from our recent investigations suggest that exposure to environmental pollutants disturbs the mitochondrial nuclear cross talk and induces a PI-3 kinase-mediated DNA damage response.[19] It has also been suggested that environmental stressors often deregulate mitochondrial functions by causing damage or mutation in the mitochondrial DNA (mtDNA).[20] Accumulation of these mutated and damaged mitochondria with time can mediate the pathophysiology of a number of diseases including metabolic disorders, neurodegenerative diseases, cardiac disorders, and cancer.[21] However, the fundamental molecular mechanisms engaged with impeded mitochondrial redox homeostasis due to landfill leachate exposure are not explicated. Therefore, in this study, we comprehensively elucidated the biological relationship that links the landfill leachate exposure to the dysfunctional mitochondria which can probably have severe health repercussions. Experiments were performed using human peripheral blood lymphocytes as cell model. These cells serve as an ideal selection to evaluate the immunotoxic potential and characterize complex cellular mechanisms associated with the exposure–response relationship.[22] Earlier studies have also shown the potential of human peripheral blood cells as a cellular model for investigating the genotoxicity of landfill leachate at different concentrations and time points.[23–25]

In this study, the assessment of oxidative stress was performed by using 5-(and -6) chloro methyl-2, 7-di-chloro-dihydro-fluorescein-di-acetate-acetyl-ester (CM-H2DCFDA) , while levels of the 8-Oxo-2′-deoxyguanosine (8-oxo-dG) modified nucleoside base were assessed as a marker for oxidative stress. Furthermore, the mitochondrial deregulation was evaluated through assessment of mtDNA copy number, mitochondrial membrane potential (JC-1 labeling), and mitochondrial genome analysis. Status of antioxidant defense was evaluated through nuclear factor erythroid 2–related factor 2 (Nrf-2) levels, a key regulator of cellular responses controlling reactive oxygen species (ROS), whereas to estimate inflammatory response, NF-κβ and levels of pro-inflammatory cytokines, ie, interleukin 6 (IL-6), interferon gamma (IFNy), and tumor necrosis factor alpha (TNFα), were assessed. The levels of caspase-3, cleaved polyadenosine diphosphate ADP-ribose polymerase (PARP), yH2AX, and bromodeoxyuridine (BrdU) were detected as the markers for DNA damage apoptosis and cell proliferation.

Material and Methods

Sample collection

"Bhanpur khanti" was selected as the sample collection site which is widespread in the area of approximately 75 acres of land situated in Bhanpur village located 11 km away from the Bhopal city. It was a primary municipal dumping site of the capital city "Bhopal" till 2018. Five soil samples weighing approximately 40 g were collected using random sampling method and were immediately taken to the laboratory for further processing.

Dose response analysis. A dose response analysis of landfill leachate–induced toxicity was conducted with concentration ranging from 1 µg/µL (1×), 10 µg/µL (10×), 100 µg/µL (100×), and 1000 µg/µL (1000×) to identify an optimum dose for the downstream experiments. The levels of cell death was assessed by Cell Death Detection kit (Roche Applied Sciences) by following all necessary instructions of the manufacturer, and the measurement of absorbance was done at 405 nm.[22]

Preparation of leachate

Samples were initially air dried, finely grounded, and sieved through 63 µm pore size sieve to obtain a homogeneous mixture. Later, leachate samples were prepared by inorganic-aqueous extraction method described by Chandra et al.[26] Briefly, 100 mg of soil sample was added to 10 mL of double distilled water; this mixture was shaken continuously at room temperature for 24 hours. It was then centrifuged at 200*g* for 10 minutes to remove coarse particulate materials, and the supernatant obtained was used as leachate. To remove the undesired microbes, the test leachate was filtered using 0.22-µm membrane. Leachate was then stored at 4°C for downstream experiments in the study. On characterization of the samples, presence of nitrates, sulfates, and heavy metals was observed (Table 1).

Lymphocyte isolation and culture

Human peripheral blood lymphocytes were isolated through density gradient centrifugation method. The cell viability assessment was done by trypan blue dye and 1×10^6 cells/plate were cultured in 4 mL RPMI 1640 media (pH: 7.4) supplemented with 24 mM/L $NaHCO_3$, 10-mM/L L-glutamine, 10 000-µL/mL penicillin, 10-mM/L Hepes, and 10 000-g/mL streptomycin and FBS. The cultured cells were then treated with 0.2 mL of phytohemagglutinin (PHA) for mitogenic stimulation and incubated at 37°C in 5% CO_2 atmosphere with 95% relative humidity for 24 hours.[22]

Assessment of oxidative stress. The amount of mitochondrial ROS generation was estimated in cells exposed to 10 µg/µL (10×) concentration for 0 to 6 h through CM-H2DCFDA labeling (Invitrogen-Thermo Fisher Scientific, USA). In brief, a freshly prepared CM-H2DCFDA (1 mM) stock solution was diluted to the final working concentration of 1 µM. The cells were then incubated with 50 µL of working CM-H2DCFDA solution for 2 hours and washed, and the measurement of the related fluorescence was done through flow cytometer (Attune NxT, Thermo Fisher Scientific, USA).[27] The quantitative levels of the 8-oxo-dG were measured in

Table 1. Characterization of the leachate sample.

PARAMETERS	CONCENTRATION
pH	7.10
Calcium (mg/L)	1600 ± 115.47
Sodium (mg/L)	750 ± 57.73
Potassium (mg/L)	180 ± 17.32
Sulfate (mg/L)	120 ± 8.66
Nitrate (mg/L)	55 ± 7.63
Chloride (mg/L)	2300 ± 208.16
Cadmium (mg/L)	0.453 ± 0.15
Copper (mg/L)	1.876 ± 0.29
Chromium (mg/L)	0.573 ± 0.19
Arsenic (mg/L)	0.099 ± 0.04
Mercury (mg/L)	0.021 ± 0.008
Lead (mg/L)	0.647 ± 0.16
Zinc (mg/L)	1.466 ± 0.32
Nickel (mg/L)	0.983 ± 0.13
Iron (mg/L)	6.830 ± 0.94
Aluminum (mg/L)	1.786 ± 0.47

Values are expressed as mean ± *SE* (n = 3).

culture supernatant of cells exposed to 10 µg/µL (10×) concentration for 0 to 24 hours using 8-oxo-dG enzyme-linked immunosorbent assay (ELISA) kit (Elabsciences, USA). In brief, samples were added onto the microtitre plate and incubated with avidin-conjugated horseradish peroxidase (HRP) for 2 hours. After incubation, substrate was added and the absorbance was recorded at 450 nm.[28] The levels of NRF-2 in cells exposed to 10 µg/µL (10×) concentration for 0 to 6 hours were analyzed through the Human Nrf-2 ELISA kit (Biocodon Technologies, USA), which works on the principal of sandwich ELISA. Briefly, samples and anti-Nrf-2 antibody labeled with biotin and streptavidin-HRP were added to the wells and incubated for 1 hour. After incubation, substrate was added and the absorbance was recorded at 450 nm.[27]

Evaluation of mitochondrial deregulation

Furthermore, the mtDNA copy number was evaluated in cells exposed to 10 µg/µL (10×) concentration for 0 to 24 hours by using 2 sets of primers for the amplification of mitochondrial NADH dehydrogenase ortholog 1 (MT-ND1) and nuclear gene human β-actin through quantitative real-time polymerase chain reaction (PCR). The values were shown as the ratio of mtDNA to the nuclear DNA (mtDNA/b-actin). The primers sequence for MT-ND1 gene was F, 5-C C C T A A A A C C C G C C A C A T C T-3 and R, 5-G A G C G A T G G T G A G A G C T A A G G T-3, while for β-actin, the primer sequences were F, TCCCTGGAGA A G A G C T A C G and R, G T A G T T T C G T G G A T G C C A C A, respectively.[29] To assess the potential of mitochondrial membrane, the cells exposed to 10 µg/µL (10×) concentration for 0 to 24 hours were stained with membrane-penetrating dye JC-1 (5, 5, 6, 6-tetra chloro-1, 1, 3, 3-tetra ethyl benzimidazol carbocyanine iodide) using MitoScreen kit (BD Biosciences, USA) as per the supplier's instructions. The cells were gated in a forward scattering (FSC)/side scattering (SSC) dot plot and the fluorescence ratio of red (590) vs green (530) was estimated.[30] The whole mitochondrial genome including hyper variable (HV) region was assessed by amplification of genome through 9 sets of overlapping primers in a thermal cycler (Applied Biosystems). In brief, 25 µL of PCR mixture for each fragment reaction was prepared by using 2× master mix at 1× concentration with 25 picomole of each primer and DNA template. The prepared reaction mixture was amplified for 30 cycles of PCR, visualized in agarose gels (0.8%) in 1× tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE), and the images were captured under ultraviolet (UV) (BioRad Gel Doc™ XR+) using Image lab software.[30]

Assessment of inflammatory response

The relation of NF-κβ activity with the leachate exposure was assessed in cells exposed to 10 µg/µL (10×) concentration for 0 to 6 hours by using the Human NF-κβ p65 and Human NF-κβ p105 ELISA kits from Biocodon Technologies, USA. For assay, samples and biotin/streptavidin-HRP conjugated anti-NF-κβ antibodies were added to the antibody pre-coated wells and incubated for 1 hour. After incubation, the wells were washed to remove unbound enzyme and substrate was added. Finally, the absorbance was recorded at 450 nm.[30] For assessment of inflammatory cytokines (IL-6, IFNγ, and TNFα), the cell supernatant collected from the culture plates of the cells exposed to 10 µg/µL (10×) concentration for 0 to 24 h and the levels were measured using ELISA kits (BD Biosciences, USA). In brief, the samples and streptavidin-HRP conjugate mixed with biotinylated anti-human cytokine antibodies were added to the wells and incubated for 1 hour. After incubation, the wells were washed and substrate was added to record the absorbance at 450 nm.[28]

Assessment of DNA damage and apopotosis

Immunoflurescence analysis was performed in cells exposed to 10 µg/µL (10×) concentration for 0 to 24 hours to assess the levels of γH2AX by following the protocol discussed by Mishra et al.[27] In brief, 10% formaldehyde and 0.1% Triton X-100 was initially used to fix and permeabilize, while 3% bovine serum

albumin was used for blocking prior to incubation with specific primary antibodies. Later, the cells were labeled with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (dilution 1:200) and DAPI (4, 6-diamidinophenylindole), and the analysis was performed.[27] The multicolor flow cytometric assay was performed in the cells exposed to 10 µg/µL (10×) concentration for 0 to 24 hours to detect cleaved PARP, γH2AX, and BrdU. The cells were initially permeabilized and fixed with BD cytofix solution for 30 minutes. The cells were then washed and treated with DNase for 1 hour to expose incorporated BrdU (during culture) followed by staining with PARP, γH2AX, and BrdU. The cells after incubation of 30 minutes were assessed in the FSC/SSC dot plot as discussed by Khan et al.[31]

For DNA laddering profile, the cells post-exposure to 10 µg/µL (10×) concentration for 0 to 6 hours were washed, and re-suspended in PBS for DNA isolation. The isolated DNA samples were then mixed with loading buffer and the electrophoresis was performed in 0.8% agarose gel at 80 V for 60 minutes with 1× TAE buffer.[27] To assess the activity of caspase-3 in cells exposed to 10 µg/µL (10×) concentration for 0 to 24 hours as a marker of mitochondrial-mediated apoptosis, Caspase-3 Apoptosis kit (BD Biosciences, USA) was used. In brief, the cells were washed and incubated in BD cytofix/cytoperm solution for 20 minutes on ice. The cells were then washed, incubated with phycoerythrin (PE) conjugated caspase-3 antibodies for 30 minutes, and analyzed by flow cytometry.[27] The levels of nucleosomes were measured through a double sandwich ELISA kit (Roche Applied Sciences) by following all necessary instructions of the manufacturer and the measurement of absorbance was done at 405 nm.[32]

Cytogenetic analysis

Analysis of cytogenetic abnormalities was performed in cells exposed to 10 µg/µL (10×) concentration for 72 hours through the method discussed earlier by Mishra et al. Briefly, metaphases were harvested, fixed, and stained with a conventional Giemsa method. A minimum of 50 metaphases were randomly selected and analyzed to find out the structural chromosome aberrations.[27]

Statistical analysis

The obtained results were represented as the mean ± standard error (*SE*). Student's *t* test or analysis of variance was used to calculate the statistical differences between the studied groups. Analysis was performed using the Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL, USA) and a *P* value of ≤ 0.05 established statistical significance.

Results

Optimum dose for treatment

On analysis, it was observed that higher concentrations, 100 µg/µL (100×) and 1000 µg/µL (1000×), induced significant toxicity in cells. The 10 µg/µL (10×) concentration was found to be a optimum dose to conduct downstream experiments as more than 75% of the exposed population was live at 6 hours exposure. It was observed that as compared to controls, leachate treatment at 1 µg/mL concentrations induces cell death processes which further increases on increasing leachate concentration to 10×. However, on further increasing the concentration, the apoptosis decreased and a toxic burst effect was observed. Thus, later experiments were conducted with constant concentration of 10 µg/µL (10×) at selective time intervals ranging from 0 to 24 hours.

Landfill leachate induces oxidative stress

The higher ROS levels observed among landfill leachate exposed cells suggested its ability to disturb mitochondrial machinery and induce ROS generation. In comparison to controls, the ROS levels depicted by resulting dichlorofluorescein (DCF) florescence were significantly higher in the cells exposed to landfill leachate suggesting its potential to disturb the mitochondrial machinery. The levels of ROS increased in a time-dependent manner after exposure to landfill leachate at 10× concentrations. The observed levels of percent DCF fluorescence in controls was 0.2 ± 0.08 while for cells exposed to leachate 10× for 15 minutes, 30 minutes, 1 hour, 3 hours, and 6 hours were $10.71 \pm 0.5\%$, $16.6 \pm 2.5\%$, $23.61 \pm 2.24\%$, $43.19 \pm 3.18\%$, and $41.12 \pm 3.18\%$, respectively (Figure 1). In addition, a time-dependent accumulation of 8-oxo-dG was observed in the exposed cells. The values expressed as the ratio of control vs treated for 1, 3, 6, 12, and 24 hours were 2.34 ± 0.15, 3.76 ± 0.21, 5.21 ± 0.63, 5.34 ± 0.54, and 5.41 ± 0.33, respectively (Figure 2). Similarly, as compared to controls, the observed levels of Nrf-2 were significantly higher in cells exposed to landfill leachate. The observed values for cells exposed to 1, 3, and 6 hours were 3.31 ± 0.98, 9.89 ± 1.35, and 15.28 ± 2.93 pg/mL, respectively, while in controls, the value was 0.62 ± 0.05 pg/mL (Figure 2).

Landfill leachate disturbs mitochondrial machinery

Furthermore, the levels of mtDNA copy number expressed as mtDNA to nuclear DNA (β-actin) ratio suggested that generated disproportionate ROS levels interact with mtDNA and cause significant damage to the exposed mitochondria. The results of quantitative PCR analysis observed a significant decline ($P \leq 0.05$) in landfill leachate exposed cells till 24 hours in a time-dependent manner. The level of mtDNA/nDNA ratio in control was 0.65 ± 0.02, while in the maximum, decline was observed among cells exposed to landfill leachate at 24 hours (0.35 ± 0.05). The other values of mtDNA/nDNA ratio analysis observed at 1, 3, 6, and 12 hours were 0.61 ± 0.01, 0.56 ± 0.03, 0.50 ± 0.02, 0.44 ± 0.03, respectively (Figure 3). The results of 9 fragment analysis suggested successful amplification of the whole mitochondrial genome in 9 fragments in

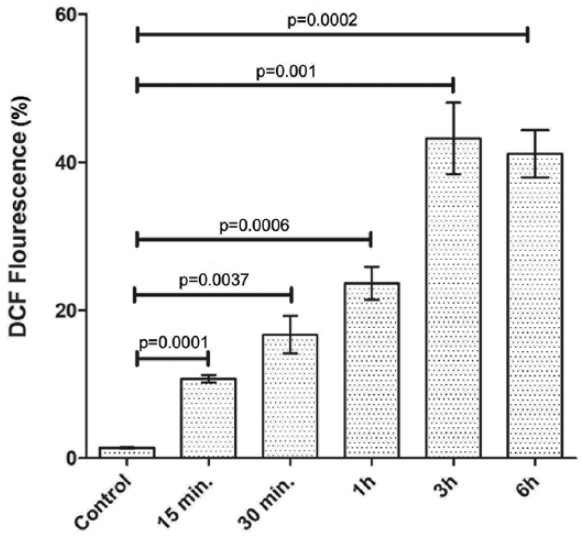

Figure 1. Generation of reactive oxygen species. Assessment of reactive oxygen species generation through flow cytometry in peripheral blood lymphocytes exposed to landfill leachate at 10 µg concentration for 15 minutes, 30 minutes, 1 hour, 3 hours, and 6 hours. Untreated cells were used as control. CM-H2DCFDA was used to label the cells and the increase in the DCF fluorescence was recorded as an indicative for generation of reactive oxygen species. Data are expressed as mean ± *SE* and $P \leq 0.05$ was considered significant.
CM-H2DCFDA, 5-(and -6) chloro methyl-2, 7-di-chloro-di-hydro-fluorescein-di-acetate-acetyl-ester; DCF, dichlorofluorescein.

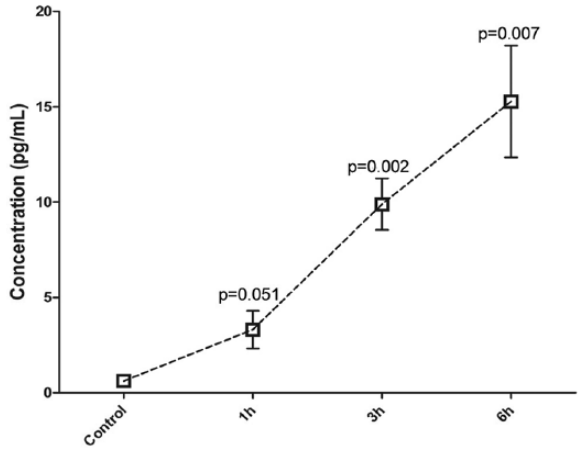

Figure 2. Levels of Nrf-2. Graph showing the Nrf-2 levels in the controls and cells exposed to landfill leachate at different time points from 1, 3, and 6 hours. Values are expressed as mean ± *SE* and $P \leq 0.05$ was considered significant.
Nrf-2, nuclear factor erythroid 2–related factor 2.

controls. However, defragmented mitochondrial bands were observed among leachate exposed cells after 24 hours suggesting the significant leachate-induced mitochondrial toxicity (Figure 4).

Landfill leachate activates inflammatory response

The observed levels of NF-κB (p65) and NF-κB (p105) were significantly higher in the cells exposed to landfill leachate.

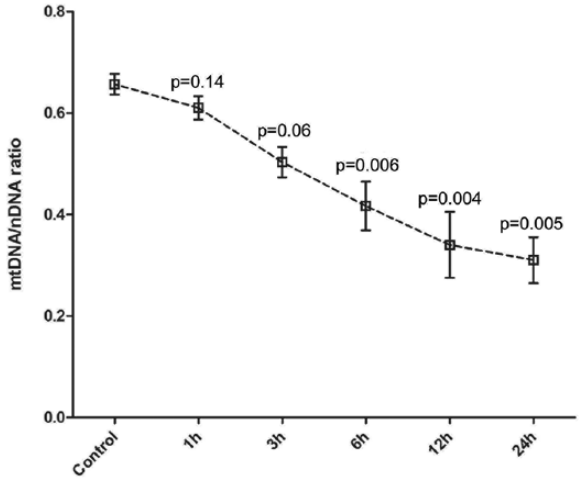

Figure 3. mtDNA/nDNA ratio. Graph showing the mtDNA/nDNA ratio in lymphocytes exposed to landfill leachate (10×) at varying time-points (1, 3, 6, 12, and 24 hours). The mtDNA content was quantified by elucidating the ratio between mean mtDNA (ND1) and mean nuclear DNA content (β-actin). Values are shown as mean ± *SE* and $P \leq 0.05$ was considered significant.
mtDNA, mitochondrial DNA.

The observed values of p65 expressed as the ratio of the control vs treated for cells exposed to leachate for 1, 3, and 6 hours were 5.05 ± 0.74, 9.72 ± 1.24, and 17.11 ± 1.03, respectively, while for p105, the observed values of the ratio were 2.68 ± 0.39, 4.06 ± 0.61, 4.68 ± 0.64, respectively. Similarly, the significant higher levels of secreted inflammatory cytokines in cultures of landfill leachate exposed cells were also observed. The maximum levels of pro-inflammatory cytokines IL-6, IFNγ, and TNFα were observed at 10× concentration and 6 hours post exposure to landfill leachate, respectively (Figure 5).

DNA damage and mitochondrial mediated apoptosis

The results of qualitative immunofluorescence analysis showed a clear accumulation of γH2AX (Ser139, phosphorylated), a prominent DNA damage response protein in leachate exposed cells. The maximum phosphorylation levels were observed among the cells exposed to leachate for 24 hours (Figure 6A). The quantitative flow cytometric analysis suggested a time-dependent incline in the levels of γH2AX among cells exposed to leachate, with the highest observed levels at post 24 hours of exposure (48.36 ± 3.89). In addition, the assessed levels of cleaved PARP, an apoptotic protein marker, and BrdU, a proliferation marker, also correlated with the results suggesting the ability of leachate to induce genotoxicity. The levels of cleaved-PARP in leachate exposed cells at 24 hours were 46.27 ± 3.54%, while the proliferation index observed in exposed cells after 24 hours was 6.62 ± 1.02%. The control levels of γH2AX and cleaved-PARP were 1.4 ± 0.30% and 1.85 ± 0.34%, whereas observed proliferative index among control cells was 34.65 ± 2.64% (Figure 6B to D).

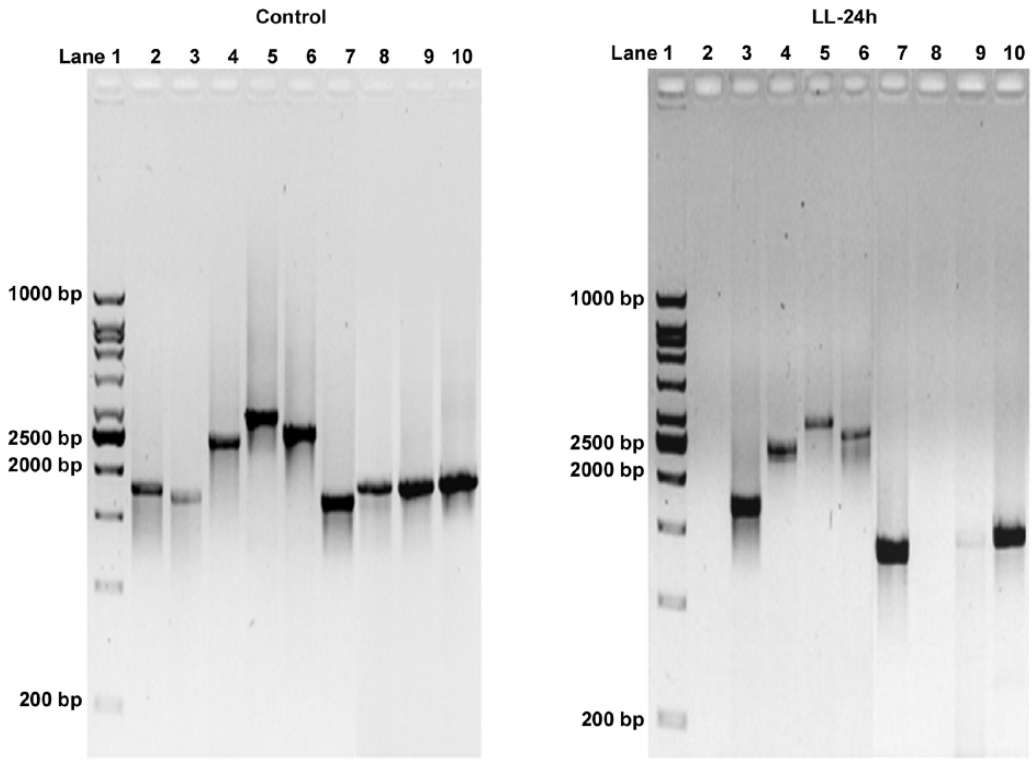

Figure 4. Mitochondrial genome analysis. Figure showing 9 fragment analysis of mitochondrial genome in controls and the cells exposed to LL for 24 hours. Lane 1: HMW marker and Lanes 2 to 10: fragments 1 to 9.
LL, landfill leachate.

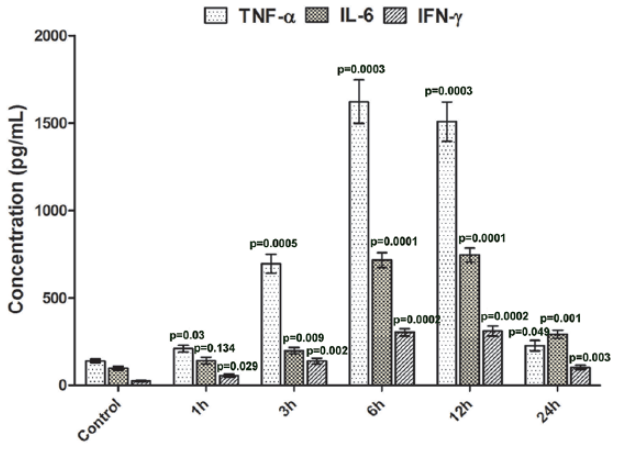

Figure 5. Pro-inflammatory cytokine response. Graph showing the secreted levels (pg/mL) of pro-inflammatory cytokines, ie, TNF-α, IL-6, and IFN-γ in controls and lymphocytes exposed to landfill leachate at different time periods. Untreated cells were used as control. Values are given as mean ± *SE* and $P \leqslant 0.05$ was considered significant.
IFNγ, interferon gamma; IL-6, interleukin 6; TNFα, tumor necrosis factor alpha.

DNA ladder analysis observed through gel electrophoresis showed a clear laddering profile suggesting DNA fragmentation in the landfill leachate exposed cells, while no such pattern was reported in the control cells. The observed results indicated the ability of landfill leachate to induce DNA fragmentation and apoptosis (Figure 7A). The observed values for apoptotic nucleosomes in cells exposed to leachate for 1, 3, 6, 12, and 24 hours were 0.26 ± 0.01, 0.835 ± 0.06, 1.91 ± 0.01, 1.935 ± 0.06, and 2.09 ± 0.04 AU, respectively, while in controls, the values were 0.11 ± 0.006 AU (Figure 7B).

In addition, the significant increase in the percent level of cells with distorted membrane potential was observed in cells exposed to 10×. The mean percentage of the cells with mitochondrial depolarization obtained was 24.67 ± 2.07% at 24 hours, while in controls, the values were 0.52 ± 0.07%. The observed values at 1, 3, 6, and 12 hours were 2.48 ± 0.09%, 4.84 ± 0.26%, 9.87 ± 1.04%, and 14.36 ± 1.93%, respectively (Figure 7C). The percent caspase-3 activity in controls was 0.35 ± 0.12% while in cells exposed to landfill leachate for 1, 3, 6, 12 and 24 hours were 1.10 ± 0.07%, 3.58 ± 0.46%, 10.11 ± 1.64%, 22.68 ± 1.16%, and 49.92 ± 3.19%, respectively (Figure 7D).

Chromosomal abbreviations

The cytogenetic analysis of the cells exposed to landfill leachate showed chromatid-type aberrations with open breaks and deletion of a chromatid fragment, whereas no such chromosomal aberrations were reported in the control subjects. These results provided a clear evidence of the clastogenic potential of landfill leachate (Figure 8).

Discussion

Improper and non-scientific management of municipal solid waste may cause serious impact on environmental and human health. The uncontrolled waste dumping of perilous waste at

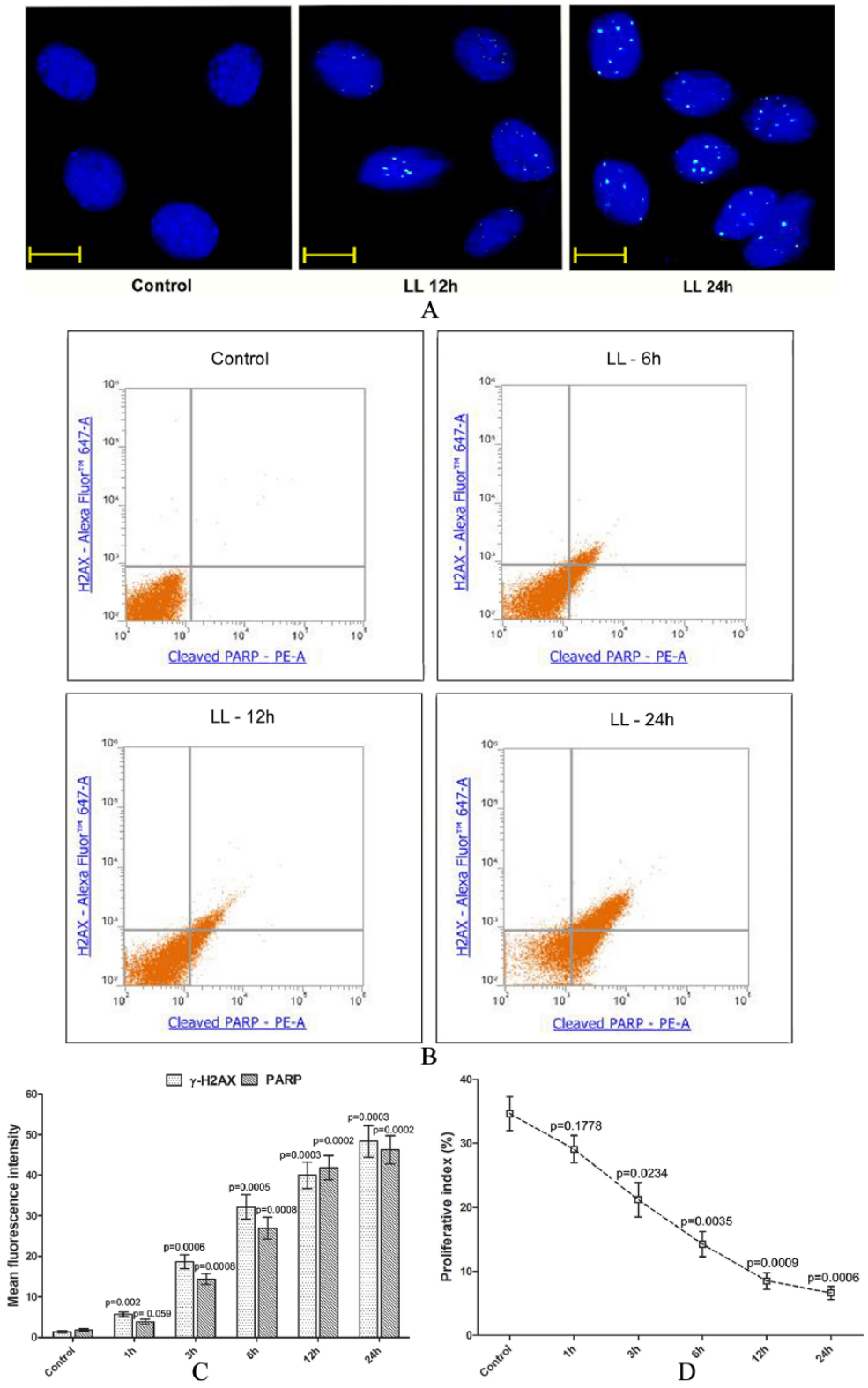

Figure 6. (A) DNA damage. Representative image of untreated control and lymphocytes exposed to landfill leachate at 12 and 24 hours. γ-H2AX foci are labeled by FITC (green) while nucleus was counterstained with DAPI (blue). (B) DNA damage and apoptosis. A representative dot plot showing the flow cytometric evaluation of cells positive for γ-H2AX (Alexa Flour-647) and cleaved PARP (PE) in controls and landfill leachate exposed cells at different time periods. The increase in the γ-H2AX and cleaved PARP activity is depicted by the shift of cells from lower left region of the quadrant (double negative) to the lower right (PARP positive), upper right (double positive), and upper left (γ-H2AX positive) regions. (C) Quantification of γ-H2AX and cleaved PARP. Graphical representation of the mean fluorescence intensity indicating the percent levels of γ-H2AX and cleaved PARP in controls and landfill leachate exposed cells at different time periods. Untreated cells were used as control. Data are shown as mean ± *SE* and $P \leqslant 0.05$ was considered significant. (D) Proliferative index. Graph showing the proliferative index of the cells exposed to landfill leachate (10×) for 1, 3, 6, 12, and 24 hours. Untreated cells were used as control while values are given as mean ± *SE* and $P \leqslant 0.05$ was considered significant.

DAPI, 4, 6-diamidinophenylindole; FITC, fluorescein isothiocyanate; LL, landfill leachate; PARP, polyadenosine diphosphate ADP-ribose polymerase.

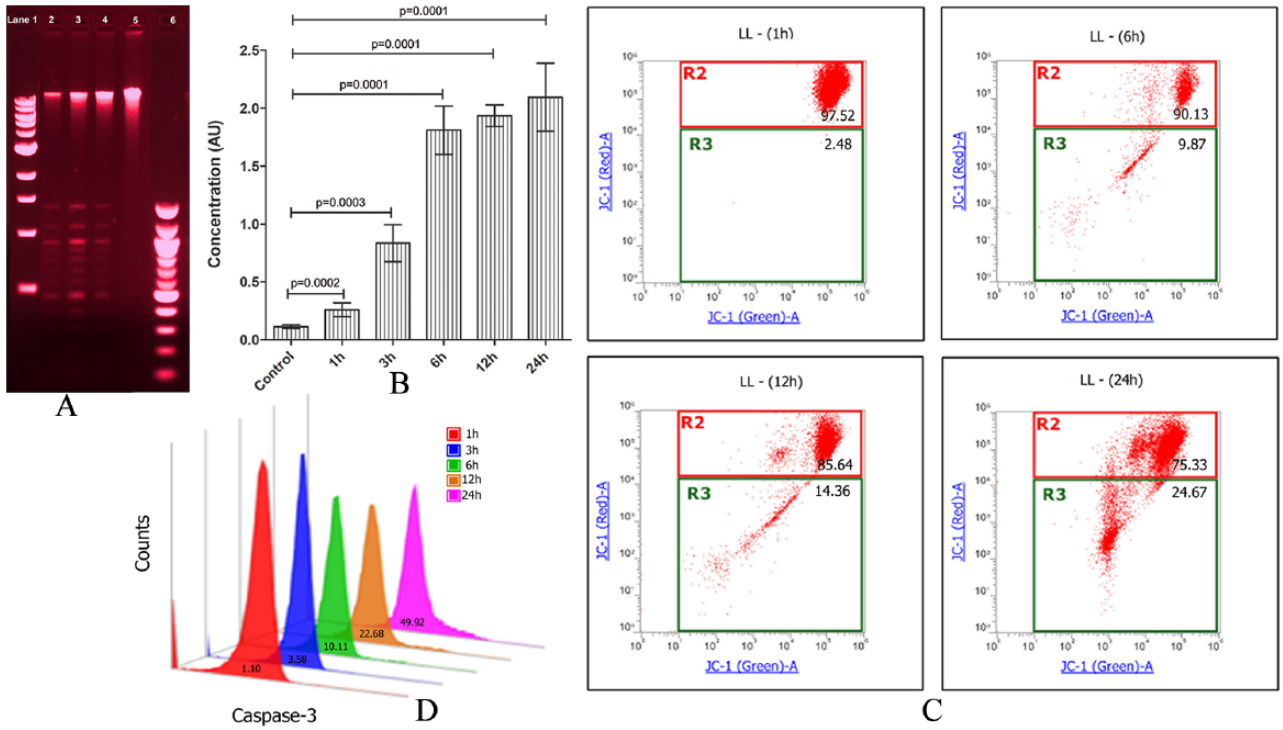

Figure 7. (A) DNA fragmentation. Figure showing DNA fragmentation profile of the cells exposed to landfill leachate. Lane 1: HMW marker; Lanes 2 to 5: cell exposed to leachate for 12, 6, 3, and 1 hour; Lane 5: LMW marker. (B) Apoptotic nucleosomes release. Graph demonstrating the comparative assessment of secreted levels of AU in the culture supernatant of untreated controls and lymphocytes exposed to the landfill leachate at varied time points from 1, 3, 6, 12, and 24 hours. Data are shown as mean ± *SE* and $P \leqslant 0.05$ was considered significant. (C) Mitochondrial membrane depolarization. Representative dot blot showing the quantitative evaluation of JC-1 indicating the mitochondrial membrane potential through flow cytometry among the cells exposed to landfill leachate (10×) for 1, 6, 12, and 24 hours. Shift in the fluorescence of JC-1 from R2 to R3 indicates the increase in the number of cells with the altered mitochondrial membrane potential. Data are shown as mean ± *SE* and $P \leqslant 0.05$ was considered significant. (D) Activity of caspase 3. Flow cytometric analysis of the activity of caspase 3, indicator of an apoptosis in cells exposed to landfill leachate (10×) for 1, 3, 6, 12, and 24 hours. An increased fluorescent signal, as indicated by the peak shift, clearly demonstrates the time-dependent increase in the activity of caspase 3. Data are shown as mean ± *SE* and $P \leqslant 0.05$ was considered significant.
AU, apoptotic nucleosomes.

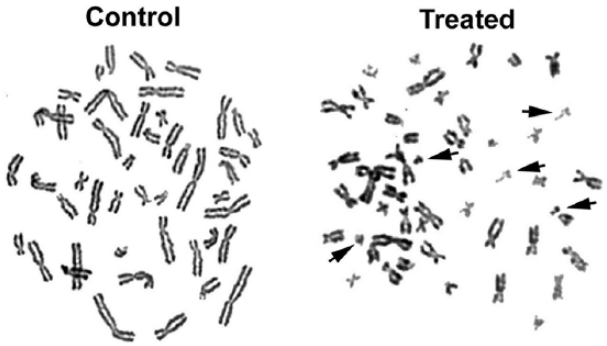

Figure 8. Cytogenetic analysis. Representative image showing cytogenetic anomalies of the cells exposed to the landfill leachate (10× concentration) for 72 hours. The arrow indicates the chromosomal abnormalities in the exposed cells.

the suburbs of the different cities creates overflowing landfills, which is a noteworthy contributing factor to the health hazards encountered by communities. These unlined sanitary landfills account for the uncontrolled discharge of toxic chemicals to nearby air and groundwater, by means of leachate and landfill gas.[33,34] The landfill leachate comprises of a blend of numerous harmful chemicals including overwhelming metallic compounds which are potentially hazardous to human well-being.[35] Studies have shown that the composite metallic configuration of the leachate significantly contributes toward the genomic impairment and leads to cellular toxicity.[36–38] Although existing studies have suggested the genotoxic and cytotoxic potential of landfill leachate, there is lack of data indicating the mechanisms underlying the landfill leachate–induced redox imbalances. Furthermore, a better comprehension of such molecular procedures will clear up the patho-physiology of various ailments related with landfill leachate exposure. This study for the first time demonstrated that exposure to landfill leachate at a minimal concentration of 10× induces significant alterations in the mitochondrial machinery and consequently triggers the mitochondria-mediated responses which finally lead to cell death.

Mitochondria are the key source of energy production and a primary target of different environmental toxicants. These toxicants have a direct impact on its functionality which can promptly reflect the changes in cellular homeostasis.[39] Moreover, presence of several diverse valence state metals such as iron,

chromium, copper, and arsenic in landfill leachate can interact with mitochondrial redox mechanisms and generate superoxide ions.[40,41] Studies indicate that the cationic metals, for instance, mercury and lead, accrue within mitochondria through calcium transporters and/or their interactions with mitochondrial pH and charge, while negatively charged mitochondrial matrix assist in the accrual of amphiphilic environmental toxins.[42,43] Individual studies on certain metals like cadmium and chromium suggested that these metals also possess the property to deregulate electron transfer chain and cause elevation in the level of ROS.[44,45] These metals possess the potential to bind to the thiol proteins present on the mitochondrial membrane, which affects the mitochondrial permeability transition, ultimately inhibiting electron transport chain (ETC).[46] In addition, the dysfunctional mitochondria have been shown to be significantly associated with several disorders including cancer, cardiovascular diseases, and metabolic disorders.[47,48] Earlier investigations have suggested that leachate primarily comprises heavy metals which are known to induce oxidative stress and cell cycle blockade in the HepG2 model.[49,50] Studies have also revealed the presence of significant contamination of nitrates, sulfates, heavy metals in the ground water, and soil samples from the area nearby Bhanpur khanti, municipal landfill site of Bhopal.[51,52] Our study observed the concurrent time dependent increase in the generation of ROS along with the increased time-period which is plausibly due to the existence of heavy metals within the leachate (Figure 1).

Conventionally, synergistic production of ROS during ETC along with ATP in mitochondria acts as a signaling molecule between mitochondria and rest of the cell in normal physiological conditions.[53,54] However, excessive ROS production due to mitochondrial dysfunctioning can lead a swing toward a pro-oxidant state and results in the induction of oxidative stress.[55,56] Although several quality control mechanisms operate which includes fusion and fission, mitophagy maintains the mitochondrial homeostasis and regulates its volume and function during the stress conditions.[57] The mtDNA mutations, copy number alterations, or defects in ETC proteins can induce "retrograde signaling" mediated through either mitochondrial ROS itself or inducing mtDNA damage and response to convey the mitochondrial conditions to the nucleus.[58] One of the most common targets of ROS during oxidative stress is the DNA which leads to DNA modification primarily at guanine nucleotide resulting in the formation of oxidized guanisine bases (8-oxo-dG)[59] which is a potential biomarker of oxidative stress. Concurrently, the results of this study clearly displayed the potential of landfill leachate to induce oxidative stress as significantly higher 8-oxo-dG levels were reported in leachate exposed cells in comparison to controls. The disturbed mitochondrial redox homeostasis further activates redox-sensitive transcription factor Nrf-2, which on stress translocate to nucleus and play an essential role in the expression of antioxidant enzymes and other stress-inducible genes to regulate the redox homeostasis.[60] In the same line, this study observed that exposure to landfill leachate was positively associated with the Nrf-2 activation, as higher levels of Nrf-2 were reported in the leachate exposed cells (Figure 2).

Moreover, mtDNA is highly susceptible to several endogenous and exogenous oxidative stresses due to their close proximity to the inner mitochondrial membrane and lack of protective histones.[61] As most of mitochondrial genes are expressed and involved in regulation of vital mechanisms, any disturbances in mitochondrial genome may lead to serious complications. This study demonstrated the significant decline in the mtDNA copy number in landfill leachate exposed cells in comparison to controls. We also observed defragmented and low intensity bands in landfill leachate exposed cells which strongly signified the leachate associated damage in the mitochondrial genome (Figures 3 and 4). These alterations in mitochondrial genome may affect mitochondrial biogenesis and other associated cellular mechanism that consecutively intensifies the associated health risks. Such dysfunctional mitochondria have been shown to be associated with pro-inflammatory responses and DNA damage.[62] Besides, mitochondrial dysfunction has been reported to be closely linked with the NF-κβ which regulates a number of genes involved in apoptosis, and inflammation. Activation of NF-κβ p65 stimulates pro-inflammatory response, which causes nuclear translocation of different downstream target proteins including COX-2 and other inflammatory cytokines. Evidences also suggest that exposure to toxic metals significantly affects the activity of NF-κβ transcription factor, which may be a probable link for generation of further cellular abnormalities.[63] Earlier reports have reported that exposure to leachate induces significant DNA damage in different cell types.[64–66] Interestingly, findings of our study also indicated that leachate-induced mitochondrial dysfunction may induce a pro-inflammatory state. In comparison to the controls, the levels of NF-κβ were significantly higher among the cells exposed to the landfill leachate, while significantly higher secreted levels of pro-inflammatory cytokines TNFα, IL-6, and IFN-γ were observed in the culture supernatant of landfill leachate exposed cells as compared with their respective controls (Figure 5). The higher accumulation of γH2AX among leachate exposed cells further suggested that this may lead to significant DNA damage indicating toward the genotoxic potential of leachate (Figure 6A and C). Notably, cytogenetic analysis in leachate exposed cells displayed aberrations, implicating the clastogenic activity of leachate (Figure 8).

Excessive generation of oxidative stress places mitochondria under immense pressure to maintain its membrane integrity.[67] However, cells possess a highly regulated defense system to trigger activation of cell death mechanisms for removing these abnormally working mitochondria. It is also known that the induced oxidative stress have been largely associated with the decrease in the mitochondrial membrane potential. Moreover, to eliminate the damaged cellular system, loss of mitochondrial potential induces the release of pro-apoptotic proteins such as cytochrome c into the cytosol that triggers the activation of certain caspase dependent apoptotic pathways leading cell toward the self-demise

mechanisms. It has been reported that leachate inhibits cell proliferation and activates cytotoxic events after prolonged exposure.[68] Therefore, this study analyzed the possible association of landfill leachate exposure with mitochondrial regulation of the cell death proteins. The fragmented DNA observed and higher release of apoptotic nucleosomes among leachate exposed cells strongly suggested the onset of apoptosis (Figure 7A and B). Besides, the observed decrease in the mitochondrial membrane potential with increases time period signifies the associated mitochondrial deregulation in landfill leachate exposed cells (Figure 7C). In addition, the increased levels of cleaved-PARP and activity of caspase-3 in landfill leachate exposed cells signifies the key loops of the mitochondrial mediated mechanism (Figures 6B, C, and 7D). These results were further correlated with the reduced cell proliferation among the exposed cells (Figure 6D).

Conclusively, the results obtained from the present examination provide insights to the comprehension of immunotoxic potential of landfill leachate at the genomic level. The heavy metals, xenobiotic compounds, and several organic and inorganic components which make the constituent of the landfill leachate have the property to get accumulated with time, leading to the extensive damage in cellular machinery. Results demonstrated that mitochondria, the most versatile organelle, are highly vulnerable to the landfill leachate leading to the onset of several molecular cascades. Eventually, our results clearly demonstrated that landfill leachate potentially induces oxidative stress via directly disturbing mitochondrial machinery, ultimately leading to apoptosis. The study suggests that the exposure of landfill leachate via contaminated surroundings can severely impact the human well-being.

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Author Contributions

PKM devised the concept, developed the methodology and supervised the experiments; PG, AB and PKM performed the flow cytometry experiments; PG and RK executed the molecular assays; NB, PKG and LL collected and analysed the leachate samples; RM performed the cytogenetics experiments; RT helped in data analysis; and AB and PKM drafted the manuscript.

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