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Long-term exposure to $PM_{2.5}$ exacerbates dopaminergic neuronal loss through CpG hypermethylation induced down-regulation of PINK1 and DJ-1 genes

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To investigate whether airborne particle ($PM_{2.5}$) aggravates Parkinson's disease (PD) and alter expression of key PD-related genes by DNA methylation. Two groups of rats were exposed to either clean air or polluted air for 3, 6, and 12 months. The neurotoxin rotenone was injected intraperitoneally to induce a Parkinson's-like disorder. Immunostaining was used to measure the number of dopaminergic neurons in substantia nigra (SN). Real-time PCR was used to measure mRNA levels of PD-related genes *PINK1* and *DJ-1* in SN. Bisulfate sequencing (BSP) was used to measure DNA methylation levels in gene promoters. In a cell-based mimic of animal experiments, SH-SY5Y cells were treated with Diesel exhaust $PM_{2.5}$ (DEP) for 1.5, 6, and 24 h. RT-PCR and BSP methods were used to measure gene expression and methylation of CpG islands in the cells. Persistent exposure to $PM_{2.5}$ significantly increased the loss of dopaminergic neurons in the SN. Prolonged $PM_{2.5}$ exposure and DEP treatment significantly reduced the mRNA levels of *PINK1* and *DJ-1*. Both $PM_{2.5}$ and DEP significantly increased the methylation level of the CpG islands in both genes. $PM_{2.5}$ induced loss of dopaminergic neurons and aggravated Parkinson's disease. $PM_{2.5}$ induced dysregulation of DNA methylation, resulting in decreased expression of the *PINK1* and *DJ-1*.

Keywords Parkinson's disease, $PM_{2.5}$, *PINK1*, *DJ-1*, CpG hypermethylation

Parkinson's disease (PD) is one of the most common neurodegenerative diseases worldwide, second only to Alzheimer's disease. The incidence, which increases with age, is 1–2% in the population over 65-year-old and 4% for people over 85-year-old[1]. With rising population, China has entered an aging society, and the morbidity of PD will increase constantly and undoubtedly be a serious burden to the economy. However, the pathogenesis of PD is not completely clear.

Fine particulate matter ($PM_{2.5}$) is the main pollutant that causes haze weather. With the rapid development of the economy, haze pollution has gradually developed into a prominent environmental problem in China. However, it was not until recently that people realized the seriousness of $PM_{2.5}$ pollution. Animal trials indicate that the olfactory mucosa may be an entry route for air pollutants to disturb the blood–brain barrier and negatively affect the central nervous system (CNS)[2,3]. Considerable evidence supports a link between $PM_{2.5}$ exposure and the development of CNS diseases, including PD and other neurodegenerative diseases[4–8]. Wang's study showed that exposure to $PM_{2.5}$ aggravated the symptom of PD in rotenone-treated mouse by inducing the dysfunction of mitochondria and *PINK1/Parkin* signaling pathways were involved in the aggravative effects of $PM_{2.5}$[9]. In addition, both Zanobetti's and Hyewon Lee's research indicated that PD patients required hospitalization after short-term exposure to elevated $PM_{2.5}$ levels[10,11].

Various PD-related genes have been identified, and knowledge about the genetics of PD has exploded[12]. The first gene associated with PD was identified in 1997 as a missense mutation in the *α-synuclein* (*SNCA*) gene; the gene is regulated by DNA methylation and the missense mutation increases the risk of PD[13]. The phosphatase and tensin homologue-induced putative kinase 1 gene (*PINK1*)[14] and the *DJ-1* gene[15] are also closely related to the pathogenesis of PD. The *PINK1* and *DJ-1* genes[16] protect mitochondrial from oxidative stress, and their

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downregulation aggravates the oxidative stress response and promote apoptosis. The *DJ-1* gene can be regulated by DNA methylation. DNA methylation, a common type of epigenetic modification, has an important function in the pathogenesis of PD. DNA methylation can silence gene expression, thereby reducing the protective effects of genes and promoting the progress of PD. Nevertheless, it remains unclear whether $PM_{2.5}$ alters DNA methylation and thereby aggravates the pathogenesis of PD by regulating expression of key genes such as *PINK1* and *DJ-1*. Study of potential gene expression changes can provide better understanding of the disease itself and may become a therapeutic target for treatment.

In this study, we evaluated the impact of $PM_{2.5}$ on expression and methylation of the key PD-related genes *PINK1* and *DJ-1*. We measured the impact of $PM_{2.5}$ on dopaminergic neurons in the substantia nigra (SN). In addition, we induced PD in rats by treating with rotenone and assessed aggravation of PD by $PM_{2.5}$ in these rats. Then, we measured expression and methylation of *PINK1* and *DJ-1* in SN after different times of exposure to $PM_{2.5}$. Finally, we verified the animal results with SH-SY5Y cells and measured the effect of $PM_{2.5}$ on misregulation of DNA methylation of the *PINK1* and *DJ-1* genes.

Results

$PM_{2.5}$ concentration description

To observe the effects of $PM_{2.5}$ on animals over different time periods, we monitored atmospheric $PM_{2.5}$ concentrations at 3, 6 and 12 months and found that the average daily concentrations of $PM_{2.5}$, which was 54.16 $\mu g/m^3$ during the first 3-month period, 52.58 $\mu g/m^3$ in the 6-month period, and 44.64 $\mu g/m^3$ in the 12-month period. Figure 1a shows the level of average concentration of $PM_{2.5}$ monitored using an individual particle monitor. All animals in the $PM_{2.5}$ group were exposed to $PM_{2.5}$ levels above China's national air quality standard (15 $\mu g/m^3$) and the World Health Organization guideline (5 $\mu g/m^3$).

Effects of $PM_{2.5}$ on body weight

The animal weights were monitored monthly, and the results showed that the $PM_{2.5}$ group experienced a significant decrease in weight gain from Month 1 to Month 9 compared to the FA group (Fig. 1b). However, there was no significant difference in weight gain between the two groups from Month 10 to Month 12.

$PM_{2.5}$ accelerated the loss of dopaminergic neurons in the substantia nigra of PD rat model

The average value of filtered air-exposed and vehicle-administered in each month was used as the control benchmark (100%). Compared with the filtered air-exposed, TH-positive cells were significantly decreased by 6.49% in the $PM_{2.5}$-exposed; compared with the vehicle-administered, TH-positive cells were significantly decreased by 22.16% in the rotenone-administered at 3 months (Fig. 2a and b). Compared with the filtered air-exposed, TH-positive cells were significantly decreased by 5.23% in the $PM_{2.5}$-exposed; compared with the vehicle-administered, TH-positive cells were significantly decreased by 52.99% in the rotenone-administered at 6 months (Fig. 2c and d). Compared with the filtered air-exposed, TH-positive cells were significantly decreased by 12.24% in the $PM_{2.5}$-exposed; compared with the vehicle-administered, TH-positive cells were significantly decreased by 60.17% in the rotenone-administered at 12 months (Fig. 2e and f).

3 months: $PM_{2.5}$-exposed (n = 12) vs filtered air-exposed (n = 12): $F(1, 20) = 16.21$, $P = 0.0007$;

rotenone-administered (n = 12) vs vehicle-administered (n = 12): $F(1, 20) = 189.1$, $P < 0.0001$; interaction effect: $F(1, 20) = 0.3146$, $P = 0.5811$.

6 months: $PM_{2.5}$-exposed (n = 12) vs filtered air-exposed (n = 12): $F(1, 20) = 3.299$, $P = 0.0844$;

rotenone-administered (n = 12) vs vehicle-administered (n = 12): $F(1, 20) = 338.1$, $P < 0.0001$; interaction effect: $F(1, 20) = 0.9893$, $P = 0.3318$.

12 months: $PM_{2.5}$-exposed (n = 12) vs filtered air-exposed (n = 12): $F(1, 20) = 34.50$, $P < 0.0001$;

rotenone-administered (n = 12) vs vehicle-administered (n = 12): $F(1, 20) = 833.9$, $P < 0.0001$; interaction effect: $F(1, 20) = 0.03124$, $P = 0.8615$.

Significance of pairwise comparisons (n = 6): ns = non-significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{**}p < 0.001$; $^{**}p < 0.0001$.

$PM_{2.5}$ decreased the levels of *DJ-1* and *PINK1* mRNAs in PD rat model

To determine whether exposure to $PM_{2.5}$ affected the mRNA levels of *PINK1* and *DJ-1*, we measured *PINK1* and *DJ-1* mRNAs in the SN. Real-time RT-PCR results showed that 3 months of exposure induced a 60% reduction in *PINK1* compared with the FA group (Fig. 3a), whereas there was no difference in *DJ-1* mRNA level (Fig. 3b). However, when the exposure time was increased to 6 months, the reduction in *PINK1* mRNA level was nearly 75% (Fig. 3c), and a significantly greater reduction (nearly 50%) was also found in *DJ-1* (Fig. 3d). We observed still further reductions of mRNA levels of *PINK1* and *DJ-1* after 12 months of exposure (Fig. 3e and f).

$PM_{2.5}$ resulted in hypermethylation of CpG islands in *PINK1* and *DJ-1* genes in PD rat model

By using the UCSC Genome Browser database, we found typical CpG islands around the promoters of the rat *PINK1* and *DJ-1* genes. There were 55 CG sites within the entire length of the CpG islands of both genes (Fig. 4a and e). Methylation status of the CpG islands was determined by bisulfite sequencing. Exposure to $PM_{2.5}$ for 6 months resulted in a 6.06% increase in methylation of the *PINK1* gene compared with 2.42% for the control (Fig. 4c). For the *DJ-1* gene, 6 months of exposure resulted in an increased methylation level from 2.4–7.27% (Fig. 4g). For 12 months exposure to $PM_{2.5}$, the methylation level was further increased, reaching 9.30% and 12.73% in the *PINK1* and *DJ-1* genes, respectively (Fig. 4d and h); 3 months exposure to $PM_{2.5}$ had no effect on the methylation level on both genes (Fig. 4b and f).

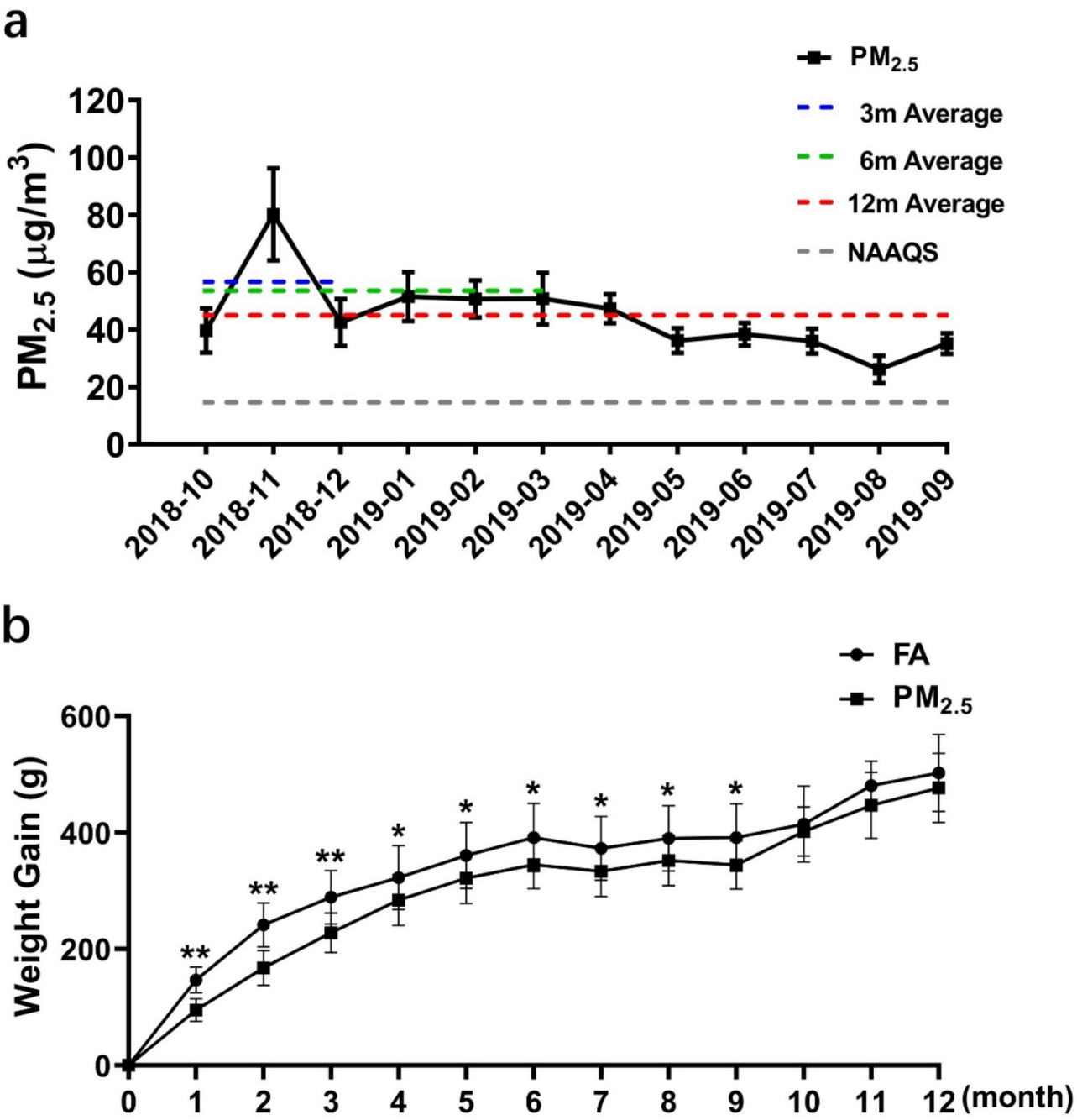

Fig. 1. The average concentration of $PM_{2.5}$ was higher than NAAQS and $PM_{2.5}$ resulted the lower body weight. (**a**) The level of average concentration of $PM_{2.5}$ monitored using an individual particle monitor. (**b**) Rat body weight gained every month in both groups (n = 20). $^{*}p < 0.05$ compared to control, $^{**}p < 0.01$ compared to control.

DEP resulted in decreased cell viability in PD cell model

We treated human neuroblastoma SH-SY5Y cells with DEP at 2.5, 5, 10, 20, 40, 80, 160, and 320 μg/ml for 24 h. The LD_{50} (median lethal dose) was 40 μg/ml by viable cells counting (Fig. 5a) and LDH assay (Fig. 5b).

DEP repressed transcription of *PINK1* and *DJ-1* genes in PD cell model

In the forgoing animal study, we found that $PM_{2.5}$ resulted in a decrease in expression of the *PINK1* and *DJ-1* genes. To confirm this effect in vitro, we performed quantitative RT-PCR to measure the *PINK1* and *DJ-1* mRNA levels in SH-SY5Y cells. Under the condition of DEP concentration of 40 g/ml, the level of *PINK1* mRNA was significantly reduced at 6 h and 24 h (Fig. 6a), while the level of DJ-1 mRNA was also significantly reduced at 24 h (Fig. 6b).

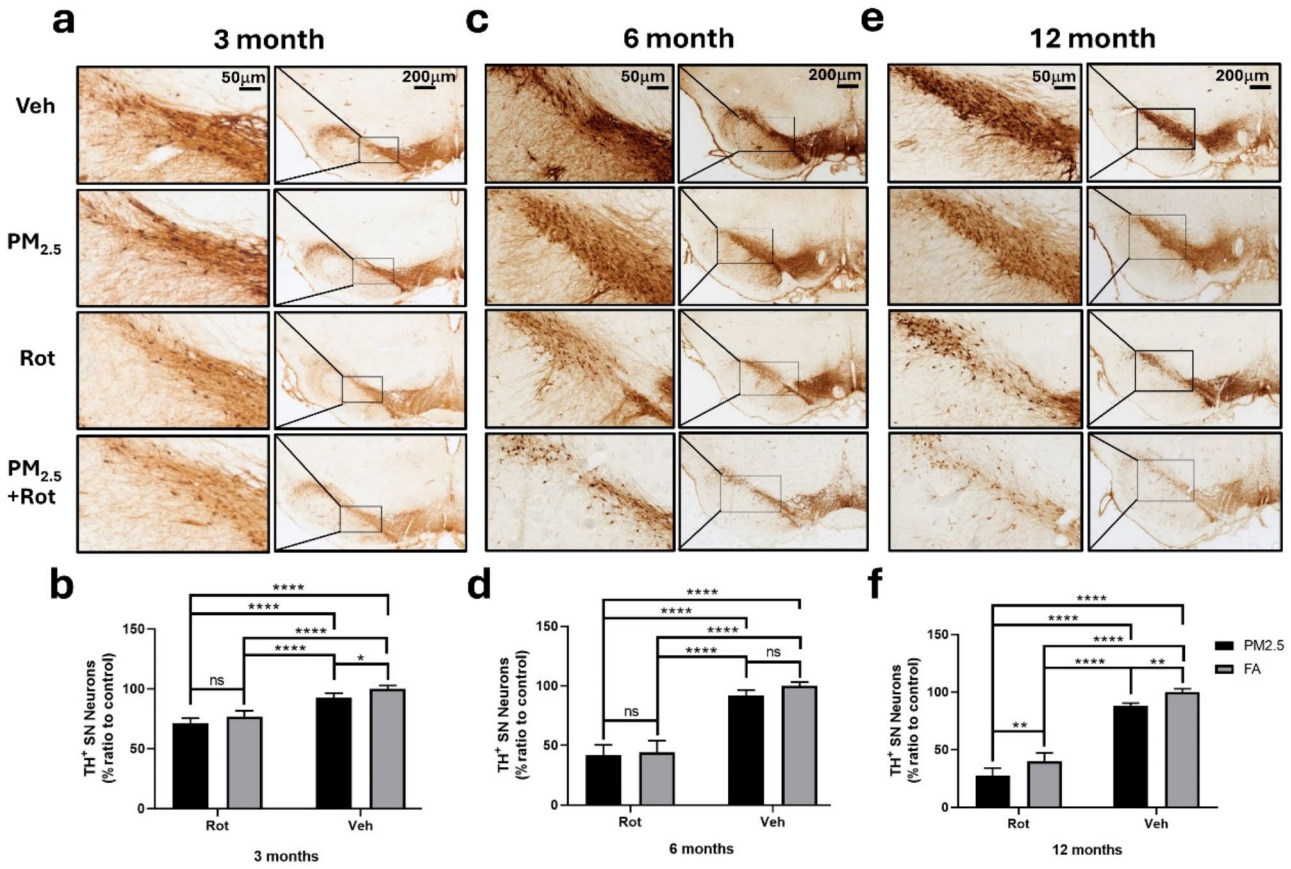

Fig. 2. $PM_{2.5}$ accelerated the loss of dopaminergic neurons in the SN. TH expression in SN sections was assessed by immune-histochemistry. (**a**, **c**, **e**) Immune-histochemistry staining results for 3, 6, and 12 m. (**b**, **d**, **f**) Quantitative analysis of TH-positive neurons. Statistical analysis was performed by two-way ANOVA.

DEP resulted in hypermethylation of CpG islands in *PINK1* and *DJ-1* genes in PD cell model

Using the UCSC Genome Browser database, we identified 69 and 55 CG sites in the entire length of human *PINK1* and *DJ-1* CpG islands (Fig. 7a and c). Compared with the methylation level of the control group (2.42%), after 6 and 24 h of DEP treatment, the methylation level of the *PINK1* gene significantly increased to 6.28% and 11.59%, respectively (Fig. 7b). Likewise, in comparison with the methylation level of the control group (1.82%), the methylation level of the *DJ-1* gene significantly rose to 8.48% after 24 h of DEP treatment (Fig. 7d).

Discussion

Epidemiological studies indicate that exposure to $PM_{2.5}$ is associated with an increased risk of developing Parkinson's disease and other neurological disorders[6,17]. Despite extensive research on the relationship between $PM_{2.5}$ and Parkinson's disease, the underlying molecular mechanisms remain largely unexplored. Therefore, our objective was to elucidate the mechanisms through which $PM_{2.5}$ exacerbates Parkinson's disease. In this study, we provide robust evidence that $PM_{2.5}$ can accelerate development of PD. The effect is based on the dysregulation of key PD-related genes, notably *PINK1* and *DJ-1*. Further, abnormal gene expression may be related to increased DNA methylation. First, we found that $PM_{2.5}$ caused the loss of dopaminergic neurons in the SN. The neurotoxin rotenone can damage dopaminergic neurons, and $PM_{2.5}$ can lead to further dopaminergic neurons loss induced by rotenone. Second, $PM_{2.5}$ decreased *PINK1* and *DJ-1* expression and increased methylation of the CpG islands of the *PINK1* and *DJ-1* genes. Third, treating SH-SY5Y cells with DEP produced similar effects as observed in animal experiment.

Our study shows that the weight of the $PM_{2.5}$ group significantly decreased during the first to ninth month, which is consistent with previous research findings. In an animal experiment using C57BL/6 J mice as in vivo models, the researchers administered $PM_{2.5}$ (2.5 mg/kg, inhalation) and rotenone (30 mg/kg, intraperitoneal injection) to the mice for 28 days[9]. The study found that the weight of the rotenone group mice was significantly lower than that of the control group from 4 to 28 days. Furthermore, there was a significant difference between the rotenone group and the $PM_{2.5}$ + rotenone group, indicating that $PM_{2.5}$ affects the body weight of PD mice. Although the observation period was only 28 days, the result is consistent with our findings. The potential mechanism by which $PM_{2.5}$ affects body weight may involve metabolic disorders caused by air pollution, which are closely related to body weight[18]. However, in epidemiological studies, the impact of air pollution on weight

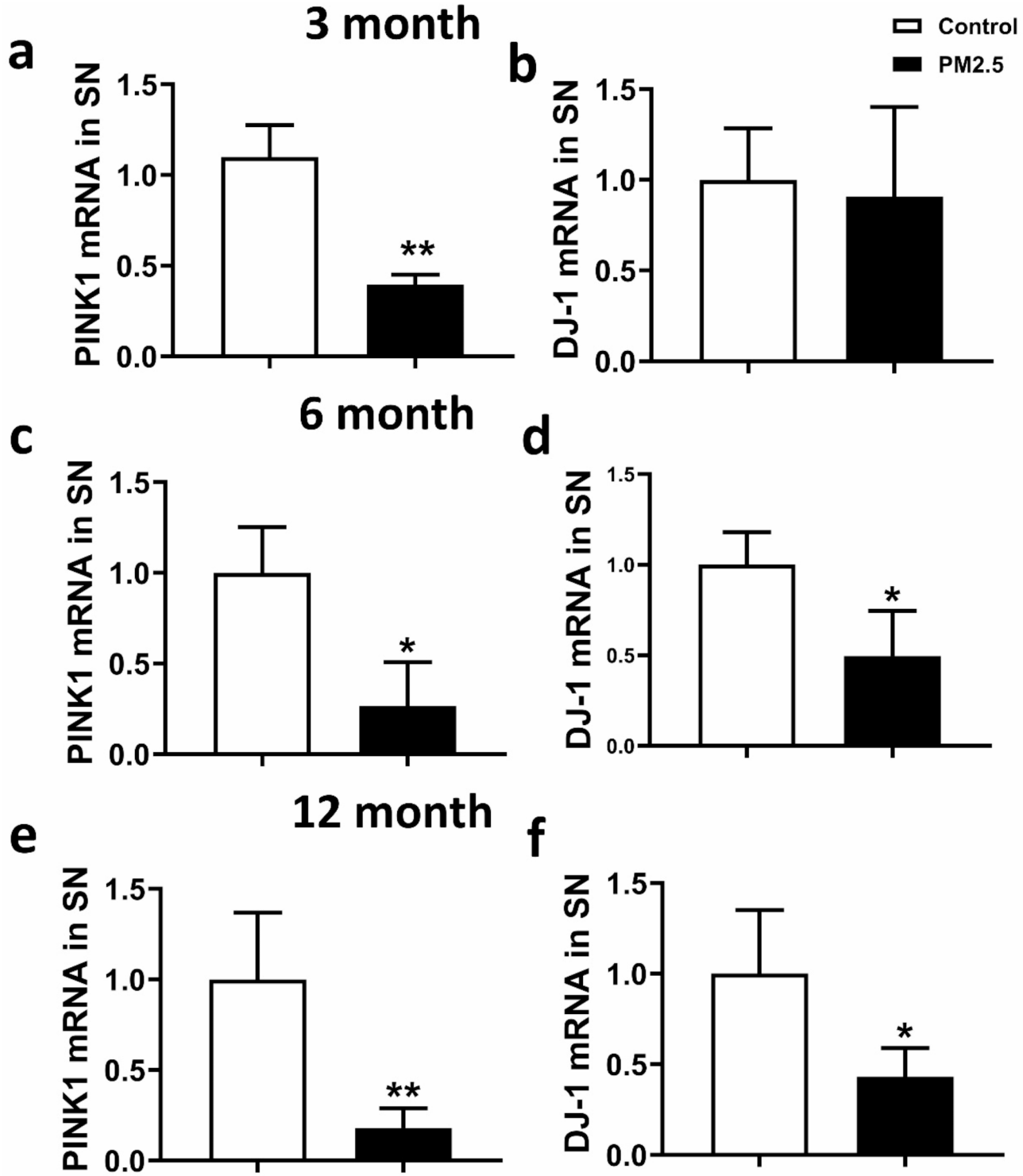

Fig. 3. $PM_{2.5}$ down-regulated the transcription of *PINK1* and *DJ-1* genes in rats' model. The mRNA levels of both genes were assessed by real-time RT-PCR. (**a**, **c**, **e**) The transcription level of *PINK1* gene at 3, 6 and 12 m in control and $PM_{2.5}$ groups. (**b**, **d**, **f**) The transcription level of *DJ-1* gene at 3, 6, and 12 m in both groups. Statistical analysis was performed by ANOVA. Significance (n = 8): $^{*}p < 0.05$ compared to control, $^{**}p < 0.01$ compared to control, $^{***}p < 0.001$ compared to control.

remains highly controversial and may vary depending on factors such as gender, age group, and type of air pollutant[19–21]. Therefore, further in-depth research is needed to clarify this issue.

Previous studies have indicated that $PM_{2.5}$ can trigger neuroinflammation, leading to the loss of dopaminergic neurons and the formation of Lewy bodies, while inducing mitochondrial dysfunction to stimulate the

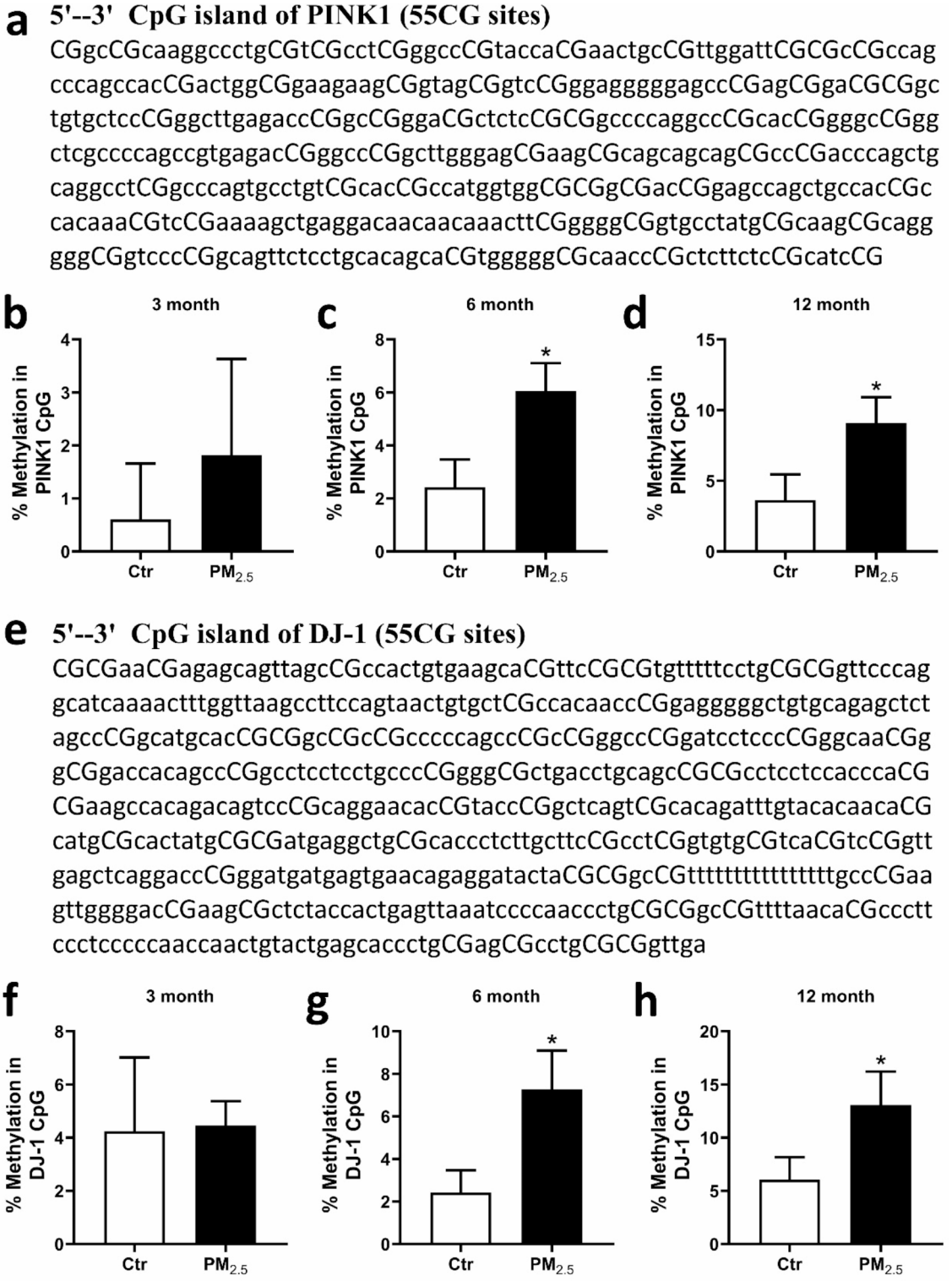

Fig. 4. The CpG islands and 5' up-streamed region around *PINK1* and *DJ-1* promoters in rats. Methylation status of the CpG islands was analyzed using Bisulfite sequencing. (**a**, **e**) 55 CpG sites in the CpG islands of *PINK1* and *DJ-1*, respectively. (**b**, **c**, **d**) Methylation level of *PINK1* CpG islands at 3, 6, and 12 m in both control and $PM_{2.5}$ groups. (**f**, **g**, **h**) Methylation level of *DJ-1* CpG islands. Significance (n = 5): $^{*}p < 0.05$ compared to control, $^{**}p < 0.01$ compared to control.

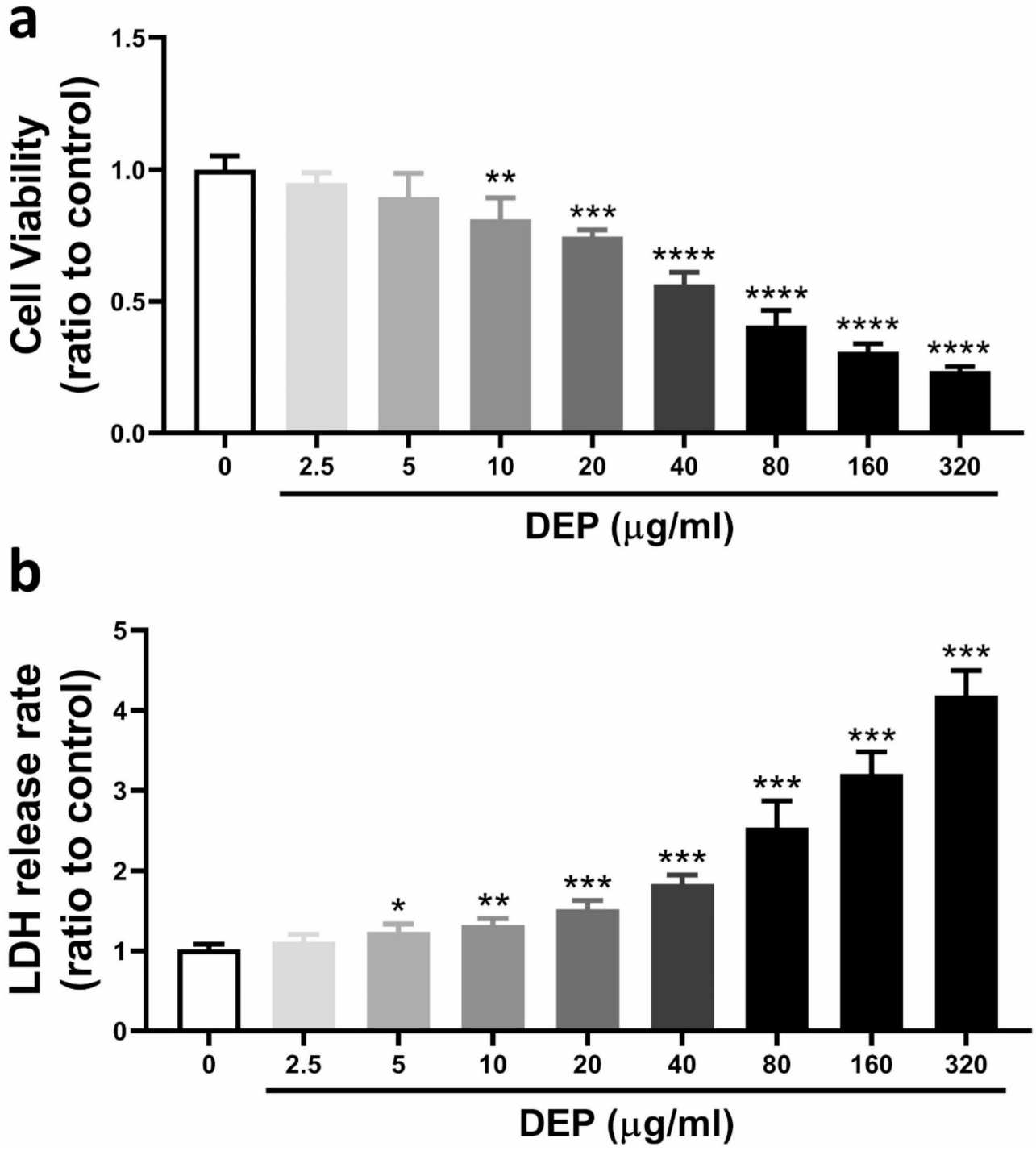

Fig. 5. DEP reduced cell viability Optimal $PM_{2.5}$ treated concentration was determined in SH-SY5Y cells by evaluating cell viability and cytotoxicity with viable cells counting and LDH assays, respectively. (**a**, **b**) Cell viability and cytotoxicity were detected in SH-SY5Y cells with viable cells counting (**a**) and LDH (**b**) assays. Significance (n = 3): $^{*}p < 0.05$ compared to control, $^{**}p < 0.01$ compared to control, $^{***}p < 0.001$ compared to control. DEP: Diesel exhaust $PM_{2.5}$.

production of reactive oxygen species (ROS), thereby aggravating neuronal damage[9,22–24]. In this study, we found that, similar to neurotoxin rotenone, $PM_{2.5}$ causes the loss of dopaminergic neurons in the SN, and $PM_{2.5}$ can further exacerbate the loss of dopaminergic neurons caused by rotenone. In addition, similar studies have observed that short-term $PM_{2.5}$ exposure can worsen animal behavior[9], but this aspect was not tested in our study. Therefore, the behavioral changes caused by long-term exposure to $PM_{2.5}$ still need further investigation.

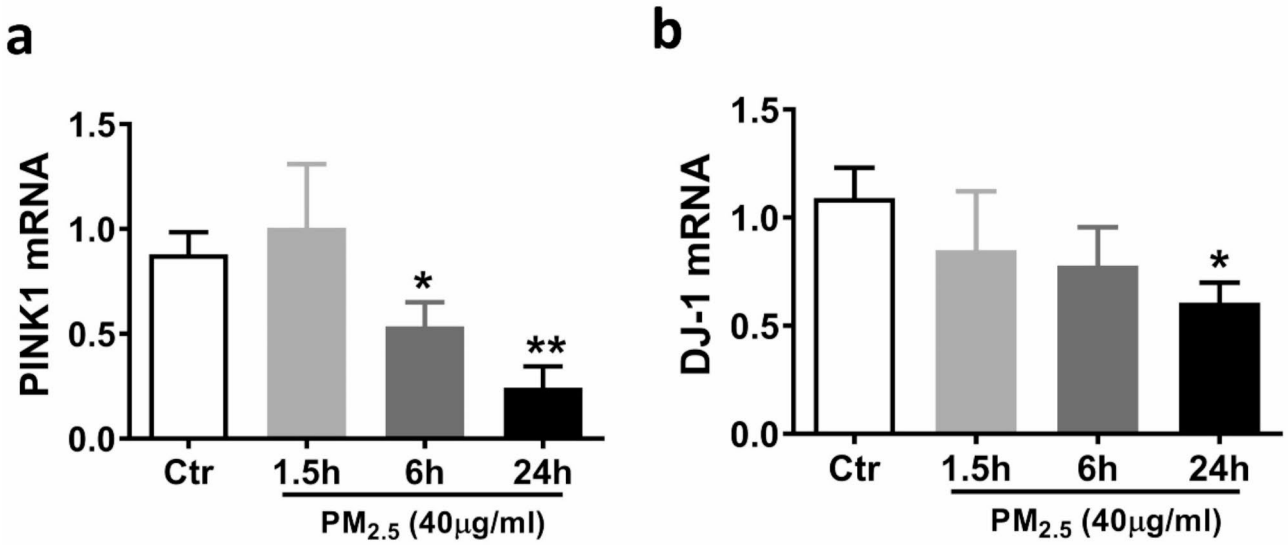

Fig. 6. DEP down-regulated the transcription of *PINK1* and *DJ-1* genes in SH-SY5Y cells. SH-SY5Y cells were treated with 40 μg/ml of $PM_{2.5}$ for 1.5, 6, and 24 h. The mRNA levels of *PINK1*(**a**) and *DJ-1(b)* were assessed by real-time RT-PCR. Statistical analysis was performed by ANOVA. Significance (n = 3): $^{*}p < 0.05$ compared to control, $^{**}p < 0.01$ compared to control, $^{***}p < 0.001$ compared to control. DEP: Diesel exhaust $PM_{2.5}$.

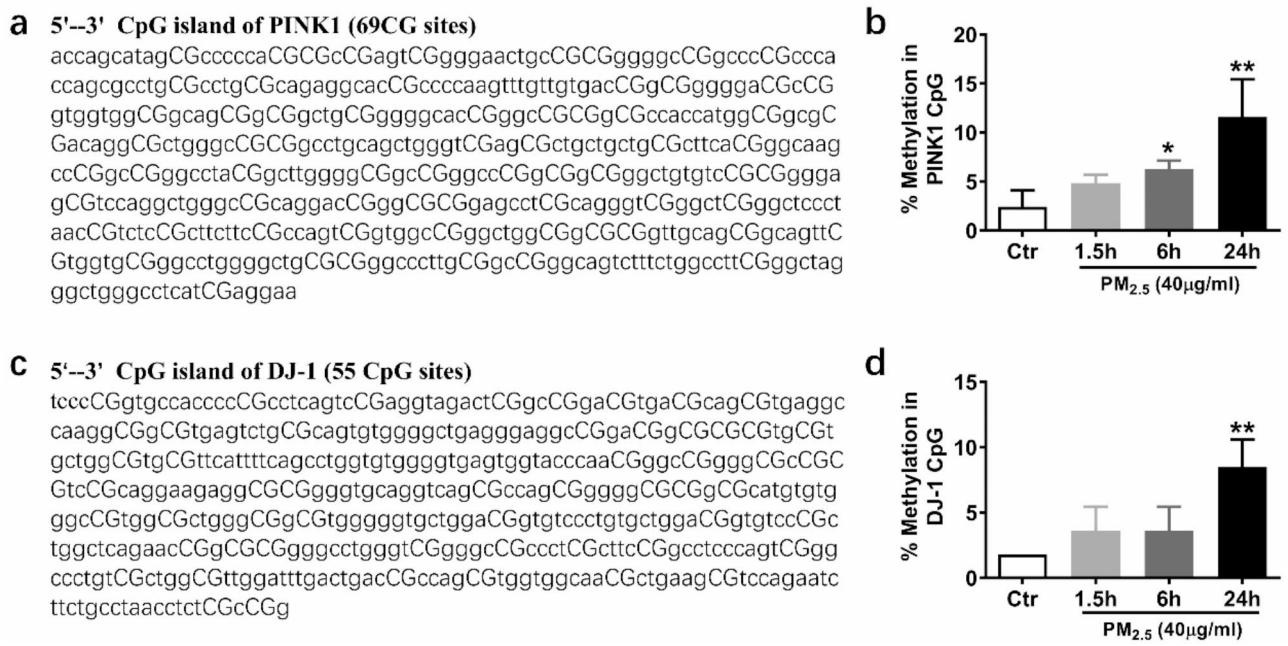

Fig. 7. The CpG islands and 5' up-streamed region around *PINK1* and *DJ-1* promoters in human cells. Methylation status of the CpG islands was analyzed using Bisulfite sequencing. (**a**, **c**) 69 and 55 CpG sites in the CpG islands of *PINK1* and *DJ-1*, respectively. (**b**, **d**) Methylation level of *PINK1* and *DJ-1* CpG islands at 1.5, 6 and 24 h treated DEP. Significance (n = 3): $^{*}p < 0.05$ compared to control, $^{**}p < 0.01$ compared to control. DEP: Diesel exhaust $PM_{2.5}$.

Our study found that exposure to $PM_{2.5}$ also leads to the loss of dopaminergic neurons, as well as downregulation of *PINK1* and *DJ-1* mRNA expression. Previous studies have found that the loss of dopaminergic neurons is correlated with low expression of *PINK1*. In previous studies on animals, cells, and organoids, *PINK1* deficiency impairs adult neurogenesis of dopaminergic neurons[25–27]. In a robust vertebrate model of *PINK1* deficiency, the *PINK1*-/- zebrafish, Th1 + neurons of the TPp and PVO are found in comparable numbers to those in young adult wild-type fish, but thereafter showed a significant decline[25]. Similarly, impaired dopaminergic neurogenesis was observed in human tissue organoid models with *PINK1* deficiency[25]. *PINK1* and *NOTCH* interact non-

classically in mitochondria, and *PINK1* deficiency significantly inhibits the proliferation of human neural stem cells (NSCs)[26]. Growth inhibition was also observed in mouse *PINK1* knockout NSCs, which was associated with mitochondrial dysfunction[32]. For *DJ-1*, previous studies have shown that reducing *DJ-1* expression can affect the function of dopaminergic neurons, and the dopaminergic neurons in the substantia nigra of *DJ-1* mutant mice are functionally impaired[28,29]. *DJ-1* protein has characteristics such as molecular chaperone, protease, glutathione peroxidase, transcriptional regulator, and *DJ-1* protein prevents neurodegenerative diseases by regulating oxidative stress and mitochondrial function, as well as its role in transcription and signal transduction[30–32]. Based on the above studies, we can conclude that the downregulation of *PINK1* mRNA expression leads to a decrease in the number of dopaminergic neurons, while the downregulation of *DJ-1* mRNA expression affects the function of dopaminergic neurons. Therefore, we hypothesize that the loss of dopaminergic neurons observed in our study, which was caused by exposure to $PM_{2.5}$, may be due to the downregulation of *PINK1* mRNA expression. In addition, $PM_{2.5}$-induced *DJ-1* mRNA is likely to exert an influence on the function of dopaminergic neurons. Previously studies have proven that DNA methylation has been shown to be pivotal to the occurrence and development of PD[33,34], including the regulatory role of PD-related genes such as *α-synuclein*, *Parkin*, *PINK1*, *DJ-1*. Yang's study showed that CpG demethylation was induced by MPP^+ mediated up-regulation of *SNCA* transcription in neurotoxin-induced PD[35]. Tarale et al. performed chronic manganese exposure of human SH-SY5Y cells and found that integration of DNA methylation data with gene expression revealed epigenetic alterations to *PINK1* and *Parkin* genes that were critical for the onset of Parkinsonism[36]. Both short and long-term exposure to ambient air pollution resulted in global changes to DNA methylation[37,38]. In our study, we found that rats which received more than 6 months of $PM_{2.5}$ exposure had elevated DNA methylation of CpG islands of the *PINK1* and *DJ-1* genes, and cells-based data showed that long-term (6 or 24 h) treated with DEP resulted in increased DNA methylation of CpG islands. Thus, our results confirmed that long-term exposure to $PM_{2.5}$ can lead to changes in DNA methylation for some genes. Further, the decreased gene expression associated with the aforesaid treatments can be explained by increased DNA methylation.

Our study had several limitations. First, in the cell-based experiment, we did not test an inhibitor of DNA methylation such as 5-Aza to determine whether DNA methylation changes could be reversed. Second, other investigators have found that neurotoxins can induce misregulation of DNA methylation[35]. However, we did not determine whether rotenone itself affected DNA methylation. Third, although much evidence confirms that DNA methylation has a key function in the occurrence and development of PD, studies on specific regulatory mechanisms are still limited. For example, we did not determine which DNA methyltransferase(s) was responsible for the increased methylation of the *PINK1* and *DJ-1* genes or which signaling pathways were involved in the process.

In summary, our study revealed that exposure to $PM_{2.5}$ leads to the loss of dopaminergic neurons in the substantia nigra, aggravates the development of PD by inducing dysregulation of DNA methylation, resulting in decreased expression of the PD-related genes *PINK1* and *DJ-1*.

Methods

PD rat model

The animal protocols in this study were approved by the Ethics Committee for Animal Experimentation at Capital Medical University (Beijing, China), and all experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. 4-week-old male Sprague–Dawley rats were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and randomly distributed into $PM_{2.5}$-exposed (n = 72) or filtered air (FA) groups (n = 72). Each group was subdivided into a rotenone-administered group (2 mg/kg) for 4 w (n = 36) or a vehicle-administered group (n = 36). The rats were housed in a controlled room at a temperature of 24 °C with a standard 12-h light/dark cycle and had free access to food and water. The rats were housed for 3 months, 6 months, and 12 months, respectively. The diagram of the experimental design is in Fig. 8.

Rotenone administration

The rotenone was dissolved in DMOS and diluted in sterile sunflower oil (2% final DMSO concentration and 98% final sunflower oil concentration)[39]. The rotenone-administered group received supplementation of rotenone (2 mg/kg/d) for 30 days before tissue extraction respectively at 3, 6 and 12 months. The rotenone solution was given by intragastric administration. The final working concentration of rotenone is re-prepared every two days and stored in a light-protected ambient temperature environment.

Exposure to $PM_{2.5}$

Animals were exposed to $PM_{2.5}$ or FA for 24/7 for 3 months (from October 11, 2018 to January 10, 2019), 6 months (from October 11, 2018 to April 10, 2019), and 12 months (from October 11, 2018 to October 10, 2019) using our real-world $PM_{2.5}$ exposure system described previously[40]. The mean daily $PM_{2.5}$ concentration in each chamber was monitored by an individual portable fine particulate monitor. $PM_{2.5}$ was monitored using a portable fine particulate monitor (pDR1500, Thermo Fisher Scientific Inc., USA) at Peking Union Medical College Hospital (located at 116°22′05″E, 39°54′50″N) in Beijing, China. The condition of FA rats in the experiment was the same as that of $PM_{2.5}$ rats in all aspects; the difference was that the improvement of air quality caused by the placement of a high-efficiency particulate air filter in the intake valve position leads to the removal of all $PM_{2.5}$ from the FA flow. The annual average $PM_{2.5}$ National Ambient Air Quality Standard (NAAQS) in China was recorded.

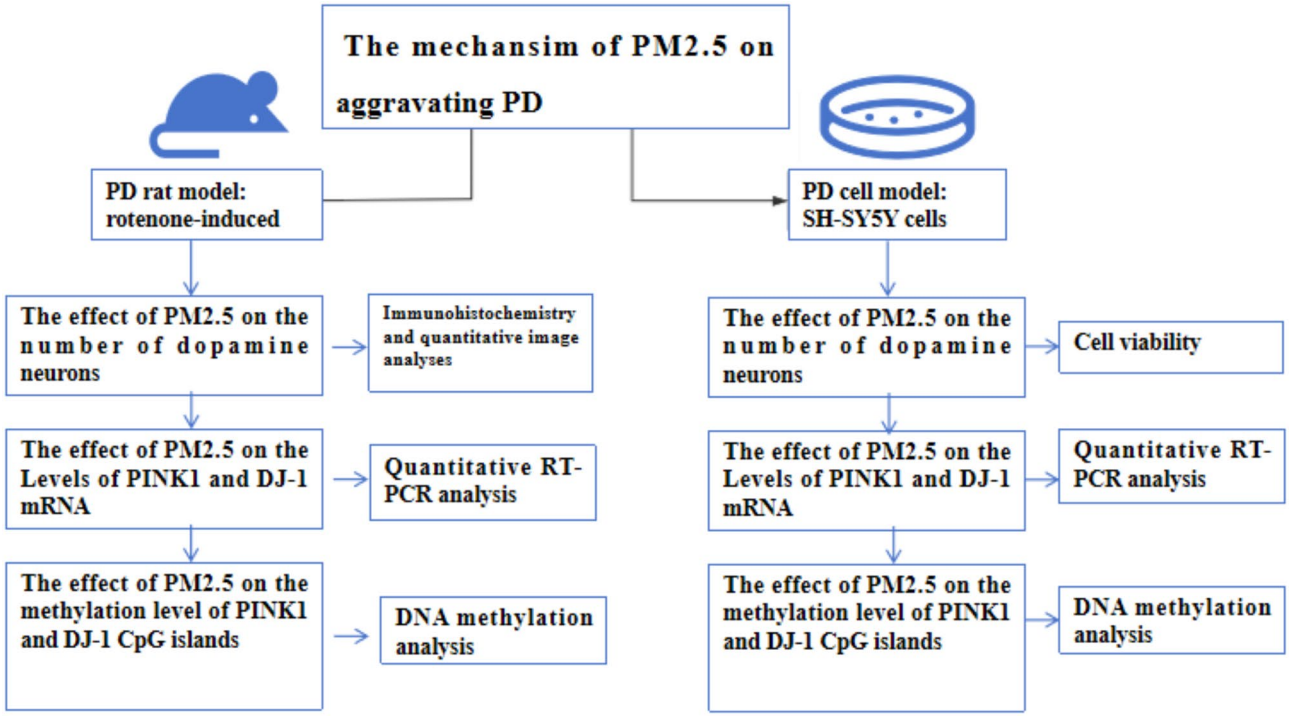

Fig. 8. The diagram of the experimental design.

Immunohistochemistry and quantitative image analyses

At the end of the treatment animals (n = 6/group) were sacrificed under general anesthesia induced by a combination of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (4 g/kg) through intraperitoneal administration and transcardially perfused with 0.9% sodium saline (room temperature), followed by 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) (pH 7.4, ice-cold). Brains were post fixed for 12 h in 4% PFA and dehydrated in 20% and 30% sucrose in PBS, respectively. Brains were frozen and sectioned coronally (30 μm) and placed into a tissue collection solution (50% 0.01 M PBS/50% glycerol) and stored at − 20 °C. The sections were permeabilized in PBS containing 0.3% Triton X-100, blocked in 5% normal horse serum, and then incubated overnight at 4 °C with an anti-TH antibody (1:2000, Sigma). After washing three times with PBS, sections were respectively incubated with a biotinylated second antibody (1:200; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) and an AB work solution (1:1:100) for 30 min at 37 °C (Vector Laboratories, USA). DAB solutions (a drop of DAB to 2 ml substrate liquid) were used to visualize the antibody. Sections were fixed on slides and cover slipped with a water-soluble mounting medium. The sections were imaged with a bright-field microscope. The estimated number of TH + cells was calculated based on the neuronal count and the corresponding sampling probabilities. The quantity of TH immunoreactive cell bodies in the SN was quantified using Stereo Investigator software, Version 2017.02.2 (09/27/2017) (MBF Bioscience, USA)[39,41,42]. The average number of SN sections was six in a 1:6 series. The number of TH cell bodies in each counting frame was determined by focusing each part with a 20-times objective lens. The total count of TH cell bodies in each section was computed by the software. A coefficient of error below 0.10 was deemed acceptable. Quantitative results for TH cell bodies were expressed as a percentage relative to the control side. Blind statistical analysis utilizing stereology methods was conducted for each experiment.

Cell culture and DEP treatment

SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% FBS (Cat# SV30087.02, Hyclone, Logan, UT, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) in a 5% CO_2 humidified atmosphere at 37 °C. The medium was routinely changed every 2 days, and cells were passaged every 4 days. Diesel exhaust $PM_{2.5}$ (DEP, a source of fine particle pollution that contains a high proportion of ultrafine particles) was obtained from the National Institute of Standards and Technology (SRM 2975, NIST, Gaithersburg, MD, USA). According to the analysis certificate of the standard reference material 2975, the average particle size distribution of DEP2975 was approximately 1.62 μm. DEP was suspended in the DMEM cell culture medium. To minimize aggregation to the greatest extent, the particle suspension was subjected to sonication for 15 min and rotation before dilution and administration.

Cell viability

Viable cells counting: Cells were harvested and stained with 0.04% Typan blue (Life Technologies, Inc., Gaithersburg, MD, USA). Viable cells were counted under a microscope using a blood cell counter. And the cell

Gene	Gene Bank ID	Sequences 5′–3′
Rat-PINK1	NM_001106694.1	Forward: GGACCGCTACCGCTTCTTC
		Reverse: CTCCTCGATCAGCCCCAAC
Rat-DJ-1	NM_001277249.1	Forward: GGTGTCGAGCGTTCGTAGC
		Reverse: CATGGAGGGCTTGCTGTAATATC
Rat-GAPDH	NM_017008.4	Forward: GGTGTCGAGCGTTCGTAGC
		Reverse: CATGGAGGGCTTGCTGTAATATC
Huam-PINK1	NM_032409.3	Forward: CTCCCTAACCGTCTCCGCTTCT
		Reverse: GGCCCCGGCTTGCTTTT
Huam-DJ-1	NM_001123377.1	Forward: CTCCCTAACCGTCTCCGCTTCT
		Reverse: GGCCCCGGCTTGCTTTT
Human-GAPDH	NM_001256799.3	Forward: CTCCCTAACCGTCTCCGCTTCT
		Reverse: GGCCCCGGCTTGCTTTT

Table 1. Primers used in RT-PCR.

Gene	
Rat-PINK1-BSP-F1	TAAGGATGTATTTTGGTTGCGTTTT
Rat-PINK1-BSP-R1	CCTAACTCAACTTCTCATCTTAACCAAA
Rat-PINK1-BSP-F2	TAGTTCGAAGGTTAGGAAGATTGTT
Rat-PINK1-BSP-R2	TCAAAATACAAACAAACTAACCCAA
Rat-DJ-1-BSP-F1	TAGGTTGGGGGAGATTAATGGTGTT
Rat-DJ-1-BSP-R1	TAATTCAAACTCTAACCCAATCTAA
Rat-DJ-1-BSP-F2	TTAATTTTGTTAGACGGTTTTGTAT
Rat-DJ-1-BSP-R2	CGTAACCACTATCCTCTTAACCTTT

Table 2. Primers used in BSP methylation assay in rat study.

viability (%) was determined as the number of living cells/total number of cells (viable + non-viable cells) × 100. The experiment was carried out in triplicate.

Lactate dehydrogenase (LDH) assays: The LDH assay was carried out using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. LDH release was measured in a 100 µl aliquot of supernatant, with 100 µl preservation solution used as a blank to correct the optical density reading at 490 nm. The experiment was carried out in triplicate.

Quantitative RT-PCR analysis

To measure *PINK1* and *DJ-1* mRNA expression, total RNA was extracted from the SH-SY5Y cells and tissues using TRIzol reagent (Cat# 15596-026, Life Technologies, California, USA) according to the manufacturer's instructions. The purified RNA was then reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047B, TaKaRa). PCR primers were designed and synthesized by Invitrogen Company; the sequences are shown in Table 1. Quantitative RT-PCR analysis of the mRNA levels of *PINK1* and *DJ-1* was performed using the SYBR®Premix Ex Taq™ II (TliRNaseH Plus), ROX plus, (RR82LR, TaKaRa). GAPDH was chosen as the housekeeping gene. The real-time PCR program steps were: 95 °C for 5 min, 45 cycles at 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 10 s, followed by 72 °C for 1 min.

DNA methylation analysis

Predication of the *PINK1* and *DJ-1* CpG islands was performed using the UCSC Genome Browser database (http://genome.ucsc.edu/). Briefly, genomic DNA was extracted using a QIAamp DNA mini kit (Cat# 51304, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The integrity and purity of DNA were spectrophotometrically examined according to its A260/A280 absorption. A total of 50 ng of genomic DNA from each sample was bisulfite-treated with the Methylamp DNA Modification Kit (Epigentek, Farmingdale, NY, USA). To assay methylation status, the CpG islands of *PINK1* and *DJ-1* were divided into three segments amplified by PCR. PCR primers were designed and synthesized by Invitrogen Company; the sequences are shown in Tables 2, 3. The quality of bisulfite conversion was evaluated using the PCR products without methyl groups as the control. The methylation status was assayed by the Sequenom Mass ARRAY platform (JIAMEI Biolab, Beijing, China). Design methylated and unmethylated primers using Meth Primer software for PCR amplification of sequence differences before and after modification. The methylation ratios were generated by Epityper software version 1.0 (Sequenom, San Diego, CA, USA). The generated data were put into the EPI 3.1 Database (EpiData Association, Odense, Denmark) and analyzed with SPSS 11.5 software (McGraw-Hill Inc, New York, NY). Methylation frequency = Number of methylation of all clones measured in each sample/total CpG of that sample[35,43].

Gene	
Cell-PINK1-BSP-F1	TGATGTTTATATTTAGGATTTGTTTGA
Cell-PINK1-BSP-R1	CTCACCTAAATCTCCTAACAAACC
Cell-PINK1-BSP-F2	GAAAGTTATTGTTAGAGGCGTTAGT
Cell-PINK1-BSP-R2	CGCCGACTCTCCGCCTATTT
Cell-DJ-1-BSP-F1	GGTTCGGGAGGTTTGGATTAGAGTT
Cell-DJ-1-BSP-R1	CTCACTACCAACGACAACAACTCAA
Cell-DJ-1-BSP-F2	GTTTTATTTAGGGTTGTTTAGTTAGAA
Cell-DJ-1-BSP-R2	TAATAAAACGACTAACCAATTCCATA

Table 3. Primers used in BSP methylation assay in cell study.

Statistical analyses

Statistical analysis was performed using Prism10 (GraphPad software, Inc., La Jolla, USA). Values were represented as means ± SEM. Data were analyzed using one-way ANOVA followed by a Tukey's multiple paired comparison test as a post hoc test. A p value of < 0.05 was considered as statistically significant.

Data availability

The authors agree to share any data on request. Any data from this study are available by contacting the corresponding author.

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Author contributions

X.G. and X.W.: study concept and design. X.D.: acquisition of data and wrote the draft. C.C.: made the figure and statistical analysis. L.G.: made a model. All authors contributed to the critical revision of the manuscript and have approved the final version of this review article.

Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

The animal protocols in this study were approved by the Ethics Committee for Animal Experimentation at Capital Medical University (Beijing, China), and all experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (ethical number: 20180712). All methods were performed in accordance with the relevant guidelines and regulations and the ARRIVE guidelines.

Additional information

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