



# Exposure of Healthy Adult Male Rats to Dust Storm Impairs Cognition, Anxiety, Locomotion and Depression-like Behaviors by Stimulation of Brain Neuroinflammation and Oxidative Stress

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## Abstract

**Background:** T Exposure of normal subjects to dust storm (DS) with different doses of ambient air-born dusty particulate matter (PM) causes memory and locomotion impairment, anxiety and depression-like behaviors. This study was designed to investigate the effect of sub-chronic exposure to DS with inhalation of ambient PM in a designed special chamber on cognition, anxiety, depression, locomotion behaviors, brain tissue inflammatory cytokines and antioxidant indices in healthy adult rats.

**Methods:** Adult male Wistar rats (250-300 g) were divided randomly into the 4 groups: Sham (clean air, contains the least dusty PM < 150 µg/m<sup>3</sup>), DS1 (200-500 µg/m<sup>3</sup> PM), DS2 (500-2000 µg/m<sup>3</sup> PM) and DS3 (2000- 8000 µg/m<sup>3</sup> PM). Rats were exposed to the clean air or different sizes and concentrations of PM in DS during the first 4 consecutive days of each week in an experimental actual-ambient dust exposure chamber.

**Results:** Sub-chronic exposing to dust storm PM impaired avoidance memory and locomotion, increased anxiety and depression like behaviors. These disturbances were in line with increased levels of inflammatory cytokines in brain tissue and suppressing the antioxidant indexes.

**Conclusion:** Current findings indicated that exposure to ambient PM due to DS caused cognitive, anxiety, depression-like and locomotion behaviors impairment by increasing the neuroinflammatory responses and suppressing the antioxidant indexes in the brain.

**Keywords:** Dust storm PM, Cognition, Locomotion, Anxiety, Depression, Inflammation, Antioxidant indexes

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## Introduction

Particulate air pollution is associated with augmented incidence of respiratory symptoms and weakened pulmonary function, but the relative impact of pollution from different domestic energy sources is not well-known or studied (1). Little is known concerning the adverse effects of chronic particulate matter (PM) inhalation on

the central nervous system (CNS) (2).

Recent studies show that exposure to airborne PM is associated with cognitive deficit, depression, anxiety, autism, and neurodegenerative diseases; however, the role of PM in the etiology of these consequences is not well-understood. Therefore, there is a need for controlled animal studies to better elucidate the causes and



mechanisms by which PM causes these health outcomes (3). Exposing the healthy young adult rats to ambient airborne dusty PM causes brain dysfunction. Dust storm containing PM (dusty PM), a component of air toxic wastes as an ecological toxicant, with a diameter between 0.5 to 10µm is a common reason of CNS disorders. An investigation has mentioned that dusty PM can impact diverse illnesses in all body organs other than CNS. A principal mechanistic hypothesis by which ambient air particles have a significant negative influence on human health is via the induction of pulmonary inflammatory responses mediated through the generation of reactive oxygen species (ROS) (4).

Investigators recommended macrophages and epithelial cells collaboration to yield maximal cytokine release in response to dusty PM exposure, which means promoting inflammatory responses. These results are consistent with a model in which dusty PM can activate tumor necrosis factor (TNF) secretion by macrophages, which in turn stimulates epithelial cells to generate pro-inflammatory cytokines (5).

Several line of studies show that exposure to ambient dusty PM causes damage to brain structure and function results in neurodegenerative diseases; However, there is still no agreement on this and there is contradictory information (6,7), while other investigations have been revealed that prolonged exposure to airborne dusty PM causes damage to brain and has the potential to alter brain inflammatory phenotype and promote development of neurodegenerative disorders pathology (8-10).

Since, little is known regarding the unfavorable effects of chronic ambient dusty PM exposure on the CNS functions, the present study planned to study how chronic exposure of different sizes and concentrations of ambient dusty PM (during experimental DS) affects the avoidance memory, locomotion, anxiety and depression-like behaviors through the brain oxidative and inflammatory processes in healthy young adult male rats.

## Materials and Methods

### Experimental animals

Sixty adult male Wistar rats (200–250 g) were obtained from animal care and breeding center, Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran. All rats were kept and managed in standard cages under standard conditions including temperature ( $22 \pm 2$  °C), humidity (50-55%) and a 12 hours light/dark cycle (light on 07:00 am) with free access to rat chow pellets and tap water *ad libitum*. All protocols were done under National Institute of Health (NIH) guidelines and approved by the Local Ethics Committee of the Animal Experiments of Ahvaz Jundishapur University of Medical Sciences (AJUMS) for Ethical Animal Use (IR. AJUMS.REC.1396.667). After three days of handling and accommodation period, the rats were divided randomly into four main groups (n=15) as follows:

1. Sham: rats were exposed to the cleaned air in a dust storm exposure chamber with a maximum concentration of  $< 150 \mu\text{g}/\text{m}^3$  ambient air dusty PMs, for 30 minutes twice daily (9 am and 4 pm), during the first four days of each week for 4 consecutive weeks.
2. DS1: rats were exposed to the ambient mild dust storm in a dust storm exposure chamber with concentration of  $200\text{-}500 \mu\text{g}/\text{m}^3$  ambient dusty PMs, including 1, 2.5, 5 and 10 µm size particles, 30 minutes two times daily (9 am and 4 pm), during the first four days of each week for 4 consecutive weeks.
3. DS2: rats were exposed to the ambient moderate dust storm in a dust storm exposure chamber with a concentration of  $500\text{-}2000 \mu\text{g}/\text{m}^3$  ambient dusty PMs.
4. DS3: rats were exposed to the ambient severe dust storm conveying high concentration dusty PMs in dust storm exposure chamber ( $2000\text{-}8000 \mu\text{g}/\text{m}^3$  ambient PMs).

All dust exposure chambers (designed and build for this research locally, Figure 1) were cleaned daily. Each main group was divided into the two sub-groups with different experiments (anxiety-like behavior, depression-like behavior, locomotion test on animals in the sub-group1 while passive avoidance task on other animals in the sub-group 2) were assessed. After performance the behavioral tests, all rats were deeply and irreversibly anesthetized using an over dose of sodium thiopental (Nesdonal, 80 mg/kg, intraperitoneally) and their brains were removed from skull gently and stored in  $-80^\circ\text{C}$  freezer up to biochemical assay. The timeline and design of experimental protocols has been illustrated in Figure 2.

### Whole-body ambient inhalational dust exposure protocol

During each experimental session, all rats were placed in the clean air ( $< 150 \mu\text{g}/\text{m}^3$ ) and dusty chambers with different dust concentrations, which were chosen according to our previous study (11) and can be seen in Table 1; Mild dust storm ( $200\text{-}500 \mu\text{g}/\text{m}^3$  as DS1), moderate dust storm ( $500\text{-}2000 \mu\text{g}/\text{m}^3$  as DS2), severe dust storm ( $2000\text{-} 5000$  or higher  $\mu\text{g}/\text{m}^3$  as DS3) for 30 minutes/ twice daily (9 am and 4 p.m.), during the first four days of each week for 4 consecutive weeks. The figure of designated and build factual ambient dust exposure chamber has been presented in Figure 1.

### Passive avoidance task

Passive avoidance task (PAT) was evaluated in shuttle box apparatus ( $27 \times 14.5 \times 14$  cm) comprised of two illuminated and dark chambers with stainless steel bars (2 mm in diameter and 1 cm in distance) in the floor and a sliding door ( $8 \times 8$  cm) between them. On the habituation day, each animal was placed in the lit chamber with the doors open and it was allowed to explore freely for 10 minutes. In acquisition phase, each rat was placed in



Figure 1. Designated experimental dust storm making chamber (designed by Prof. Sarkaki A., Made by Omid Iranian Co. Tehran and Aref. Jam Co. Ahvaz, Iran).

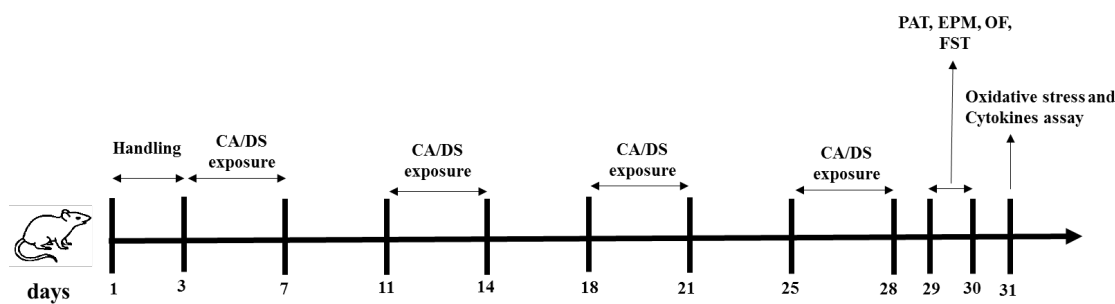


Figure 2. Time- line of the experimental protocols.

the lit chamber facing away from the guillotine door, and 10 s later, the door was elevated. The initial latency (IL) was recorded through an entering delay score to the dark chamber. Then, the guillotine door was closed and a mild electrical foot-shock (50 Hz, 1.2 mA for 3 seconds) was delivered through the grid floor with a stimulator. The rats were retained in the dark chamber for 2 min of consolidation time. On the next day, the retention trial was performed to evaluate the passive avoidance memory as step-through latency of rats. cut-off time was set at 300 s while no shock was delivered in this trial (12).

**Anxiety-like behavior evaluation**

Anxiety was evaluated through elevated plus maze (EPM) test. The maze consisted of two open arms (50 × 10 × 0.5 cm) and two closed arms (50 × 10 × 40 cm), extending

from a central quadrangle (10 × 10 cm) which was placed in a bright room, and the maze was elevated 50 cm above the floor. The maze was elevated 50 cm above the ground and a video camera was fixed above the maze to record the movements for analysis. The behavioral experiments were conducted in a quiet room illuminated by a dim light (50 lx). For the test, each animal was placed in Central Square facing an open arm and the behavior of animals was recorded for 5 minutes by a digital camera which was fixed above the apparatus. Then, time spent in the open and closed arms of EPM and entry numbers into closed and open arms were recorded (13).

**Locomotion activity test**

Locomotion and exploratory behaviors of animals were assessed by the open field (OF) test. Basically, the OF

Table 1. Dust storm classification method.

Category	Visibility (m)	Wind speed (m s <sup>-1</sup> )	Hourly averaged PM <sub>10</sub> (µg.m <sup>-3</sup> )
Normal air (DA)	haze	-	50-200
Light dust storm (DS1)	<2000	-	200-500
Dust storm (DS2)	<1000	>17	500-2000
Strong dust storm (DS3)	<200	>20	2000-5000
Serious strong DS (DS4)	<50	>25	>5000



device was conducted in clear black Plexiglas boxes 50 × 50 × 40 cm (length × width × height), which was subdivided into 25 equal squares by white color lines respectively. The rats were placed in the center of the apparatus to assess locomotion (number of line crossing) and frequency of rearing (standing on hind limbs to search the outside of maze) and grooming (Scratching and cleaning the face and body) actions were recorded for 5 minutes with a video camera device (Maze router, Tabriz, Iran) (14).

### **Depression-like evaluation**

In this test, for depression-like behavior, a rat was dropped into a Plexiglas cylinder (60 cm height and 25 cm in diameter) filled with tap water ( $24 \pm 1^\circ\text{C}$ ) with a depth of 30 cm. The durations of swimming (the animal making horizontal movements in the water), climbing (vertical movements along the wall of the beaker), and immobility (floating in the water without struggling) were recorded for a time period of 5 minutes (15).

### **Cytokines levels of hippocampal tissue**

At the end of the behavioral tests, six rats were randomly selected from each group and anesthetized deeply and irreversibly with a ketamine-xylazine mixture (100/9 mg/kg, ip). The brains were rapidly removed, and hippocampal tissues were quickly dissected on ice, rinsed with saline, and frozen at  $-80^\circ\text{C}$  until they were used. Then, the brain tissues were homogenized with phosphate buffer solution (pH 7.4) and a protease inhibitor cocktail (100 mg tissue/1.2 mL of the buffer). The samples were centrifuged at  $10\,000 \times g$  for 20 min at  $4^\circ\text{C}$ , and consequently, clear supernatants were collected carefully, divided into aliquots, and stored immediately at  $-80^\circ\text{C}$  until analysis. Total protein concentrations of the supernatants were measured using a Bio-Rad protein assay kit according to the manufacturer's protocols.

The brain tissue content of inflammatory interleukins- $1\beta$  and interleukins-6 (IL- $1\beta$ , IL-6) and anti-inflammatory IL-4 were measured using the specific ELISA kits, (Zell Bio, GmbH, Germany). Briefly, the specimens were poured into wells that contained anti IL- $1\beta$  antibody. Then, the conjugated secondary antibody was added to the medium with biotin. After adding streptavidin-HRP, it was incubated at  $37^\circ\text{C}$  for 60 minutes. Then, it was rinsed 5 times by saline. The chromogen was added to the medium, and 30 minutes later the stopping solution was added to the medium. The changed color of chromogen was read in the 450 nm optical range. Brain content of IL- $1\beta$ , IL-6 and IL-4 were expressed in picograms in 1 mg of protein (pg/mg protein) (5).

### **Estimation of enzymatic and non-enzymatic antioxidants**

#### *Evaluation of superoxide dismutase and catalase*

Superoxide dismutase (SOD) activity was assessed

indirectly by a coupled reaction with glutathione (GSH) reductase, which was done by the SOD assay kit (16). Catalase (CAT) activity was measured by a specific kit, according to the producer's directions. In brief, CAT activity was assayed by monitoring the  $\text{H}_2\text{O}_2$  decomposition, which was measured spectrophotometrically via the decrease in absorbance rate at 240 nm and was expressed as U/mg of protein (17).

#### *Evaluation of glutathione*

GSH was assayed according to the method of Sedlak and Lindsay in which DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) was used as the reagent. Briefly, aliquots of 250  $\mu\text{L}$  of hippocampus tissue homogenates were mixed with 750  $\mu\text{L}$  of 0.2 M Tris buffer (pH 8.2) and 50  $\mu\text{L}$  of 0.01 M DTNB. Then, 3.95 mL of absolute methanol was added to obtain the final 124 volume of 5 mL. The absorbance rates of the supernatants were read at 412 nm and the concentrations were stated as nmol/mg of protein (18).

### **Statistical analysis**

All data were presented as mean  $\pm$  SEM and the data normality was assessed using Kolmogorov-Smirnov test. Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P* value less than 0.05 was considered as significant difference. All statistical analyses were done by the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, USA).

## **Results**

### **Passive avoidance task**

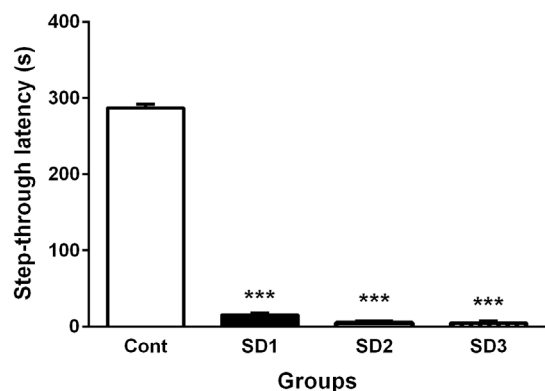
As shown in Figure 3, the memory in groups exposed with mild, moderate and severe ambient dust storm was significantly increased during PAT compared to the sham group ( $P < 0.001$ ). There was no significant difference in initial latency among the tested groups.

### **Anxiety-like behavior**

Figure 4 illustrates the number entrance and time spent in open arm during the evaluation of anxiety-like behavior in EPM. As shown in Figure 4A and 4B, the number of entrance and time spent in open arm decreased significantly in DS groups exposed to mild to severe dust storm with low to high concentrations of dusty PM compared to the sham group ( $P < 0.001$ ).

### **Locomotion**

As shown in Figure 5, all parameters in OF test including total distance travelled (the number of line crossing or ambulation; panel A), rearing (standing on hind limbs to search the outside of maze; panel B) and grooming (scratching and cleaning the face and body; panel C) decreased significantly in all groups exposed to dusty PM compared to the sham group ( $P < 0.001$ ).



**Figure 3.** Passive avoidance memory evaluation for different tested groups in shuttle box device. Data represented as mean±SEM. DS1: mild dust storm, DS2: moderate dust storm and DS3: severe dust storm. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. \*\*\*  $P < 0.001$  vs. Sham

### Depression-like behavior

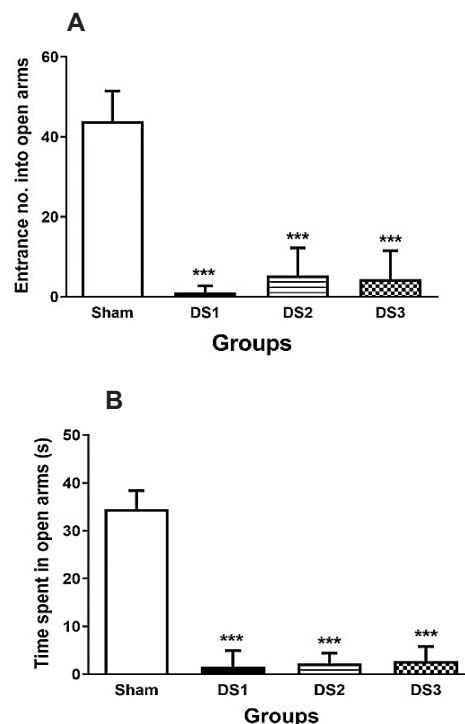
Figure 6 shows the immobility time in fast swimming test as an index of depression-like behavior in the different tested groups. The immobility time in DS groups exposed to mild to severe dust storm with low to high concentrations of dusty PM increased, but only in DS2 and DS3 groups, this difference was significant compared to the sham group ( $P < 0.05$ ).

### Hippocampal level of cytokines

In Figure 7, the levels of inflammatory and anti-inflammatory cytokines in brain hippocampi tissues in different tested groups have been demonstrated. The IL-1 $\beta$  in DS2 and DS3 groups exposed to moderate and severe concentrations of dusty PM during dust storm respectively increased significantly compared to the sham group ( $P < 0.001$ , Figure 7A). As shown in Figure 7B, the level of IL-6 in hippocampal tissue also increased significantly in DS2 and DS3 groups in comparison to the sham group ( $P < 0.01$ ). In contrast, the level of IL-4, an anti-inflammatory cytokine, decreased significantly in DS2 and DS3 groups compared to the sham group ( $P < 0.001$ , Figure 7C).

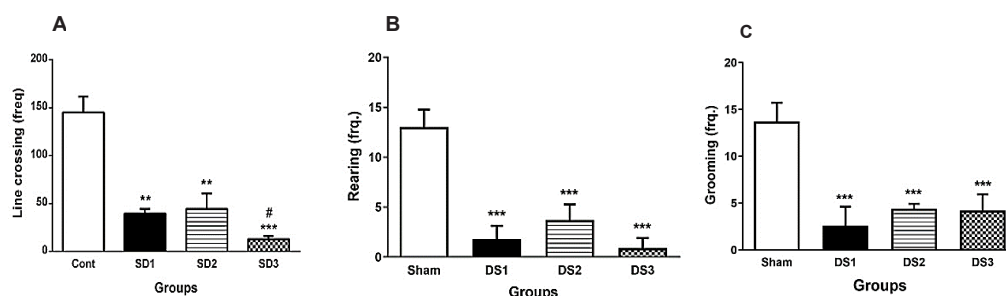
### Anti-oxidants in brain hippocampus tissue

Figure 8 shows the activity of SOD, CAT and GSH, as

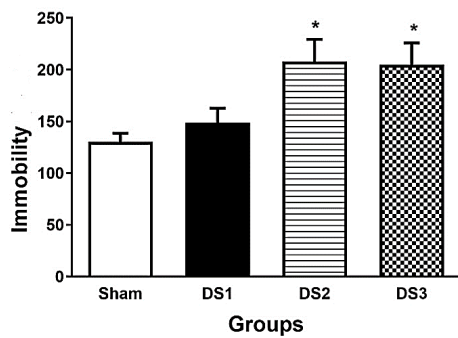


**Figure 4.** A) Percentage of time spent in open arms, B) number of open arms entries. Data are presented as Mean±SEM. DS1: mild dust storm, DS2: moderate dust storm and DS3: severe dust storm. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. \*\*\*  $P < 0.001$  vs. Sham

anti-oxidant systems. In all DS groups, SOD decreased significantly compared to the sham group in a dose-dependent manner ( $P < 0.001$ ). The decrease of activity of enzyme in DS2 and DS3 groups was significant compared to the DS1 group ( $P < 0.001$ , Figure 8A). CAT decreased in all DS groups significantly and in a dose-dependent manner compared to the sham group ( $P < 0.01$  and  $P < 0.001$ ). The decrease of enzyme activity in the DS2 and DS3 groups was significant in comparison to the DS1 group ( $P < 0.5$  and  $P < 0.01$  respectively, Figure 8B). GSH concentration decreased in all DS groups significantly in a dose-dependent manner compared to the sham group ( $P < 0.001$ ). The decrease of GSH level in hippocampal tissue of DS2 and DS3 groups was significant compared to the DS1 group ( $P < 0.5$  and  $P < 0.01$  respectively, Figure 8C).



**Figure 5.** Locomotor activity in the OF test. (A) Total traveled distance (Line crossing), (B) Number of rearing, (C) Number of grooming. Data are presented as Mean±SEM. DS1: mild dust storm, DS2: moderate dust storm and DS3: severe dust storm. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. #  $P < 0.05$  vs. Sham, \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  vs. Sham



**Figure 6.** Total immobility time in the FST. Data are presented as Mean ± SEM, DS1: mild dust storm, DS2: moderate dust storm and DS3: sever dust storm. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. \*  $P < 0.05$  vs. Sham.

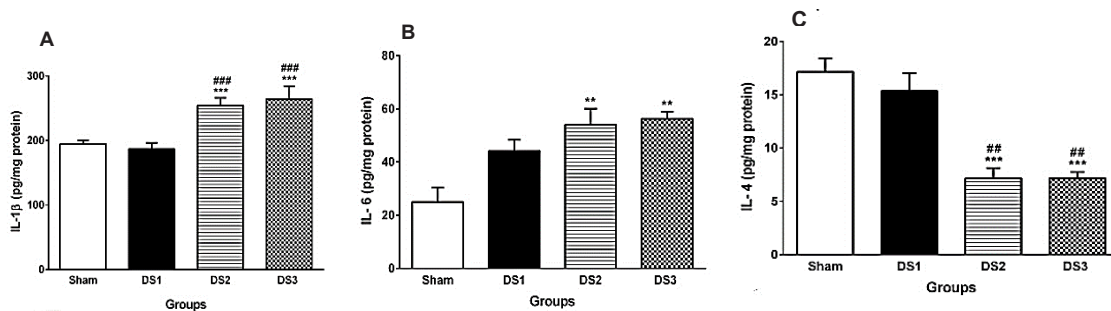
## Discussion

In the present study, exposure to ambient dusty PM produced a wide range of behavioral deficits, including anxiety-like behaviors in EPM, locomotor/exploratory impairments in OFT, memory deficiency in PAT, and depression-like behaviors in FST. These behavioral impairments were associated with increase of inflammatory and oxidative indexes in the brain tissue.

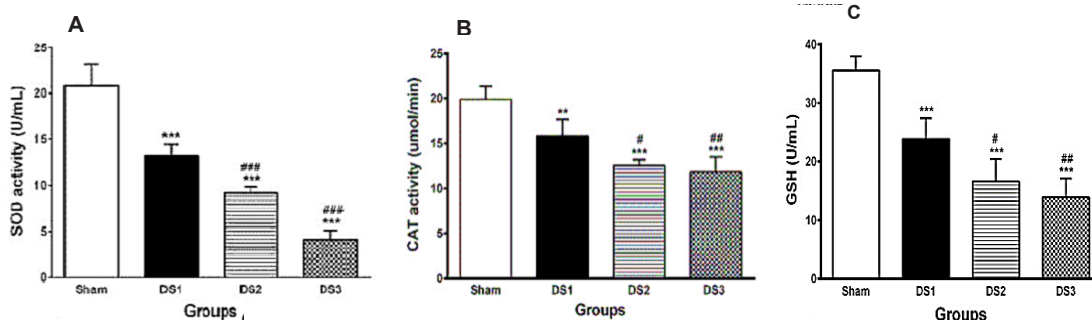
Over the past decade, it has been reported that exposure to ambient air pollution is associated with deleterious changes in the brain tissue (19,20), and cognitive decline (21-25). Recently, we demonstrated that exposure of healthy adult male rats to ambient dusty PM could disrupt the BBB, and induce brain edema, which was associated

with cognitive and behavioral deficits (11). In the present study, exposure to ambient PM induced anxiety related behaviors in EPM, which was demonstrated by decrease of the time spent in open arms and entrance number into open arms in the ambient dusty PM exposure rats. In our previous study, too, anxiety-like behaviors in EPM increased in ischemic rats exposed to dusty PM (26), which confirms the current findings. In addition, our findings showed that locomotion activity (line crossing), exploratory (rearing) and anxiety-like behavior (grooming) in the OFT were significantly lower in the dusty PM groups. Furthermore, depression like behavior in the FST group increased following exposure to the ambient dusty PM. These finding were consistent with previous studies, which indicated that ambient PM could induce different behavioral deficits (11,26).

It was also evidencing that PM exposure is resulted in a significant reduction of cognitive ability in mice, which might be induced by BBB disruption and neuroinflammatory reactions. Hence, inflammation in the hypothalamus and olfactory bulb tissue were also demonstrated (27). In the current study, exposure to ambient dusty PM caused poor performance of rats in the PAT, which was demonstrated by decreasing in step through latency time. In this test, animals learned that entering into the dark box related with an electrical shock. Therefore, avoiding dark box entrance showing the cognitive ability of the rat (28). Cognitive impairment in the PAT was evidenced by a significant decrease in the step-through latency of the PM-exposed rats. Our



**Figure 7.** Brain tissue levels of pro-inflammatory and anti-inflammatory cytokines. (A) IL-1 $\beta$ , (B) IL-6 and (C) IL-4. Data are presented as Mean ± SEM, DS1: mild dust storm, DS2: moderate dust storm and DS3: sever dust storm.



**Figure 8.** A-C) SOD, CAT and GSH activity levels in hippocampus. Data were presented as Mean ± SEM (B), DS1: mild dust storm, DS2: moderate dust storm and DS3: sever dust storm. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. #  $P < 0.05$ , ##  $P < 0.01$ , ###  $p < 0.001$  vs DS1, \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  vs. Sham

results were in line with other studies, which showed that ambient dusty PM caused serious cognitive decline in animal model (27).

Inflammation and oxidative stress have been evidenced to have an essential role in the pathogenesis of air pollution-induced disorders through increasing the production of proinflammatory mediators and ROS production and affecting brain functions (29). Epidemiological studies have demonstrated a link between air pollutant exposure and adverse brain effects (30). Our findings showed that oxidative stress in the brain tissue significantly increased following exposure to ambient dusty PM, which represented by decreasing the activity of SOD and CAT activities, and contents of GSH in the brain tissue. In consistent with our results, it has been demonstrated that ambient PM induces oxidative stress and pro-inflammatory responses in human bronchial epithelial cells (31). Dusty PM was demonstrated to trigger ROS production and inhibited anti-oxidant enzymes, and has a significant negative effect on human health (32).

It has been shown that PM exposure increases ROS and inflammatory responses in the brain (33,34), which were accompanied by BBB disruption (35,36), and microglial activation that releasing inflammatory cytokines (37). The current data showed that the brain contents of IL-1 $\beta$  and IL-6 were significantly increased following exposure to ambient dusty PM. This observation was in agreement with our previous reports, which shows that expression of TNF- $\alpha$ , as an inflammatory cytokine, was increased in the brain tissue after ambient dusty PM-exposure (11). The increased levels of IL-1 $\beta$  and IL-6 may be due to the lessening of the IL-4 levels (as an anti-inflammatory cytokine) in the brain tissue, which was demonstrated in the current study. We showed that brain tissue contents of IL-4 decreased in response to ambient dusty PM-exposure. Previously, it was also evidenced that anti-inflammatory contents of IL-10 decreased in the brain tissue after exposure to ambient dusty PM (11). It has been suggested that dust particles could reach the brain tissue through olfactory gateways, and increased glial responses and TNF- $\alpha$  levels in the brain regions (38). It also demonstrated that PM exposure induced injuries in the brain tissue through endothelial dysfunction and inflammatory responses (39). In addition, exposure to ambient air pollution can have adverse effects on cognitive decline and impairment (40), which may cause by amplification of oxidative stress and neuroinflammatory markers following dust exposure (41).

In several studies, airborne dust exposure has affected cognitive function in older adults (42), and demonstrated worse cognitive function (43). In addition, other studies showed that exposure to ambient dust storm associated with neurodegenerative diseases, by the possible mechanisms includes inflammatory responses, mitochondrial dysfunction and oxidative stress (39,44).

Therefore, according to our results, exposure to ambient airborne dust may lead to memory impairment and neurodegenerative diseases in adults.

## Conclusion

Overall, the findings of the present investigation showed that exposure to the ambient dusty PM induced cognitive impairments, increased anxiety and depression-like behaviors and locomotor/exploratory deficiency in male rats. These behavioral impairments might be caused by increase of inflammatory and oxidative indexes in the brain tissue following exposure to the ambient PM. These findings suggest that ambient PM may have deleterious effects on brain function, and put one's life in serious danger.

## Acknowledgments

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## Authors' Contribution

SH and BS performed behavioral tests and analyzed data. EKH contributed in writing the manuscript. YF and NB were responsible for monitoring and approving behavioral tests in different experimental groups. HM was responsible for dust exposing to rats, and GG was responsible for GRIMM (GRIMM Co. Germany) spectrometer control. MR performed and analyzed the biochemical factors examination. AS designed, guided and supervised the project.

## Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Conflict of Interests

The authors declare that there is no conflict of interest.

## Ethical Approval

All experiment protocols were done under National Institute of Health (NIH) guidelines and approved by the Local Ethics Committee of the Animal Experiments of Ahvaz Jundishapur University of Medical Sciences (AJUMS) for Ethical Animal Use (IR. AJUMS.REC.1396.667).

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